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# Regulators of G-Protein Signaling and Their $G\alpha$ Substrates: Promises and Challenges in Their Use as Drug Discovery Targets

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Abstract	728
I. Introduction	729
A. Biological and pharmaceutical importance of G-protein coupled receptor signaling	729
B. The classic guanine nucleotide cycle of heterotrimeric G-protein subunits	729
C. Structural determinants of G-protein subunit function	730
1. $G\alpha$ subunit	730
2. $G\beta\gamma$ dimer	730
3. Structural basis for intrinsic GTP hydrolysis activity by $G\alpha$ subunits	730
4. Structural features of Regulators of G-protein Signaling—the $G\alpha$ GTPase-accelerating proteins	730
II. Selected functional vignettes among the complement of Regulators of G-protein Signaling	733
A. The utility of the “Regulators of G-protein Signaling-insensitivity” point mutation	733
B. Regulators of G-protein Signaling 1, 2, and 13 in immune system regulation	734
C. Regulators of G-protein Signaling 2, 4, 5, and 6 in cardiovascular system regulation	735
III. Pursuing chemical probes for Regulators of G-protein Signaling GTPase-accelerating protein activity	736
A. GTPase-accelerating protein activity as the sole determinant of Regulator of G-protein Signaling action on G-protein-coupled receptor signaling sensitivity and kinetics	736
B. The potential for allosteric control over Regulator of G-protein Signaling GTPase-accelerating protein activity	738
C. Current state of identifying chemical probes that modulate Regulator of G-protein Signaling protein activity	739
1. Pharmaceutical company activities published in the literature	739
2. Academic laboratory activities with binding assays	740
3. An enzymatic assay using rate-altered $G\alpha$ and fluorescence polarization detection of GDP generation	741
IV. Activated $G\alpha_{q/11}$ point mutants as targets for ocular melanoma therapeutic strategies	742
A. Regulator of G-protein Signaling domain-based gene therapy?	742
B. Gene- or peptide-based strategies?	743
Acknowledgments	745
References	745

**Abstract**—Because G-protein coupled receptors (GPCRs) continue to represent excellent targets for the

discovery and development of small-molecule therapeutics, it is posited that additional protein components of the signal transduction pathways emanating from activated GPCRs themselves are attractive as drug discovery targets. This review considers the drug discovery potential of two such components: members of the “regulators of G-protein signaling” (RGS protein) superfamily, as well as their substrates, the heterotrimeric G-protein  $\alpha$  subunits. Highlighted are recent advances, stemming from mouse knockout studies and

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the use of “RGS-insensitivity” and fast-hydrolysis mutations to G $\alpha$ , in our understanding of how RGS proteins selectively act in (patho)physiologic conditions controlled by GPCR signaling and how they act on the nucleotide cycling of heterotrimeric G-proteins in shaping the kinetics and sensitivity of GPCR signaling. Progress is documented regarding recent activities along the path to devising screening assays and chemical probes for the RGS protein target, not only in pursuits of inhibitors of RGS domain-mediated ac-

celeration of G $\alpha$  GTP hydrolysis but also to embrace the potential of finding allosteric activators of this RGS protein action. The review concludes in considering the G $\alpha$  subunit itself as a drug target, as brought to focus by recent reports of activating mutations to GNAQ and GNA11 in ocular (uveal) melanoma. We consider the likelihood of several strategies for antagonizing the function of these oncogene alleles and their gene products, including the use of RGS proteins with G $\alpha_q$  selectivity.

## I. Introduction

### A. Biological and Pharmaceutical Importance of G-Protein Coupled Receptor Signaling

For a cell to adapt to its environment, it must be able to receive extracellular cues and then elicit an appropriate intracellular response to those cues. Although there are multiple receptor families (i.e., receptor tyrosine kinases, ion channels, nuclear receptors), G-protein-coupled receptors (GPCRs<sup>1</sup>) represent the largest and most pharmacologically important family. Approximately 1% of the human genome is dedicated to these receptors (Takeda et al., 2002; Fredriksson et al., 2003; Vassilatis et al., 2003), and nearly a third of the pharmaceuticals currently on the market target one or more of these receptors (Jacoby et al., 2006; Overington et al., 2006; Lagerström and Schiöth, 2008). In addition to being the largest component of the “druggable” proteome, GPCRs are also responsible for our ability to perceive the visual, olfactory, and gustatory cues in our environment. Missense or truncation mutations to individual codons in genes encoding GPCRs result in a myriad of pathological conditions, including color blindness, retinitis pigmentosa, pseudohermaphroditism, and Hirschsprung’s disease (Spiegel and Weinstein, 2004). Given the importance of GPCRs in both pathologic conditions and treatment of disease, it is critical that we comprehensively understand these receptors and their downstream signaling components.

<sup>1</sup>Abbreviations: CaM, calmodulin; CCG-4986, methyl-N-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfonimidoate; CCG-50014, 4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione; CCG-63802, (2E)-2-(1,3-benzothiazol-2-yl)-3-[9-methyl-2-(3-methylphenoxy)-4-oxo-4H-pyrido[1,2-a]pyrimidin-3-yl]prop-2-enitrile; CCG-63808, (2E)-2-(1,3-benzothiazol-2-yl)-3-[9-methyl-2-(4-fluorophenoxy)-4-oxo-4H-pyrido[1,2-a]pyrimidin-3-yl]prop-2-enitrile; FP, fluorescence polarization; GAP, GTPase-accelerating protein; GEF, guanine nucleotide exchange factor; GIRK, G-protein coupled inwardly rectifying potassium channel; GoLoco, G $\alpha_{i/o}$ -Loco interaction motif; GPA1, yeast G $\alpha$  subunit; GPCR, G-protein-coupled receptor; HTS, high-throughput screen; IL, interleukin; mAChR, muscarinic acetylcholine receptor; PDB, Protein Data Bank; PIP<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; PLC, phospholipase C; RGS, regulator of G-protein signaling; siRNA, small interfering RNA; Sst, supersensitive; YM-254890, (1R)-1-[(3S,6S,9S,12S,18R,21S,22R)-21-acetamido-18-benzyl-3-[(1R)-1-methoxyethyl]-4,9,10,12,16,22-hexamethyl-15-methylene-2,5,8,11,14,17,-20-hepta-oxo-1,19-dioxo-4,7,10,13,16-pentaazacyclodocosan-6-yl]-2-methylpropyl rel-(2S,3R)-2-acetamido-3-hydroxy-4-methylpentanoate.

At the most basic level, GPCRs consist of seven  $\alpha$ -helical transmembrane stretches with an extracellular N terminus and an intracellular C terminus. These diverse receptors can be further divided into subfamilies named by their hallmark member: glutamate-, rhodopsin-, adhesion-, frizzled-, and secretin-like (Fredriksson et al., 2003; Perez, 2003). Although the precise mechanism of activation of the heterotrimeric G-protein probably varies from family to family and remains elusive, in simplest terms upon binding of a hormone, neurotransmitter, ion, or other stimuli, the GPCR undergoes conformational changes that allow the activation of the G $\alpha$ -GDP/G $\beta\gamma$  complex. Upon the binding of an activating ligand, the GPCR catalyzes the release of GDP and subsequent binding of GTP on the G $\alpha$  subunit (Gilman, 1987; Johnston and Siderovski, 2007; Oldham and Hamm, 2008).

### B. The Classic Guanine Nucleotide Cycle of Heterotrimeric G-Protein Subunits

Heterotrimeric G-proteins act as molecular switches that are considered in the off state when bound to GDP and in the on state (“activated”) when GTP-bound. In the basal state, the GDP-bound G $\alpha$  subunit is in complex with the G $\beta\gamma$  dimer (Fig. 1). The G $\alpha$ /G $\beta\gamma$  interaction serves to enhance localization to the membrane, to enhance coupling, and to slow the spontaneous dissociation of GDP (so-called “GDP dissociation inhibitory” function that reduces basal activity) (Brandt and Ross, 1985; Higashijima et al., 1987; Robillard et al., 2000; Evanko et al., 2001). Upon an agonist-induced conformational change, the receptor acts as a GEF resulting in the displacement of GDP and subsequent binding of GTP (which is in higher abundance). The nucleotide pocket of the heterotrimeric G-protein  $\alpha$  subunit is surrounded by three flexible switch regions that undergo dramatic conformational changes depending on nucleotide state (Bohm et al., 1997; Wall et al., 1998). The binding of GTP and subsequent change in the switch regions results in the dissociation of the GTP-bound G $\alpha$  from G $\beta\gamma$ . At this point, the activated G $\alpha$  subunit and the G $\beta\gamma$  obligate heterodimer are able to interact with effectors such as adenylyl cyclase, phospholipase C isoforms, RhoGEFs, and ion channels (Clapham and Neer, 1997; Kozasa et al., 1998; Simonds, 1999; Kammermeier et al., 2000; Rhee, 2001; Lutz et al., 2007).

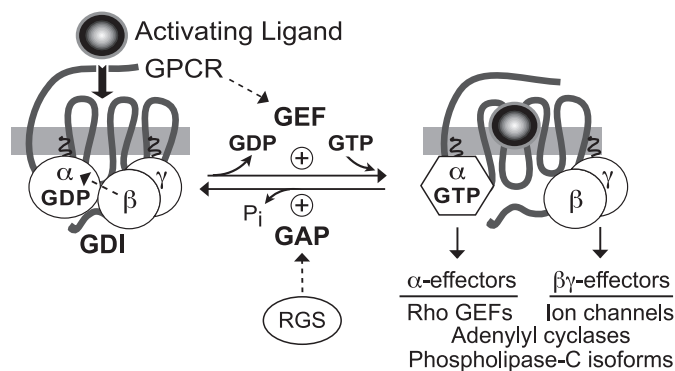


FIG. 1. The standard model of guanine nucleotide cycle of G-protein coupled receptors. The heterotrimeric G-protein consists of a GDP-bound  $G\alpha$  subunit associated with the  $G\beta\gamma$  heterodimer. The  $G\beta\gamma$  serves not only to assist the coupling of  $G\alpha$  to the GPCR, but also as a guanine nucleotide dissociation inhibitor (GDI) for  $G\alpha$ , preventing the release of GDP. Upon binding of an activating ligand to the receptor, conformation changes result in the GPCR acting as a GEF, causing the release of GDP and subsequent binding of GTP. This exchange of bound nucleotide results in the dissociation of  $G\beta\gamma$  and both  $G\alpha$ -GTP and  $G\beta\gamma$  are free to signal to downstream effectors. Downstream effectors are activated until the GTP is hydrolyzed by the intrinsic GTP hydrolysis activity of the  $G\alpha$  subunit [which can be further accelerated by particular downstream effectors such as PLC $\beta$ 1 and p115RhoGEF (Berstein et al., 1992; Kozasa et al., 1998)]. Upon hydrolysis of GTP,  $G\alpha$ -GDP rebinds  $G\beta\gamma$  and the system returns to the inactive state. The rate of GTP hydrolysis can be dramatically enhanced by RGS proteins, which serve as GAPs for  $G\alpha$  subunits in vitro (Berman et al., 1996) and in vivo (Lambert et al., 2010).

### C. Structural Determinants of G-Protein Subunit Function

**1.  $G\alpha$  Subunit.** The  $G\alpha$  subunit, in its inactive state, binds GDP within a nucleotide-binding pocket circumscribed by residues derived from both of its constituent domains: a Ras-like domain (resembling the structural fold of “small” G-proteins) and an all  $\alpha$ -helical domain unique to the “large”  $G\alpha$  family, comprising a structurally distinct six-helix bundle (Fig. 2A). An extended N-terminal  $\alpha$ -helix is modified by covalent attachment of the fatty acids myristate and/or palmitate, which facilitates membrane targeting as well as assembly with  $G\beta\gamma$  subunits (Wedegaertner et al., 1995). Exchange of GDP for GTP is catalyzed in a poorly understood process by an activated GPCR acting as a GEF for the  $G\alpha$ -GDP/ $G\beta\gamma$  heterotrimer (Johnston and Siderovski, 2007; Oldham and Hamm, 2008). This receptor-catalyzed nucleotide exchange results in nucleotide-pocket residues interacting with the  $\gamma$ -phosphoryl group of the newly bound GTP (Lambright et al., 1994; Posner et al., 1998) and causes a structural rearrangement within three switch regions (I–III) of  $G\alpha$  (Fig. 2B). The particular conformations of these three switch regions are critical to the protein/protein interactions that  $G\alpha$  makes with its nucleotide-state-selective binding partners, such as  $G\beta\gamma$ , effectors, RGS proteins, and GoLoco motifs (Bohm et al., 1997; Tesmer et al., 1997a; Wall et al., 1998; Willard et al., 2004b).

**2.  $G\beta\gamma$  Dimer.**  $G\beta$  and  $G\gamma$  subunits form tightly associated heterodimers (Fig. 2C).  $G\beta$  begins with an extended N-terminal  $\alpha$ -helix and is composed mainly of a  $\beta$ -propeller fold formed by seven individual segments of

a  $\sim$ 40-amino acid sequence known as the WD-40 repeat.  $G\gamma$  is an extended stretch of two  $\alpha$ -helices joined by an intervening loop. Assuming no significant tertiary structure on its own, the N terminus of  $G\gamma$  participates in a coiled-coil interaction with the N-terminal  $\alpha$ -helix of  $G\beta$  (Fig. 3); much of the remainder of  $G\gamma$  binds along the outer edge of the  $G\beta$  toroid (Wall et al., 1995; Sondek et al., 1996).  $G\gamma$  is prenylated post-translationally on a cysteine residue found four amino acids from the C terminus. Most  $G\gamma$  subunits receive a 20-carbon geranylgeranyl group at this position (as illustrated in Fig. 2C), whereas  $G\gamma$ 1,  $G\gamma$ 8, and  $G\gamma$ 11 receive a 15-carbon farnesyl group instead (Wedegaertner et al., 1995). Such lipid modification facilitates membrane localization of the  $G\beta\gamma$  heterodimer that is important to receptor coupling. GDP-bound  $G\alpha$  and the  $G\beta\gamma$  dimer form the G-protein heterotrimer via two principal sites of interaction: 1) extensive burial of the  $\beta$ 3/ $\alpha$ 2 loop and  $\alpha$ 2 helix (switch II) of  $G\alpha$  within six of the seven WD repeats of  $G\beta$ , and 2) contact between the side of the first  $\beta$ -propeller blade of  $G\beta$  and the extended N-terminal helix of  $G\alpha$  (see Fig. 2C) (Bohm et al., 1997; Wall et al., 1998). These extensive interactions form the basis for competition for  $G\beta\gamma$  binding between  $G\alpha$ -GDP and  $\beta\gamma$ -effectors. Structures of  $G\beta\gamma$  bound to the regulatory protein phosphodiesterase, the receptor kinase (and “ $\beta\gamma$ -effector”) GRK2, and SIRK/SIGK [peptides capable of disrupting  $\beta\gamma$ -effector activation (Scott et al., 2001)] have shown that the effector-binding site on  $G\beta\gamma$  overlaps significantly with the region responsible for binding switch II of  $G\alpha$  near the central pore of the  $G\beta$  torus (Gaudet et al., 1996; Lodowski et al., 2003; Bonacci et al., 2006).

**3. Structural Basis for Intrinsic GTP Hydrolysis Activity by  $G\alpha$  Subunits.** The mechanism of GTP hydrolysis by  $G\alpha$  has been discerned from X-ray diffraction crystallographic structures, especially of the  $G\alpha$  transition state-mimetic form (i.e.,  $G\alpha$  bound to GDP and  $AlF_4^-$ ) (Coleman et al., 1994), as well as hydrolysis reaction intermediates, including  $G\alpha$  bound to guanosine 5'-( $\beta$ - $\gamma$ -imido)triphosphate or GDP plus inorganic phosphate (Raw et al., 1997; Coleman and Sprang, 1999). The GTP hydrolysis reaction is mediated by three conserved  $G\alpha$  amino acids (Fig. 2D). Gln-204 in switch II (residues numbered as found in  $G\alpha_{i1}$ ) coordinates the critical nucleophilic water molecule responsible for hydrolysis of the  $\gamma$ -phosphate, whereas Arg-178 and Thr-181 (both from switch I) help to stabilize the leaving group (as mimicked by the planar anion  $AlF_4^-$ ), the latter residue coordinating a bound  $Mg^{2+}$  ion (Coleman et al., 1994).

**4. Structural Features of Regulators of G-Protein Signaling—the  $G\alpha$  GTPase-Accelerating Proteins.** The GTP hydrolysis activity intrinsic to the  $G\alpha$  subunit was initially thought to control the lifetime of G-protein  $\alpha$  subunits in their GTP-bound state and the in vitro kinetics of GTP hydrolysis observed by  $G\alpha_s$  supported this hypothesis (Cassel et al., 1979); however, intrinsic rates of GTP hy-



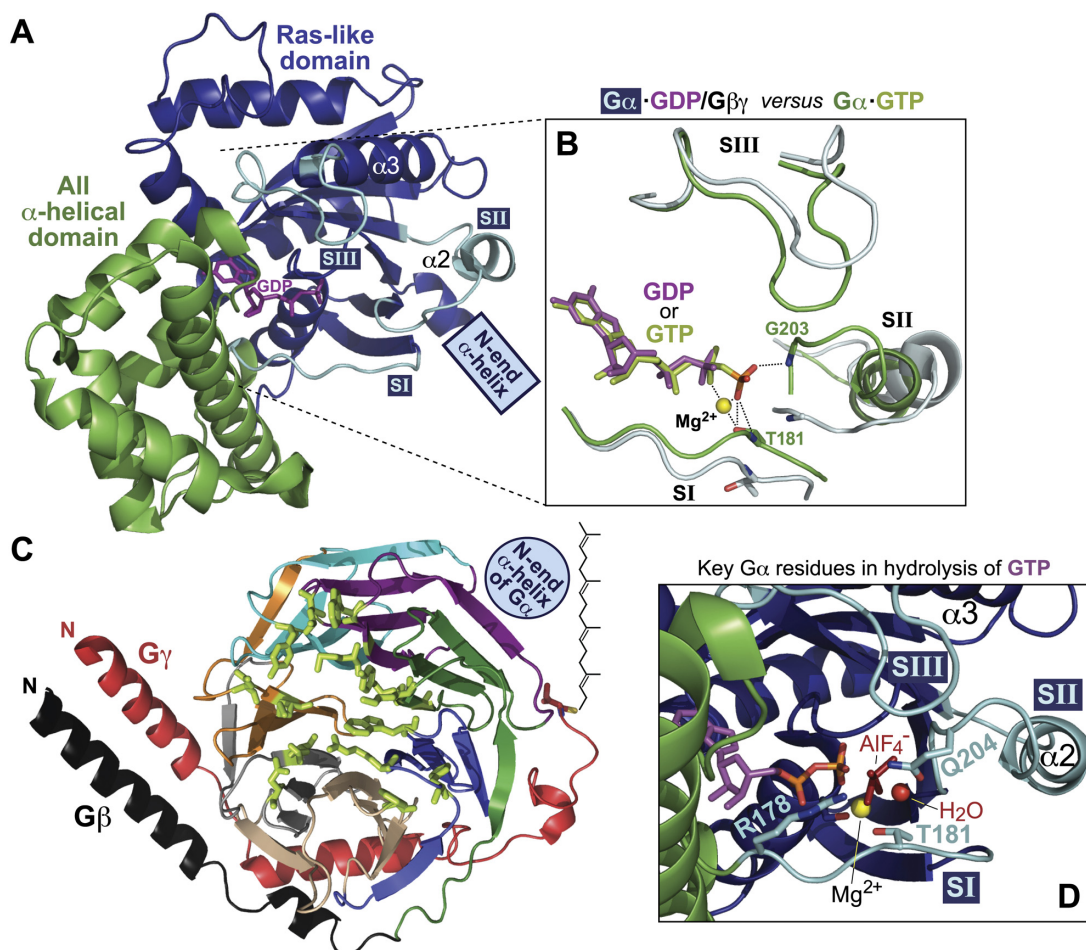


FIG. 2. Structural features of the heterotrimeric G-protein subunits. A, overall structural fold of the heterotrimeric G-protein  $G\alpha$  subunit in its inactive, GDP-bound form. The  $G\alpha$  subunit (PDB number 1GP2) is composed of a Ras-like domain (blue) and an all  $\alpha$ -helical domain (green), between which is found the guanine nucleotide binding pocket (GDP in magenta). The three flexible switch regions (SI, SII, and SIII) are highlighted in cyan. B, details of structural differences between GDP- and GTP-bound states. The additional (third) phosphoryl group (orange and red) of bound GTP establishes contacts with residues Thr-181 and Gly-203 of switches I and II, respectively, thus leading to changes in all three switch regions (green; PDB number 1G1A) versus their conformation in the GDP-bound state (cyan; PDB number 1GP2). Magnesium cation is highlighted in yellow. C, overall structural fold of the  $G\beta\gamma$  heterodimer. The  $G\beta\gamma$  subunit (PDB number 1OMW) is colored to highlight the seven WD40 repeats that comprise the  $\beta$ -propeller fold: WD1, green; WD2, purple; WD3, cyan; WD4, orange; WD5, gray; WD6, wheat; and WD7, blue. The cysteine residue within  $G\beta\gamma$  (red) that receives post-translational geranylgeranylation is highlighted in sticks configuration. The relative positioning of the N-terminal  $\alpha$ -helix of the  $G\alpha$  subunit (when in the  $G\alpha$ -GDP/ $G\beta\gamma$  heterotrimeric complex) is also highlighted. D, structural basis of GTP hydrolysis by  $G\alpha$ . Residues within  $G\alpha$  that are critical to the GTP hydrolysis mechanism include Arg-178 and Thr-181 from switch I and Gln-204 from switch II (colored as in A and numbered as in  $G\alpha_{i1}$ ; coordinates are from PDB number 1GFI). Magnesium cation is highlighted in yellow. The planar anion  $AlF_4^-$ , which mimics the  $\gamma$ -phosphate leaving group of the  $GTP \rightarrow GDP + P_i$  hydrolysis reaction, is depicted in metallic red.

hydrolysis measured in vitro could not account for the fast deactivation kinetics seen with other G-proteins in the cellular context. For instance, purified transducin, which is the heterotrimeric G-protein that couples to the photo-transduction GPCR rhodopsin, hydrolyzes GTP with a  $t_{1/2}$  of  $\sim 15$  s; however, the rate of retinal deactivation is  $< 1$  s (Vuong and Chabre, 1991). In addition, G-protein coupled inwardly rectifying potassium channels (GIRKs), which are activated by  $G\beta\gamma$  freed from  $G\alpha_i$  subunits, are deactivated 100 times faster than would be predicted based on the intrinsic GTP hydrolysis rate exhibited by  $G\alpha_i$  subunits in vitro (Breitwieser and Szabo, 1988; Yatani et al., 1988). The first evidence that the cycle of nucleotide binding and hydrolysis by  $G\alpha$  subunits could be modulated by binding partners other than  $G\beta\gamma$  came from the report of Bernstein et al. (1992) demonstrating that the  $G\alpha_{q11}$  effec-

tor phospholipase C (PLC)  $\beta 1$  could also increase the rate of GTP hydrolysis by  $G\alpha_{q11}$ . Although PLC $\beta 1$  seemed to have paradoxical roles, being both an effector and a GTPase-accelerating protein (GAP) for  $G\alpha_{q11}$ , this report provided an early demonstration of a GAP for heterotrimeric G-protein  $\alpha$  subunits, although GAPs had been known for Ras-family GTPases for at least 5 years before this (Trahey and McCormick, 1987). The first evidence of noneffector GAPs for heterotrimeric G-proteins came from a yeast-based genetic screen for mutants that increased sensitivity of *Saccharomyces cerevisiae* to  $\alpha$ -factor pheromone. These screens identified two primary factors, supersensitive-1 (Sst1) and supersensitive-2 (Sst2), that made yeast supersensitive to  $\alpha$ -factor (Chan and Otte, 1982a,b). In these initial studies, Sst1 acted as a "barrier" inhibiting the diffusion of  $\alpha$ -factor in solution (Chan and Otte, 1982b).

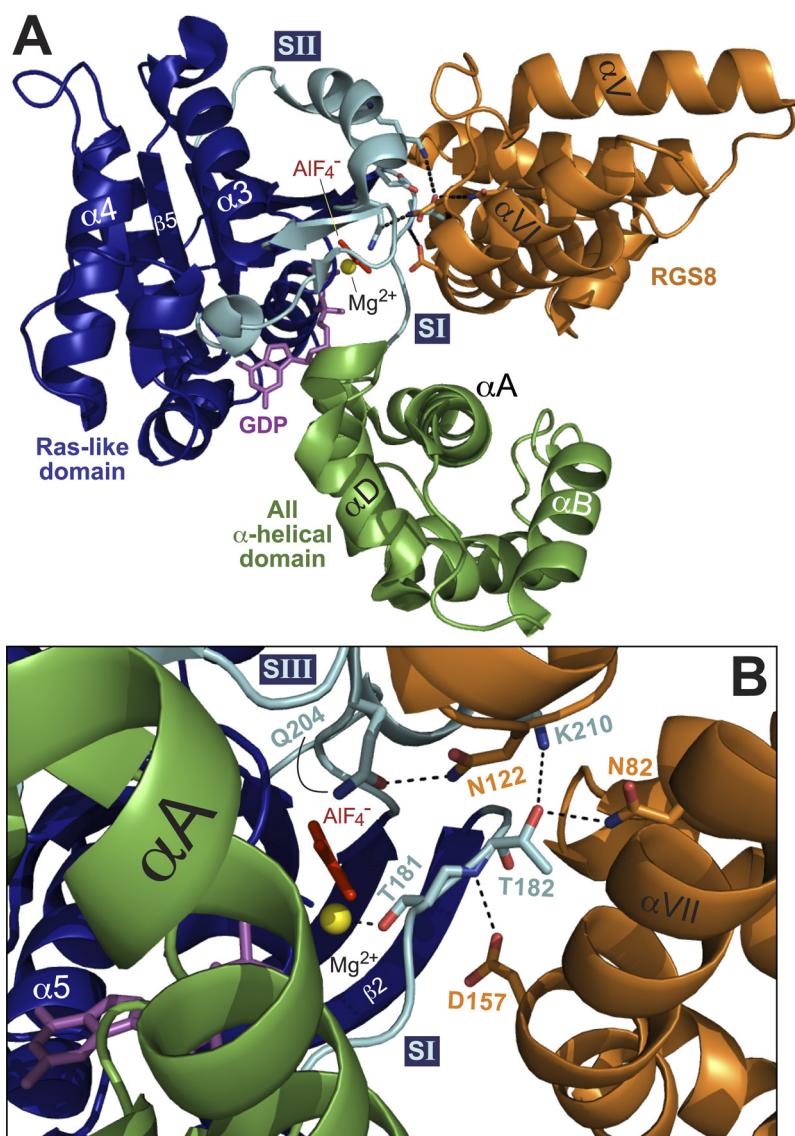


FIG. 3. RGS proteins stabilize the transition state of  $G\alpha$  subunits and coordinate the positioning of the  $G\alpha$  catalytic glutamate residue that is critical to intrinsic GTPase activity. Diagram of the RGS8/ $G\alpha_{13}$  structure [PDB number 2ODE (Soundararajan et al., 2008)], as rendered using PyMOL (Schrödinger, Inc., Portland, OR). The all-helical subdomain of  $G\alpha_{13}$  is shown in green, whereas the Ras-like nucleotide binding domain is shown in dark blue. The three flexible switch regions (SI, SII, and SIII) are highlighted in cyan. The guanine nucleotide,  $\text{AlF}_4^-$ , and  $\text{Mg}^{2+}$  are highlighted in magenta, red, and yellow, respectively, whereas RGS8 is illustrated in orange. A, the RGS8  $G\alpha$ -binding interface consists primarily of the SI and SII regions of  $G\alpha_{13}$ . B, the Asn-122 amide forms a hydrogen bond with Gln-204 of  $G\alpha_{13}$ , orienting it to help stabilize the planar leaving group, whereas the Asn-82 of RGS8 forms contacts with side-chain carbonyl of Thr-182, allowing the side-chain carbonyl to make a contact with the SII Lys-210 of  $G\alpha_{13}$ , stabilizing SI and SII in their transition state orientations. In addition, Asp-157 of RGS8 stabilizes the backbone amine of Thr-182, allowing the Thr-181 side-chain hydroxyl group to stabilize the  $\text{Mg}^{2+}$  ion (yellow).

Consistent with this initial description, Sst1 (also known as Bar1) is now known to encode an extracellular protease that degrades  $\alpha$ -factor in the environment (MacKay et al., 1988). Although the molecular details of Sst2's function remained enigmatic for 7 more years (unlike Sst1's protease activity), Sst2 was speculated to inhibit the pheromone response in the intracellular compartment (Chan and Otte, 1982b). Once the components of the pheromone pathway had been rigorously elucidated (Hartwell, 1980; Dietzel and Kurjan, 1987; Miyajima et al., 1987; Nakayama et al., 1988), work by Dohlman et al. (1995) demonstrated that the overexpression of the yeast  $G\alpha$  subunit (GPA1) suppressed the pheromone supersensitivity of Sst2

mutant yeast, but overexpression of  $G\beta$  subunit was not able to suppress the phenotype. Although these experiments were not able to demonstrate conclusively a direct binary interaction between Sst2 and GPA1, they helped establish the groundwork for discovery of a then-novel family of negative regulators of GPCR signaling by multiple groups studying multiple different systems, from *Caenorhabditis elegans* body-bending and egg-laying behaviors to human T- and B-lymphocyte immediate-early gene activation programs (De Vries et al., 1995; Berman et al., 1996; Druey et al., 1996; Hunt et al., 1996; Koelle and Horvitz, 1996; Siderovski et al., 1996; Watson et al., 1996).



This large family of G $\alpha$ -directed GAPs, the “regulators of G-protein signaling” (RGS proteins), is characterized by the presence of a nine- $\alpha$ -helix bundle that binds most avidly to the G $\alpha$  transition state for GTP hydrolysis (Tesmer et al., 1997a; Slep et al., 2001; Soundararajan et al., 2008). The nine helices are subdivided into two subdomains, the first of which is composed of helices  $\alpha$ I, II, III, VIII, and IX; the remaining subdomain comprises helices  $\alpha$ IV, V, VI, and VII, with each subdomain arranged in antiparallel helical bundles (Tesmer et al., 1997a; Slep et al., 2001; Soundararajan et al., 2008). Unlike the GAPs for small G-proteins that contribute a critical arginine (or other residue) into the active site for nucleotide hydrolysis (Vetter and Wittinghofer, 2001), RGS proteins do not contribute any single residue to the nucleotide binding pocket that is directly necessary for the catalytic mechanism (and that can be eliminated yet still retain G $\alpha$ -binding affinity). Their catalytic activity has been established by X-ray diffraction crystallography and NMR structures of isolated RGS proteins, as well as RGS protein/G $\alpha$  protein complexes (Tesmer et al., 1997a; Moy et al., 2000; Slep et al., 2001; Slep et al., 2008; Soundararajan et al., 2008; Kimple et al., 2009). RGS proteins are selective for binding to the transition state of G $\alpha$ (GTP  $\rightarrow$  GDP + P $_i$ ), which can be mimicked by G $\alpha$ -GDP bound with the planar ion aluminum tetrafluoride (AlF $_4^-$ ) (Sondek et al., 1994; Berman et al., 1996; Popov et al., 1997). Three critical contacts are formed between RGS proteins and their G $\alpha$  partners (Tesmer et al., 1997a; Slep et al., 2001; Slep et al., 2008; Soundararajan et al., 2008). The highly conserved amide of Asn-122 (Fig. 3; residue numbered as in human RGS8) forms a hydrogen bond with the critical glutamine of G $\alpha$  responsible for GTP hydrolysis (Gln-204 of G $\alpha_{i3}$ ). This helps orient the glutamine residue to stabilize the terminal phosphate that is being hydrolyzed from GTP (as mimicked by the AlF $_4^-$  ion). A second Asn (Asn-82 of RGS8) contacts the side-chain hydroxyl of a switch I threonine (Thr-182 in G $\alpha_{i3}$ ), allowing the side-chain hydroxyl to contact the switch II Lys (Lys-210 in G $\alpha_{i3}$ ). This locks switches I and II into their transition state conformations, promoting accelerated GTPase activity. In addition, an aspartate residue in the C terminus of the RGS domain (Asp-157 in RGS8), which is conserved in all RGS proteins except RGS2, serves to stabilize the backbone amine of the G $\alpha$  switch I Thr-182 (allowing the neighboring Thr-181 side-chain hydroxyl group to stabilize the Mg $^{2+}$  cation; Fig. 3).

## II. Selected Functional Vignettes among the Complement of Regulators of G-Protein Signaling

### A. The Utility of the “Regulators of G-Protein Signaling-Insensitivity” Point Mutation

The numerous contacts made by Thr-182 as described above (Fig. 3B) highlight the importance of this switch I region in G $\alpha$  in stabilizing the RGS domain/G $\alpha$  interaction and, in addition, explain the profound loss of bind-

ing and GAP activity that occurs when the neighboring glycine (Gly-183 in G $\alpha_{i3}$ ) is subtly changed to serine. This “RGS-insensitivity” point mutation (glycine to serine) was originally identified by DiBello et al. (1998) in GPA1, the G $\alpha$  subunit of the yeast *S. cerevisiae*, functions equivalently in mammalian G $\alpha$  subunits such as G $\alpha_{i1}$ , G $\alpha_o$ , and G $\alpha_q$  (Lan et al., 1998; Clark and Traynor, 2004) and has also been shown to leave all other functions of G $\alpha$  intact, including intrinsic nucleotide binding and hydrolysis activities, as well as coupling to G $\beta\gamma$ , receptor, and effectors (Lan et al., 1998; Chen et al., 2004; Day et al., 2004; Fu et al., 2004; Ikeda and Jeong, 2004). Separate mouse strains bearing this RGS-insensitivity point mutation (G184S) within their G $\alpha_o$  or G $\alpha_{i2}$  gene loci, respectively, have been generated (Fu et al., 2004; Huang et al., 2006); these mice possess select changes in various organ system functions controlled by GPCR signaling, including central nervous system, cardiovascular, and endocrine functions (Fu et al., 2006, 2007; Huang et al., 2006, 2008; Goldenstein et al., 2009; Icaza et al., 2009; Signarvic et al., 2010; Talbot et al., 2010). The most recent findings of Ruiz de Azua et al. (2010), that RGS4 is a negative regulator of M3 mAChR signaling to insulin secretion in pancreatic  $\beta$ -cells, articulate well with the findings of Huang et al. (2008) that RGS-insensitive G $\alpha_{i2}$ (G184S) knock-in mice exhibit increased glucose tolerance when on a high-fat diet.

Collectively, the results obtained thus far with the RGS-insensitivity mutation have highlighted the importance of RGS protein action on G $\alpha$  nucleotide cycling in the context of the whole organism but do not necessarily identify the specific member(s) of the RGS protein superfamily at play in these various organ systems that could be directly exploited as a drug discovery target. Thirty-seven RGS proteins are encoded by gene loci in the human genome; this collection of related proteins has been divided into 10 different subfamilies based on the relatedness of their RGS domain sequence and their multiple domain architectures (Fig. 4). Several excellent recent reviews have been published regarding the specialized physiological functions now known for many of these individual RGS proteins, including in the central nervous system (Hooks et al., 2008; Anderson et al., 2009; Sjögren and Neubig, 2010; Traynor, 2010) and in cancer (Hurst and Hooks, 2009; Sjögren et al., 2010). Thus, to complement these recent publications, we have chosen to highlight below several vignettes regarding appreciation of the roles of RGS proteins in cardiovascular and immune system functions. These highlights are in no way meant to be comprehensive but are intended to emphasize the potential for proteins from among this large family as targets for therapeutic exploitation.

The largest RGS protein subfamily is known as the R4 family and contains 10 members: RGS1, -2, -3, -4, -5, -8, -13, -16, -18, and -21. R4 family members represent some of the smallest and simplest of the RGS proteins and,

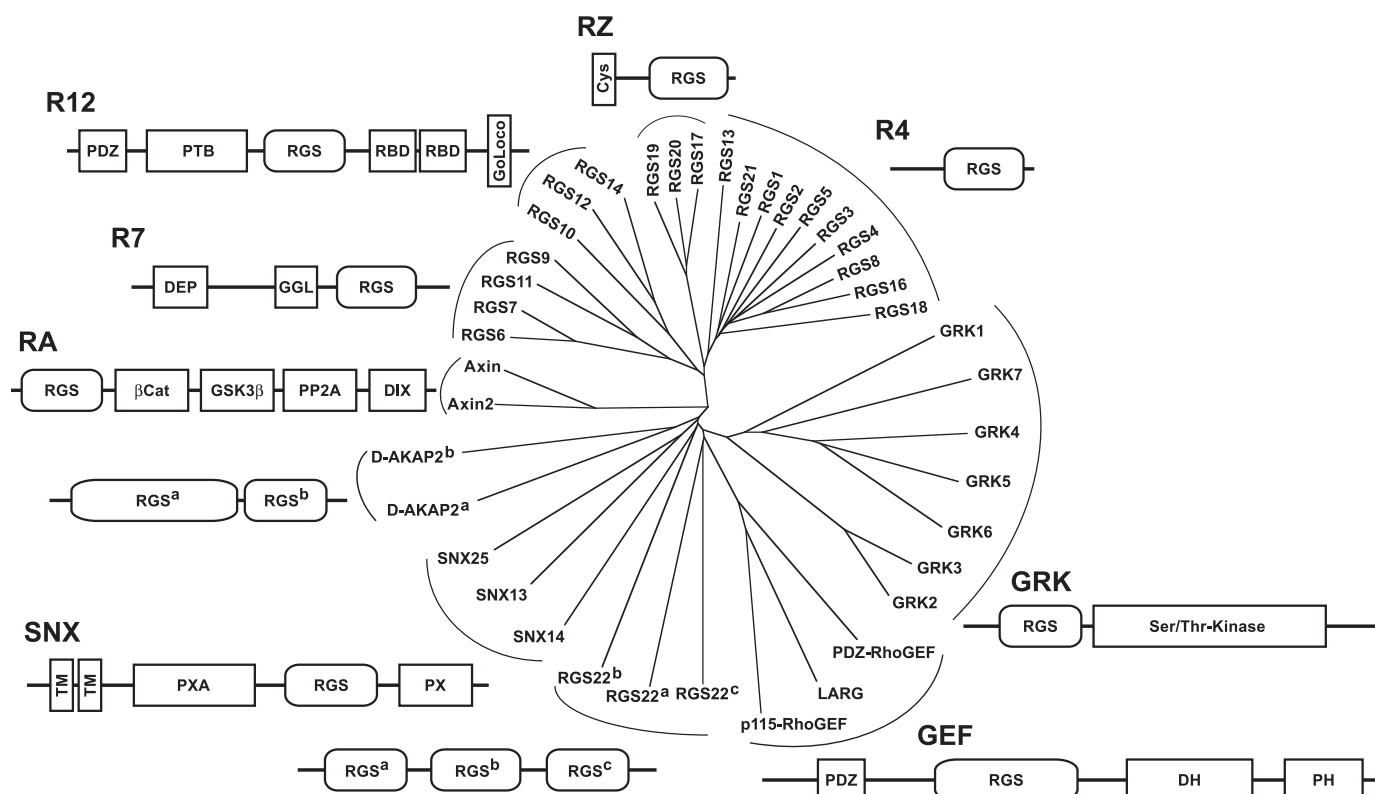


FIG. 4. Subfamily categorizations of the 37 RGS domain-containing proteins identified in humans, based on sequence similarities and domain architectures. An unrooted dendrogram was generated using ClustalW (<http://www.clustal.org>; Thompson et al., 1994) and visualized using TreeView (Page, 1996). Domain boundaries were predicted using the SMART database (Letunic et al., 2009). PDZ, PSD-95/Dlg/ZO-1 domain; PTB, phosphotyrosine-binding domain; RBD, Ras-binding domain; DEP, Dishevelled/EGL-10/Pleckstrin domain; GGL, G $\gamma$ -like domain;  $\beta$ Cat,  $\beta$ -catenin interaction region; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$  interaction region; PP2A, protein phosphatase 2A; DIX, Dishevelled interaction region; SNX, sorting nexin; TM, transmembrane domain; PXA, domain associated with a PX domain; PX, p40/p47-Phox homology domain; DH, Dbl-homology domain; PH, Pleckstrin-homology domain.

with the exception of RGS3 (Kehrl et al., 2002), consist of a single RGS domain with minimal additional amino acids at their N and C termini. With the exception of RGS2 (Heximer et al., 1997b; Kimple et al., 2009), members of the R4 family accelerate the hydrolysis of GTP by both G $_i$  and G $_q$  family G $\alpha$  subunits (Arshavsky et al., 2002; Soundararajan et al., 2008). With little biochemical selectivity between G $\alpha_{11}$ , G $\alpha_{12}$ , G $\alpha_{13}$ , G $\alpha_o$ , and G $\alpha_q$  substrates, and no additional regions containing obvious domain structures, members of this subfamily would be predicted to act promiscuously as negative regulators of G $_i$ - and G $_q$ -coupled GPCRs; however, early work by Zeng et al. (1998) and Xu et al. (1999) demonstrated that the N termini of R4 family members, outside of the canonical RGS domain, can provide specificity to the *in vivo* potency of R4 protein GAP activity on specific receptors. Although it is not entirely clear how these terminal extensions on R4 family RGS domains enhance specificity, it has been suggested that selectivity toward particular GPCR signaling pathways is mediated by the binding of adaptor proteins such as spinophilin (Wang et al., 2005) or through direct interactions with GPCRs (Xu et al., 1999; Bernstein et al., 2004; Wang et al., 2009). In addition to receptor specificity that is dependent on the N terminus, point mutations have been identified that

affect the overall *in vivo* stability of RGS protein in overexpression studies (Bodenstein et al., 2007). The physiological relevance of the N terminus in regulating degradation of RGS proteins is supported by the identification of a hypertensive cohort that had a single nucleotide polymorphism in the gene loci of RGS2 that results in a Gln-2-Leu mutation resulting in destabilization of RGS2 and subsequent hypertension (Yang et al., 2005).

#### B. Regulators of G-Protein Signaling 1, 2, and 13 in Immune System Regulation

Although the biochemical role of RGS proteins as GTPase-accelerating proteins has been well characterized (Berman et al., 1996; Apanovitch et al., 1998; Snow et al., 1998a), and the cellular role of RGS proteins in attenuating GPCR-mediated signaling is also established (Doupnik et al., 1997; Saitoh et al., 1997; He et al., 1998; Lambert et al., 2010), it has remained a more arduous task to characterize the specific roles of RGS proteins in the context of whole-organism homeostasis and pathophysiology. Several mouse knockout strains of R4 family members have been published to date that have shed light on this issue. Using an RGS1-deficient mouse strain, Moratz et al. (2004) reported on the im-

portance of RGS1 in negatively regulating CXCR4 and CXCR5 chemokine receptor signaling in B-lymphocytes and the necessity of RGS1 expression for the proper maturation of germinal centers. Bansal et al. (2008) identified a different immune system phenotype in RGS13-deficient mice. Their research demonstrated that loss of RGS13 resulted in increased mast-cell degranulation and anaphylaxis. Deregulation typically occurs when the antigen-bound IgE interacts with the IgE receptor (Fc $\epsilon$ RI), which is not a G-protein coupled receptor (Kinet, 1999; Rivera and Gilfillan, 2006; Metz and Maurer, 2007; Gilfillan and Rivera, 2009); however, Bansal et al. (2008) showed that RGS13 acts in a GAP-independent manner to negatively regulate IgE-mediated degranulation. They determined that the amino-terminal 51 amino acids (outside of the RGS domain) bind the p85 $\alpha$  regulatory subunit of phosphatidylinositol-3-OH kinase, preventing the activation of antigen-induced phosphatidylinositol 3-kinase-mediated degranulation.

In addition to their role in B cells and mast cells, RGS proteins are also important in other immune system functions. RGS2 was originally identified as “G0-switch gene-8” (*G0S8*), a gene up-regulated upon activation of blood mononuclear cells by the plant lectin concanavalin A or treated with cycloheximide (Siderovski et al., 1990, 1994; Heximer et al., 1997a). In studies of RGS2-deficient mice, these authors were able to show that, unlike RGS1-deficient mice, RGS2-deficient mice have normal B cell quantities and differentiation; however, RGS2-deficient mice were unable to mount a robust T-cell mediated immune response. RGS2-deficient T cells, compared with wild-type T cells, were impaired in their ability to proliferate in response to T-cell receptor engagement, to treatment with phorbol 12-myristate 13-acetate and Ca<sup>2+</sup> ionophores, or to anti-CD3 $\epsilon$  cross-linking, with or without CD28 coreceptor engagement (Oliveira-Dos-Santos et al., 2000). In addition, T cells had an impaired ability to secrete interleukin-2 (IL-2) in response to an immune challenge. Although the diminished IL-2 secretion could account for the decreased proliferation (Cantrell and Smith, 1984), supplementation of IL-2 was unable to stimulate T-cell proliferation to levels seen in wild-type T-cells, suggesting that the observed phenotype was not the result of decreased IL-2 production (Oliveira-Dos-Santos et al., 2000).

### C. Regulators of G-Protein Signaling 2, 4, 5, and 6 in Cardiovascular System Regulation

In addition to their roles in modulating immune responses, R4 family RGS proteins have been shown to regulate cardiovascular development and physiology (Manzur and Ganss, 2009). Maintenance of vascular perfusion of the entire body is a delicate balance. If arterial pressure is significantly decreased, regional hypoxia and coagulative necrosis will destroy tissue; however, if arterial pressure is elevated, the risk of

heart failure, stroke, and kidney disease are dramatically increased (Harris et al., 2008). One crucial component to maintaining normotension is vascular resistance, which is dynamically modulated by the vascular smooth muscle that lines blood vessels. Given that vessel resistance (R) is inversely proportional to vessel radius (r) ( $R \propto 1/r^4$ ), small changes in the lumen of a vessel can have dramatic change in the resistance and thus the vascular pressure (Levy et al., 2007). GPCRs are crucial mediators of vasodilation and vasoconstriction (Brinks and Eckhart, 2010); for example, angiotensin II, norepinephrine, vasopressin, and acetylcholine cause vasoconstriction by activating GPCRs coupled to G $\alpha_{q/11}$ , which subsequently activate PLC.

The unique in vitro specificity of RGS2 toward G $\alpha_q$  (Heximer et al., 1997b; Kimple et al., 2009) and the multitude of G $\alpha_q$ -coupled GPCRs that control vasoconstriction suggest that RGS2 might be an important negative regulator in inhibiting vascular smooth muscle constriction. The clearest demonstration of the importance of RGS2 in regulating blood pressure came from studies of RGS2-deficient mice (Oliveira-Dos-Santos et al., 2000) that characterized RGS2-deficient animals as having constitutive hypertension (Heximer et al., 2003; Tang et al., 2003; Gu et al., 2008). Further evidence supporting the role of RGS2 in maintaining normostatic blood pressure has come from human population-based studies of hypertensive cohorts. These studies have identified single nucleotide polymorphisms within the coding region of RGS2 that result in a decrease of proper localization of RGS2 to the plasma membrane and a resultant decrease of its inhibitory influence on G $\alpha_q$ -mediated vasoconstrictive hormone signal transduction (Yang et al., 2005; Gu et al., 2008). Promoter polymorphisms in the *RGS2* gene locus and renal actions of RGS2 have also been highlighted as contributing factors to the intrinsic homeostatic and extrinsic therapeutic control of blood pressure maintenance (Gurley et al., 2010; Semplicini et al., 2010; Sugimoto et al., 2010). In addition, the RGS2-deficient mouse was instrumental in demonstrating the role for RGS2 protein action in cardiac compensation to blood pressure overload and the antihypertrophic effects of PDE5 inhibition (Takimoto et al., 2009).

Although the loss of RGS2 results in constitutive hypertension, mice deficient in RGS5, which is highly expressed in pericytes, exhibit constitutive hypotension, suggesting that RGS5 might be a critical negative regulator of vasodilatory signaling or vascular development (Cho et al., 2008). Although the mechanism by which RGS5 assists in the maintenance of normal blood pressure remains to be established, RGS5 has been observed to be highly expressed in vascular smooth muscle and pericytes (Bondjers et al., 2003; Cho et al., 2003). The high expression of RGS5 in pericytes of angiogenic tumor vessels (Berger et al., 2005) led Hamzah et al. (2008) to cross *rgs5*-deficient mice with a tumorigenic



mouse strain that rapidly develops insulinomas. The tortuosity and dilated nature of the vessels characteristic of insulinomas derived from wild-type mice were lost in the RGS5-deficient line. Instead, the blood supply in RGS5-deficient insulinomas had a regular appearance with normal branching reminiscent of normal developmental angiogenesis in organs (Ryschich et al., 2002; Hamzah et al., 2008). Although the precise role that RGS5 is playing in the neovascularization of tumors is unclear, it is apparent that RGS5 is a critical component of maintaining normal blood pressure and proper angiogenesis.

An additional RGS-deficient mouse model identified to have a cardiovascular phenotype is the RGS4-deficient mouse (Grillet et al., 2005). In this mouse, the RGS4 promoter was used to drive the expression of  $\beta$ -galactosidase, thereby allowing expression of the *Rgs4* gene to be characterized by histochemical staining. The authors reported high levels of expression of the *Rgs4* gene locus in the sinoatrial node (Cifelli et al., 2008), an anatomical region of the heart that serves to initiate and control the timing of cardiac contractions (Efimov et al., 2010). In the absence of RGS4 expression, basal heart rates were identical to those of wild type; however, upon activation of the parasympathetic system by the administration of carbachol, RGS4-deficient mice had an exaggerated decrease in heart rate compared with wild-type control mice (Cifelli et al., 2008). In examining isolated sinoatrial myocytes from RGS4-deficient and control animals, Cifelli et al. (2008) also observed a decreased frequency of action potential initiation in response to activation of the parasympathetic nervous system by carbachol administration.

More recently, RGS6-deficient mice have been generated by independent groups, highlighting a role for this particular R7 family RGS protein in modulating parasympathetic regulation of heart rate (Posokhova et al., 2010; Yang et al., 2010). Reduction in heart rate by parasympathetic innervation is known to involve the sequential activation of m2 muscarinic acetylcholine receptors (M2-mAChR), heterotrimeric G proteins of the  $G_{i/o}$  subfamily, and the atrial potassium channel  $I_{KACH}$ , composed of GIRK1 and GIRK4 channel subunits. RGS6 deficiency was found to yield a profound delay in M2-mAChR/ $I_{KACH}$  deactivation kinetics in both adult sinoatrial nodal cells and neonatal atrial myocytes; mice lacking RGS6 have a mild resting bradycardia and altered heart rate responses to pharmacological manipulations consistent with enhanced M2-mAChR/ $I_{KACH}$  signaling. This function for RGS6 is consistent with its obligate association with the unique  $G\beta_5$  subunit (Posner et al., 1999; Snow et al., 1999; Zhang and Simonds, 2000) (an association shared with the other R7 family members RGS7, -9, and -11 (Cabrera et al., 1998; Snow et al., 1998b; Makino et al., 1999); it has recently been suggested that this  $G\beta_5$  association can recruit R7 family RGS proteins to GIRK channels and

thereby regulate the kinetics of neuronal signaling through the receptor/G-protein/channel axis (Xie et al., 2010).

### III. Pursuing Chemical Probes for Regulators of G-Protein Signaling GTPase-Accelerating Protein Activity

#### A. GTPase-Accelerating Protein Activity as the Sole Determinant of Regulator of G-Protein Signaling Action on G-Protein-Coupled Receptor Signaling Sensitivity and Kinetics

As the above passage regarding R7 family members illustrates, many RGS proteins bear additional protein-protein interaction domains beyond their signature RGS domain with  $G\alpha$  GAP activity (see Fig. 4). As another example, two of the R12 family members (RGS12 and RGS14) contain a second,  $G\alpha$ -GDP-interaction motif [alternatively known as the GoLoco motif or GPR motif (Ponting, 1999; Kimple et al., 2001; Lanier, 2004)] and additionally coordinate components of the Ras/Raf/mitogen-activated protein kinase signaling pathway (Traver et al., 2000; Mittal and Linder, 2006; Willard et al., 2007, 2009; Shu et al., 2010). These additional interaction points, and their logical implication of signaling protein "physical scaffolding" (e.g., Benians et al., 2005), have been thought to underlie a "paradoxical" function of RGS proteins observed in reconstituted cellular systems early after their initial discovery: namely, acceleration of the onset of GPCR signaling without a demonstrable change in activating ligand potency per se (Doupnik et al., 1997; Saitoh et al., 1997; Zerangue and Jan, 1998). To address this long-standing paradox, Lambert et al. (2010) recently revisited the cellular reconstitution studies of Doupnik et al. (1997) and Saitoh et al. (1997) but used a rapid, bioluminescence-based measure of  $G\beta\gamma$  liberation from activated receptors (rather than whole-cell electrophysiological measurements of GIRK channel activity) and employed the classic  $G\alpha$  "RGS-insensitivity" point mutation (section II.A) in combination with a second, *cis*-acting "fast-hydrolysis" point mutation that accelerates the intrinsic GTPase activity of  $G\alpha$  subunits (Thomas et al., 2004) (schematized in Fig. 5). X-ray diffraction crystallography was performed to validate that the *cis*-acting fast-hydrolysis point mutation poises the  $G\alpha$  in a pretransition state for GTP hydrolysis and thereby accelerates what normally the *trans*-acting RGS domain performs for the  $G\alpha$  (Lambert et al., 2010). In both yeast pheromone response assays and measurements of reconstituted neurotransmitter GPCR signaling, observed increases in receptor sensitivity to respective activating ligands (i.e., leftward shifts in  $EC_{50}$ ) upon using "RGS-insensitive"  $G\alpha$  subunits (e.g., Fig. 5B) (Lambert et al., 2010) reaffirmed the prevailing notion that the critical function of endogenous RGS proteins is to regulate GPCR agonist sensitivity (Neubig and Siderovski, 2002; Ishii and Kurachi, 2003). Moreover, adding the fast-hydrolysis mu-

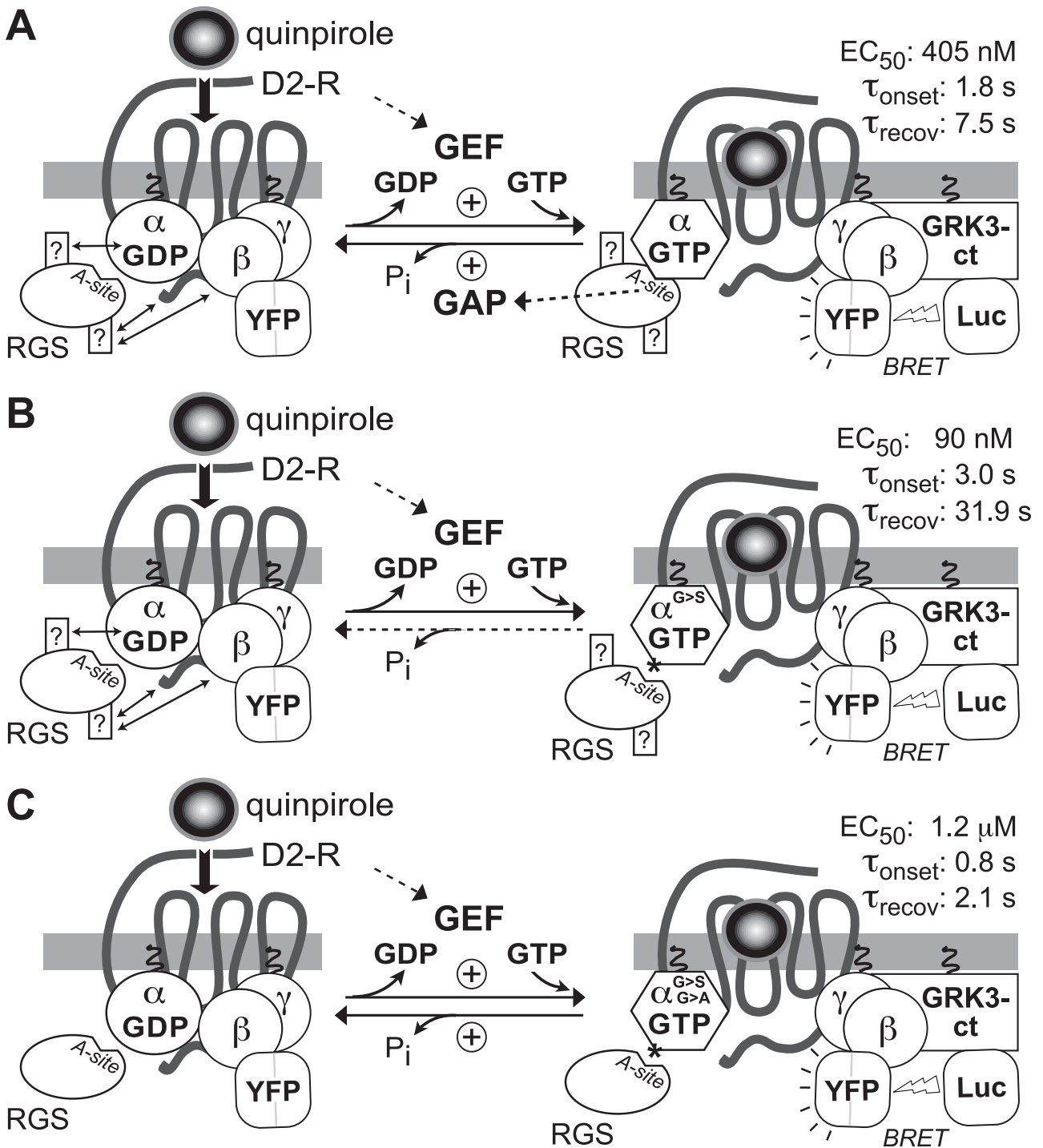


FIG. 5. Use of the RGS-insensitivity (“G>S”) and fast-hydrolysis (“G>A”) point mutants of  $G\alpha$  in establishing the central role of GTPase acceleration by RGS proteins in their modulatory actions on GPCR signaling kinetics and sensitivity. A cell-based system employing  $G\alpha$  reconstitution, along with a temporally sensitive and -reversible measure of  $G\alpha\beta\gamma$  activation, was established to interrogate whether “non-GAP” activities of RGS proteins (illustrated as question marks in rectangles) exist that influence the kinetics of signal onset ( $\tau_{onset}$ ) and receptor sensitivity ( $EC_{50}$  for agonist) beyond the GAP activity embodied by the RGS domain A-site. In this experimental system set up by Lambert et al. (2010), G-protein heterotrimer activation, by the binding of the agonist quinpirole to the dopamine D2 receptor (“D2-R”), increases bioluminescence resonance energy transfer (BRET) between the  $G\beta\gamma$ -binding reporter protein masGRK3ct-Rluc8 (GRK3-ct Luc) and  $G\beta_1\gamma_2$ -Venus (YFP). Human embryonic kidney 293 cells were pretreated with pertussis toxin to inactivate native  $G\alpha_{i/o}$  subunits, and so quinpirole responses were mediated by heterotrimers composed of ectopically expressed, pertussis toxin-insensitive  $G\alpha_{\alpha A}$  (wt; A), PTX- and RGS-insensitive  $G\alpha_{\alpha A}$  (G184S; called “G>S” in B and denoted with an asterisk), or additionally containing the fast hydrolysis switch II point mutation (G203A; called “G>A” in C). The glycine-to-alanine switch II mutation (G203A, “fast-hydrolysis”) was seen to blunt agonist sensitivity [ $EC_{50}$  of 1.2  $\mu$ M, nearer to that of wild-type  $G\alpha_{\alpha A}$  ( $EC_{50}$  of 405 nM)] over the more sensitive responses mediated by the use of RGS-insensitive  $G\alpha_{\alpha A}$  alone (G184S;  $EC_{50}$  of 90 nM). In addition, normalized BRET plotted against time during sequential addition of the agonist quinpirole (30  $\mu$ M) and the antagonist haloperidol (10  $\mu$ M) indicated that the fast-hydrolysis mutation (“G>A”) restored rapid onset and recovery kinetics ( $\tau_{onset}$  and  $\tau_{recov}$  values nearer to that of wild-type  $G\alpha_{\alpha A}$  use) over the more languid responses mediated by the use of RGS-insensitive (“G>S”)  $G\alpha_{\alpha A}$  alone. These results served to negate the necessity of evoking non-GAP activities of RGS proteins to explain earlier observations of RGS proteins leading to accelerated GPCR signaling onset without demonstrable changes in activating ligand potency (Doupnik et al., 1997; Saitoh et al., 1997; Zerangue and Jan, 1998), hence the removal of the question marks in rectangles from the RGS protein in the final panel.

tation on top of the “RGS-insensitive” mutation restored normal agonist sensitivity and kinetics of both signal onset and decay to that observed from wild-type  $G\alpha$  acted on by endogenous RGS proteins (Lambert et al., 2010); indeed, addition of faster-than-intrinsic GTPase activity by the *cis*-acting mutation produced slightly faster onset and recovery kinetics than the wild-type situation as well as a rightward shift in  $EC_{50}$  (e.g., Fig. 5C). This restoration of “normalcy” to GPCR signaling by the  $G\alpha$  fast-hydrolysis mutant strongly suggests that the critical function for RGS proteins in regulating kinetics and (ultimately) agonist sensitivity of GPCR signaling comes solely from GTPase acceleration (Lambert et al., 2010), suggesting that kinetic (and not physical) events are the likely mechanism of RGS protein action in this context (Zhong et al., 2003; Turcotte et al., 2008) and supporting the idea (Neubig and Siderovski, 2002; Chasse and Dohlman, 2003; Cho et al., 2004; Liebmann, 2004) that modulating the GAP activity of RGS domains by small molecules will be a valuable new approach in changing GPCR responsiveness in pathophysiological settings.

### B. The Potential for Allosteric Control over Regulator of G-Protein Signaling GTPase-Accelerating Protein Activity

As previously stated, GPCRs are the single largest target for currently prescribed pharmaceuticals, and RGS proteins are potent negative regulators of GPCR-mediated signaling. RGS proteins thus provide an attractive target to either modulate the action of currently prescribed pharmaceuticals or modulate tonic signaling in a pathway-dependent manner (Neubig and Siderovski, 2002; Cho et al., 2004; Liebmann, 2004; Riddle et al., 2005). The specific targeting of RGS proteins by small molecules is still in its infancy (see discussion in section III.C), yet the “druggability” of RGS domains was suggested early on the basis of observations from the first crystal structure of the RGS4/ $G\alpha_{i1}$  complex (Tesmer et al., 1997a), coupled with discovery of the RGS-insensitivity point mutation on  $G\alpha$  (DiBello et al., 1998). As shown in Fig. 6, threonine-182 of switch I within  $G\alpha_{i1}$ , among all switch region contacts, experiences the largest change in accessible surface area upon complex formation (Tesmer et al., 1997a) by becoming buried within a depression on the  $G\alpha$ -interacting “A-site” of the RGS4 RGS domain; the critical nature of this burial of threonine-182 is underscored by the profound loss of GAP activity upon subtle substitution of the neighboring glycine (Gly-183) within  $G\alpha_{i1}$  to serine. Thus, early speculations have been made (e.g., Neubig and Siderovski, 2002) that an inhibitor binding within this pocket of the RGS domain should block the Thr-182 interaction and thus abrogate the  $G\alpha$ /RGS domain binding event.

Although a small molecule that binds to the A-site depression on an RGS domain and blocks its interaction with  $G\alpha$  would be an invaluable proof of principle for the

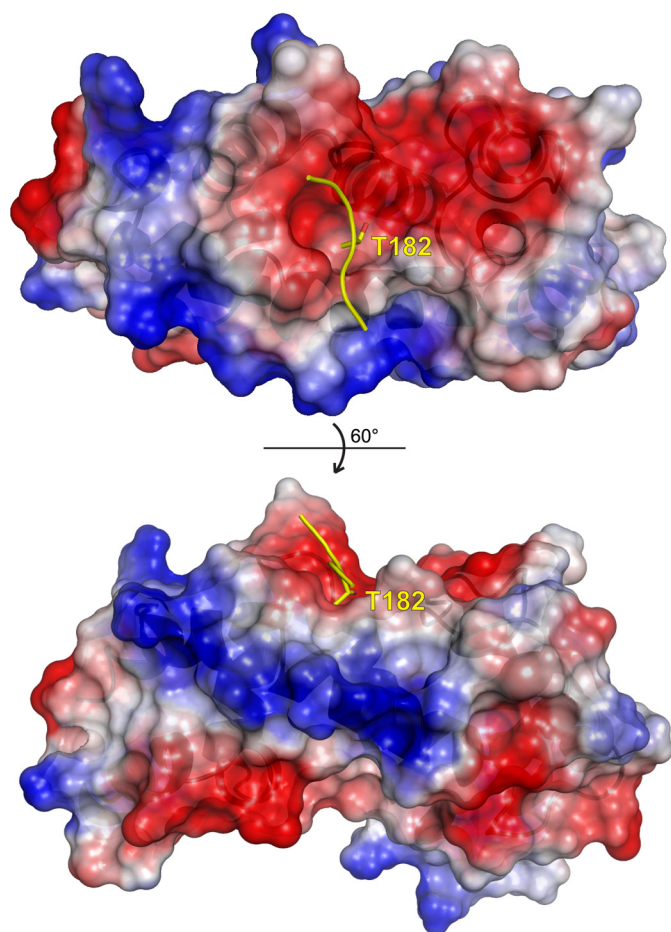


FIG. 6. The  $G\alpha_{i1}$  interaction surface (“A-site”) of the RGS4 RGS domain contains a charged depression. Electrostatic surface rendering of RGS4 (PDB number 1AGR; potentials contoured at  $\pm 5$  kT/e), highlighting the electronegative region (red) into which the threonine-182 residue of  $G\alpha_{i1}$  is buried. Electropositive potential is highlighted in blue.

“druggability” of RGS proteins, it would be equally useful to have a small molecule that could allosterically enhance the GAP function of endogenous RGS proteins [i.e., in pathological states in which an enhancement in RGS protein GAP activity is desired; for example, as believed to be the case with RGS4 in schizophrenia (Mirnics et al., 2001; Volk et al., 2010)]. Bioinformatic methods (Sowa et al., 2000) and mutagenesis (Popov et al., 2000) have implicated a region between helices IV and V in RGS4 as an allosteric site on the RGS domain responsible for the influences of phosphatidylinositol-3,4,5-trisphosphate ( $PIP_3$ ) and  $Ca^{2+}$ /calmodulin (CaM) on GAP activity. This allosteric site (“B-site”), flanked by key basic residues (Fig. 7), is distinct from the  $G\alpha$ -interacting A-site and, upon binding of  $PIP_3$ , decreases GAP activity *in vitro*. In a  $Ca^{2+}$ -dependent manner, CaM can competitively inhibit  $PIP_3$ -mediated GAP inhibition (Popov et al., 2000; Ishii et al., 2001; Ishii et al., 2002). Modulation of GAP activity via  $PIP_3$  and  $Ca^{2+}$ /CaM is also seen in cellular assays using cardiac myocytes in electrophysiological recordings of GPCR signaling to ion channel gating (Ishii et al., 2002, 2005; Ishii



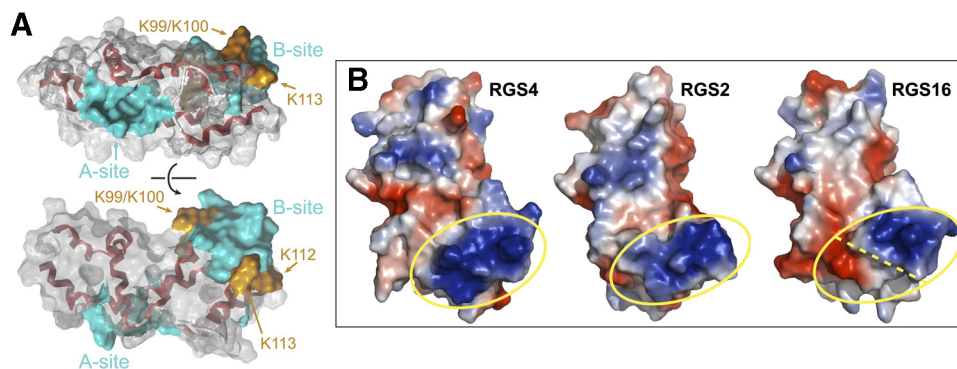


FIG. 7. Predicted structural determinants within RGS4 that enable allosteric control over G $\alpha$ -directed GAP activity by PIP<sub>3</sub> and Ca<sup>2+</sup>/CaM. A, structural coordinates of the RGS domain of RGS4 were rendered in PyMOL using data from the RGS4/G $\alpha_{i1}$ -GDP-AlF<sub>4</sub><sup>-</sup> complex [PDB number 1AGR (Tesmer et al., 1997a)]. The conserved RGS domain fold is composed of nine  $\alpha$ -helices (displayed in red). Orange regions depict lysines thought to be required for PIP<sub>3</sub> binding, whereas solid cyan areas depict the proposed A- and B-sites. Rotation about the horizontal axis by 90° is shown in the lower panel. B, electrostatic surface rendering of RGS4 (PDB number 1AGR), RGS2 (PDB number 2AF0), and RGS16 (PDB number 2BT2) using PyMol, highlighting the electropositive (blue) region considered the putative CaM-binding B-site within RGS2 and RGS4 (yellow oval). RGS16, shown to be insensitive to PIP<sub>3</sub> and CaM modulation (Tu and Wilkie, 2004), has less electropositive potential in this region, as well as a patch of electronegative potential (red).

and Kurachi, 2004; Ishii et al.). Based on sequence conservation in the B-site, it is possible that the allosteric modulation of RGS1, -2, -10, and -19 also occurs (Tu and Wilkie, 2004); however, this remains to be experimentally validated. This B-site could potentially be exploited by small molecules to either mimic the effect of PIP<sub>3</sub> in inhibiting GAP activity or the effect of Ca<sup>2+</sup>/CaM in preventing the allosteric inhibition of GAP activity by PIP<sub>3</sub>.

### C. Current State of Identifying Chemical Probes that Modulate Regulator of G-Protein Signaling Protein Activity

The most direct means to disrupt the RGS domain/G $\alpha$  interaction is via point mutations on either protein's interaction surface. Single amino acid substitutions on either side of the interface can completely abolish binding and the catalytic activity of RGS proteins (e.g., DiBello et al., 1998; Srinivasa et al., 1998; Wieland et al., 2000; Willard et al., 2005; Kimple et al., 2009). The ability to disrupt this large protein/protein interface (1290 Å<sup>2</sup>; Tesmer et al., 1997a) with single point mutations suggests that small perturbations in the RGS domain A-site surface, such as those caused by a bound small molecule, could have dramatic results in inhibiting RGS domain GAP activity.

Measuring RGS domain-mediated acceleration of GTP hydrolysis in vitro, as part of a compound library screening campaign, for example, is complicated by the fact that GDP release by G $\alpha$  (not GTP hydrolysis per se) is the rate-limiting step in steady-state nucleotide cycling by G $\alpha$  subunits (Higashijima et al., 1987; Ross, 2002). Thus, to quantify the effects of RGS domain GAP activity, one typically preloads the G $\alpha$  subunit with radiolabeled GTP (in the absence of the critical cofactor for hydrolysis: Mg<sup>2+</sup>) and then measures one round of hydrolysis in a so-called "single-turnover" assay (e.g., Berman et al., 1996; Hunt et al., 1996; Watson et al., 1996;

Snow et al., 1998a). This experimental design requires one to establish a pool of G $\alpha$ -([ $\gamma$ -<sup>32</sup>P]GTP), initiate the assay at time 0 with the addition of Mg<sup>2+</sup>, sample aliquots from this reaction over time, precipitate all unhydrolyzed [ $\gamma$ -<sup>32</sup>P]GTP with charcoal, separate the charcoal, and then quantify the inorganic <sup>32</sup>P-phosphate that was produced (and that resides in the supernatant) using liquid scintillation counting. This cumbersome assay design is not readily amenable to rapid automation, so alternative, fluorescence-based assays for RGS domain GAP activity have been developed that should be more suitable for high-throughput screening (HTS) of compound libraries (e.g., Willard and Siderovski, 2004; Willard et al., 2004a, 2005; Roman et al., 2007, 2009; Blazer et al., 2010).

1. *Pharmaceutical Company Activities Published in the Literature.* Wyeth Laboratory has published a yeast two-hybrid assay-based screening method for identifying RGS4 and RGS20 inhibitors (Nieuwenhuijsen et al., 2003; Wang and Young, 2004). Although their screen was reported to have identified small molecule inhibitors, these compounds have not yet been made public. In a functional screen to identify novel treatments for urinary incontinence using ex vivo rat bladder smooth muscle cultures, a Bristol-Myers Squibb group identified two compounds (BMS-192364 and BMS-195270) that had no known molecular target yet resulted in relaxation of bladder tone (Fitzgerald et al., 2006). Using a nematode genetics approach to identifying the target of these two drugs, this group concluded that these two compounds targeted the G $\alpha$ /RGS domain interaction and specifically locked the pair in an unproductive complex (Fitzgerald et al., 2006). There is precedence for such a proposed mechanism of action, given that brefeldin A, a naturally occurring antibiotic, can trap the Ras-family GTPase ARF1 in an unproductive complex with the ARF1 GEF, Sec7 (Mossessova et al., 2003). However, no confirmatory report has yet appeared in the literature

indicating that these two BMS compounds directly target the  $G\alpha$ /RGS domain interaction.

**2. Academic Laboratory Activities with Binding Assays.** In addition to the efforts that are ongoing by the pharmaceutical industry, academic laboratories have been developing novel high-throughput screening assays for the RGS domain/ $G\alpha$  interaction target and searching for small-molecule modulators of RGS protein GAP activity. A high-throughput flow cytometry method has been described for screening for small molecules that can disrupt the binding of RGS proteins to  $G\alpha$  subunits (Roman et al., 2007, 2009). This elegant assay design uses fluorescently labeled  $G\alpha_0$  protein and a LumAvidin microsphere-coupled RGS protein to look for compounds that disrupted their interaction. The advantage of this assay is the ability to multiplex different biotinylated RGS proteins to different LumAvidin microspheres (Roman et al., 2007, 2009). Results of an initial “in house” screening of a small, ~3000-compound collection from ChemBridge have been published (Roman et al., 2007); a single compound from this collection [methyl-*N*-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfinimidoate (CCG-4986)] was reported to inhibit the primary *in vitro* RGS4/ $G\alpha_0$  binding assay as well as RGS4-mediated acceleration of  $G\alpha_0$  GTPase activity in the *in vitro* single-turnover assay format. This compound was reported to lack activity in assays of intact, RGS4-transfected cells but was shown to work on permeabilized C6 glioma cells in potentiating [D-Ala<sup>2</sup>,*N*-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin-mediated inhibition of forskolin-stimulated cAMP production (i.e., negating the inhibitory influence of added recombinant RGS4 on activated,  $G_{i/o}$ -coupled  $\mu$ -opioid receptor signaling to the inhibition of adenylyl cyclase).

The lack of effect of CCG-4986 on intact cellular GPCR signaling was originally interpreted as reflecting a lack of cell permeability by the compound (Roman et al., 2007). This issue of cell permeability is a general concern for any future chemical probes identified as specific to the  $G\alpha$ /RGS domain interaction because, unlike the orthosteric and allosteric binding sites of GPCRs (May et al., 2007; Smith et al., 2011), the RGS protein target is obviously intracellular. However, subsequent work with this particular compound suggested that the lack of action of CCG-4986 on intact cells is more likely to be due to its sensitivity to a reducing environment (e.g., as is present inside the cytosol of intact, nonpermeabilized cells). CCG-4986 was thus categorized as a generic, thiol-reactive compound likely to be ill-suited for further development as a proof-of-principle chemical probe for modulating RGS domain GAP activity in a cellular context (Kimple et al., 2007); results from mutagenesis and mass spectrometry studies have suggested that CCG-4986 works in an indiscriminate fashion, covalently modifying more than one cysteine within recombinant RGS4 (including Cys-132, present in the  $G\alpha$ -binding A-site) and thereby blocking the binding of its  $G\alpha$  substrate by steric hindrance (Fig. 8).

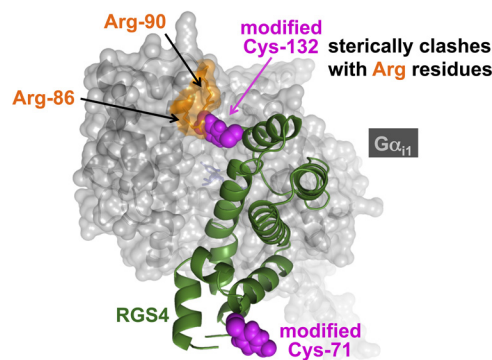


FIG. 8. CCG-4986 is a generic thiol-reactive compound and should not be considered an RGS4-selective GAP-inhibitory drug. Mass spectrometry data from recombinant RGS4 (green) incubated with CCG-4986 (Kimple et al., 2007) indicates that at least two, surface-exposed cysteine residues (Cys-71 and Cys-132) become covalently modified with a 153-Da moiety (magenta) derived from the 4-nitrobenzenethiol radical liberated from CCG-4986. One of these positions (Cys-132) is near two arginines (Arg-86, Arg-90; orange) within the all  $\alpha$ -helical domain of the  $G\alpha$  subunit (rendered here in a gray, space-filling translucent cloud overlying the  $G\alpha$  ribbon trace). Figure was rendered in PyMol based on the structural coordinates of the RGS4/ $G\alpha_{11}$ -GDP-AlF<sub>4</sub><sup>-</sup> complex (PDB id 1AGR).

More recently, a different binding assay (involving time-resolved FRET with terbium-labeled  $G\alpha_0$  and Alexa Fluor 488-labeled RGS4) was employed to identify two additional candidate RGS4 inhibitory compounds: (2*E*)-2-(1,3-benzothiazol-2-yl)-3-[9-methyl-2-(3-methylphenoxy)-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-3-yl]prop-2-enenitrile (CCG-63802) and (2*E*)-2-(1,3-benzothiazol-2-yl)-3-[9-methyl-2-(4-fluorophenoxy)-4-oxo-4*H*-pyrido[1,2-*a*]pyrimidin-3-yl]prop-2-enenitrile (CCG-63808) (Blazer et al., 2010). These two compounds are highly related and share a soft electrophilic functionality ( $\alpha,\beta$ -unsaturated nitrile) that probably explains their cysteine dependence for *in vitro* activity on RGS4 (akin to that of CCG-4986) (Blazer et al., 2010); no congeners of CCG-63802 and CCG-63808 have yet been reported to have activity in RGS protein inhibition without also having this reactive acrylonitrile (vinyl cyanide) group. The likely reactive nature of these two compounds complicates their further development as an RGS domain-selective probe in a cellular context. To quote a recent review by Frye on what constitutes a high-quality chemical probe worthy of further pursuit in cell-based assays: “[E]lectrophiles... must be credentialed with extensive mechanistic studies to demonstrate specificity” (Frye, 2010). However, further *in vitro* work with CCG-63802 and CCG-63808 could provide valuable insight into what one should consider “druggable” targets within the RGS domain, especially if structural studies such as X-ray crystallography or NMR spectroscopy could establish the disposition of these compounds within the RGS domain to high-resolution. This also holds true for the compound 4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione (CCG-50014) (Blazer et al., 2011), the most recent cysteine-reactive chemical probe identified as a covalent modifier and GAP-activity inhibitor of RGS4 and RGS8; a high-resolution structure of one of

these RGS protein targets in complex with CCG-50014 would hopefully support existing computational docking studies (Blazer et al., 2011) that this compound inhibits GAP activity through direct actions on the allosteric B-site of the RGS domain (section III.B).

Both the Wyeth yeast two-hybrid screen and the fluorescence-based binding assays described previously are unable to measure the actual catalytic activity of RGS domains *in vitro*. Instead, it has been common practice in RGS protein assay design and high-throughput screens to use binding of G $\alpha$  to the RGS domain as a surrogate for GAP activity. Based on the mechanism by which RGS proteins stabilize the switch regions in their transition state conformation, this is a valid assumption; however, using binding as a surrogate for GAP activity has some potential pitfalls. A compound such as brefeldin-A (which traps the G-protein in an unproductive complex with its regulatory partner) would be missed. It is possible that, for the RGS domain/G $\alpha$  target, a small molecule might inhibit RGS domain-mediated stabilization of the switch regions in a conformation that facilitates hydrolysis or otherwise traps the RGS domain/G $\alpha$  complex. In addition, it is possible that, relying on binding rather than enzymatic activity in a compound library screen, one may have false negatives given weak binding of a low-potency inhibitor that would be lost to the noise of the binding assay. Instead, if one were able to read out successive rounds of GTP hydrolysis by G $\alpha$ , and acceleration of that steady-state hydrolysis by the RGS protein, the effects of such weak inhibitors may become apparent. Finally, small molecules might exist that can selectively bind and allosterically potentiate the GAP activity of RGS proteins. With this logic in mind, a true enzymatic assay for RGS domain GAP activity that is amenable to HTS automation has recently been developed.

**3. An Enzymatic Assay Using Rate-Altered G $\alpha$  and Fluorescence Polarization Detection of GDP Generation.** To facilitate an enzymatic approach to HTS for RGS domain GAP activity modulators, the nucleotide cycle of G $\alpha_{i1}$  was first changed so that GTP hydrolysis [ $k_{\text{cat}}(\text{GTPase})$ ], not spontaneous GDP release [ $k_{\text{off}}(\text{GDP})$ ], would become the rate-limiting step. G $\alpha_{i1}$  and closely related G $\alpha$  proteins have been the focus of extensive structure/function studies (Coleman et al., 1994; Sondek et al., 1994; Nishina et al., 1995; Posner et al., 1998), and point mutations have been identified previously (Coleman et al., 1994; Nishina et al., 1995; Posner et al., 1998; Marin et al., 2001) that affect both  $k_{\text{off}}(\text{GDP})$  and  $k_{\text{cat}}(\text{GTPase})$  without affecting functional interaction with the RGS domain. Two of the most striking G $\alpha$  mutations have been made to the highly conserved active-site arginine, which greatly reduces intrinsic GTPase activity but does not eliminate RGS domain-mediated GTPase acceleration [Arg-178 in G $\alpha_{i1}$ , Arg-183 in G $\alpha_q$ ; e.g., Fig. 2D (Coleman et al., 1994; Berman et al., 1996)], and to the alanine residue within the conserved TCAT loop known

to contact the guanine ring of bound nucleotide (A326S; Posner et al., 1998), which results in a ~25-fold increase in  $k_{\text{off}}(\text{GDP})$  relative to wild-type G $\alpha$ . In testing these and other rate-altering mutations (Zielinski et al., 2009), a combination of R178M and A326S substitutions to G $\alpha_{i1}$  was identified that produces sufficient alteration in nucleotide binding and cycling rates to allow the steady-state measurement of RGS domain-mediated acceleration of [ $\gamma$ - $^{32}\text{P}$ ]GTP hydrolysis. It is noteworthy that multiple RGS proteins in binding and GTP hydrolysis assays were used to ascertain that these two substitutions within the guanine nucleotide binding pocket of G $\alpha_{i1}$  had no effect on either the affinity or the specificity of the RGS domain interaction (Zielinski et al., 2009).

To detect RGS protein-accelerated GTPase activity without the use of radioactivity, a monoclonal antibody and fluorescent tracer system, previously developed by Bellbrook Labs for their Transcreeper ADP assay (Kleman-Leyer et al., 2009), was adapted for selective immunodetection of GDP with a fluorescence polarization (FP) readout (Fig. 9). Measuring GTPase activity through the detection of generated GDP product using this “mix and measure” FP assay overcomes the low signal-to-noise ratio and other limitations of inorganic phosphate detection methods [e.g., malachite green and phosphate-binding protein assays (Van Veldhoven and Mannaerts, 1987; Geladopoulos et al., 1991; Willard and Siderovski, 2004; Shutes and Der, 2005)] and has previously been validated as a robust HTS method in the case of ADP detection for kinases and ATPases (Huss et al., 2007; Liu et al., 2007; Klink et al., 2008). Moreover, because this Transcreeper GDP-based assay for the G $\alpha$ /RGS domain interaction is one based on enzymatic activity, it should enable detection of all types of modulators of RGS domain GAP activity, including those that

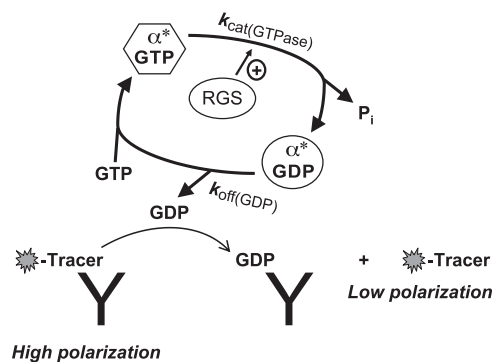


FIG. 9. HTS-compatible enzymatic assay developed for the G $\alpha$ /RGS domain target using a rate-altered G $\alpha$  mutant and a fluorescence polarization immunoassay for the detection of generated GDP. The G $\alpha$  subunit used in this assay bears two point mutations (denoted with asterisk) that dampen intrinsic GTPase and enhance spontaneous GDP release, respectively, thereby shifting the rate-limiting step in steady-state GTP hydrolysis away from product release [ $k_{\text{off}}(\text{GDP})$ ] toward GTP hydrolysis [ $k_{\text{cat}}(\text{GTPase})$ ] so that the influence of RGS domain GAP activity can be observed (section III.C.3). Fluorescent tracer is illustrated with a jagged oval; when bound to the GDP-selective monoclonal antibody, emitted light remains polarized, whereas there is low polarization of emitted light when tracer is displaced by free GDP as generated by the reaction.



bind at allosteric sites and affect RGS domain catalytic activity without directly targeting the RGS domain  $G\alpha$  binding-site per se (see section III.B). In a pilot 960-compound library screen in the 384-well form factor with the rate-altered  $G\alpha_{i1}$ (R178M/A326S) mutant (Zielinski et al., 2009), wild-type (i.e., surface cysteine-containing) RGS4 protein was employed so that the thiol-reactive compound CCG-4986 could serve as a positive control for RGS4 inhibition; subsequent screens of larger compound collections should necessarily use a cysteine-less mutant RGS4 [e.g., RGS4(C71N/C132E)] and/or addition of reductant, as has previously been considered (Kimple et al., 2007; Blazer et al., 2010; Roman et al., 2010) to avoid additional thiol-reactive hits. In addition, a counter-screen using rate-altered  $G\alpha_{i1}$ (R178M/A326S) mutant without any input RGS protein, as has been demonstrated previously in a pilot screen (Zielinski et al., 2009), will be necessary to exclude hits that act directly on the intrinsic GTPase activity of the  $G\alpha$  subunit or otherwise interfere with assay components outside of the desired RGS protein target. However, a compound that has selective modulatory activity on the intrinsic GTPase activity of  $G\alpha$  subunit(s), especially one capable of enhancing weak or dormant intrinsic GTPase activity, may be valuable in its own right as a lead for future therapeutics (e.g., see next section below).

#### IV. Activated $G\alpha_{q/11}$ Point Mutants as Targets for Ocular Melanoma Therapeutic Strategies

As previously mentioned, recent reviews (Hurst and Hooks, 2009; Sjögren et al., 2010) have surveyed the burgeoning evidence that RGS proteins can be involved in aberrant survival, growth, and motility/invasiveness signal transduction pathways that underlie tumorigenesis and metastasis, including correlative data from gene microarray studies of cancerous versus normal tissue indicating differential *RGS* transcript expression patterns and from single-nucleotide polymorphism analyses of *RGS* gene loci indicating altered risks for cancer development. These findings are consistent with the idea that the predominant activity of RGS proteins is negative regulation of GPCR signaling, coupled with the ample existing evidence that GPCRs can be involved in dysregulated autocrine/paracrine signaling (or frankly hijacked by oncogenic viruses) to drive abnormal cell growth, survival, and motility (Sodhi et al., 2004; Dorssam and Gutkind, 2007; Spiegelberg and Hamm, 2007). Further support for the carcinogenic potential of dysregulated RGS protein GAP function comes from early evidence that GTPase-inactivating point mutations to their substrates (i.e.,  $G\alpha$  subunits) are found in human tumors [e.g., pituitary and endocrine tumors (Landis et al., 1989; Lyons et al., 1990)] and can transform rodent fibroblasts cells upon ectopic overexpression (for review, see Dhanasekaran et al., 1995; Fukuhara et al., 2001). More recently, a somatic mutation to the *GNAO1* gene

locus ( $G\alpha_o$ ) was described in breast carcinoma (Kan et al., 2010) as leading to a missense mutation (R243H) of unknown functional consequence; Garcia-Marcos and colleagues have since identified that the R243H substitution renders  $G\alpha_o$  constitutively active by enhancing spontaneous nucleotide exchange [rather than by affecting either intrinsic or GAP-accelerated GTPase activity per se (Garcia-Marcos et al., 2011)]. Here, in this final section, we consider another recent finding of  $G\alpha$  subunit involvement in cancer—that of  $G\alpha_q$  and  $G\alpha_{11}$  in ocular (or “uveal”) melanoma—and how the field’s existing knowledge base, including the structural determinants of  $G\alpha_{q/11}$  signal regulation, could be exploited for therapeutic development for this particular cancer.

Recent genetic analyses of ocular melanoma have revealed a high percentage (>80%) of activating point mutations in either the *GNAQ* ( $G\alpha_q$ ) or *GNA11* ( $G\alpha_{11}$ ) gene loci (Onken et al., 2008; Van Raamsdonk et al., 2009, 2010), most predominantly a Q209L (“exon 5”) mutation that completely cripples the intrinsic GTPase activity of these  $G\alpha$  subunits and thereby creates a persistent GTP-bound, activated state (Kleuss et al., 1994). The Gln-209 of  $G\alpha_q$  and  $G\alpha_{11}$  is analogous to the Gln-204 residue of  $G\alpha_i$  depicted in Figs. 2D and 3B as critical to GTP hydrolysis, to the Gln-227 of  $G\alpha_s$  found mutated in pituitary tumors (Landis et al., 1989), and to the Gln-61 residue of the small GTPase Ras found mutated in multiple cancers (Feig and Cooper, 1988; Cox and Der, 2002). Such constitutive activation of mutant  $G\alpha_q$  or  $G\alpha_{11}$  in melanocytes is thought to drive inappropriate proliferative signaling through the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling cascade in a manner similar to that of the activating mutations to B-Raf that are observed with high frequency in cutaneous melanoma [e.g., B-Raf(V600E) (Davies et al., 2002)].

With the recent success of targeting activated B-Raf in cutaneous melanoma using novel chemotherapeutic agents such as the kinase inhibitor PLX4032 (Bollag et al., 2010; Flaherty et al., 2010), developing similar therapeutic strategies toward the oncogenic alleles of  $G\alpha_q$  and  $G\alpha_{11}$  might have equal success in uveal melanoma treatment. This is most germane for developing new treatments for the life-threatening metastatic dissemination of the melanoma to the liver (Vahrmeijer et al., 2008); although debilitating, eye removal (“enucleation”) because of the primary tumor is not life-threatening per se.

##### A. Regulator of G-Protein Signaling Domain-Based Gene Therapy?

In the current absence of small molecules able to reactivate the GTPase activity of  $G\alpha$  mutants (Ja et al., 2006; Siderovski et al., 2009), what other strategies could be employed to antagonize constitutively activated  $G\alpha_q$  and  $G\alpha_{11}$  in uveal melanoma? Given the theme of this review, some targeted gene-therapy application of

the RGS proteins might spring to mind. Indeed, one member of the R4 family, RGS2, exhibits a unique selectivity in its *in vitro* GAP activity for G $\alpha$  subunits of the G $\alpha_q$  family (Heximer et al., 1997b). X-ray diffraction crystallography was recently used to elucidate the particular structural determinants that engender this unique selectivity shown by RGS2 (Kimple et al., 2009). Unfortunately, RGS domain GAP activity has no effect on G $\alpha$  subunits lacking the critical hydrolytic glutamine absent in the Q209L alleles of G $\alpha_q$  and G $\alpha_{11}$ ; this lack of activity was first established in the original description of RGS proteins as G $\alpha$ -directed GAPs (Berman et al., 1996). Fortunately, RGS domain GAP activity is able to accelerate GTP hydrolysis by other “GTPase-deficient” G $\alpha$  point mutants (Berman et al., 1996) that bear a less-crippling mutation to the arginine residue that also contributes to the hydrolysis reaction, known as Arg-183 in G $\alpha_q$  and G $\alpha_{11}$  and highlighted as Arg-178 of G $\alpha_{i1}$  in Fig. 2D. This particular mutation (R183Q or R183C in exon 4) is seen in G $\alpha_q$  and G $\alpha_{11}$  in uveal melanoma, but is considered less potent as an oncogenic allele and is less frequently observed (<10%) versus the Q209L mutation [>70% (Van Raamsdonk et al., 2010)].

RGS domains not only possess GAP activity but also bind the active (GTP-bound) species of G $\alpha$  subunits and thus can curtail downstream signal transduction in cells by a process of “effector antagonism” (e.g., Hepler et al., 1997; Anger et al., 2004). Indeed, the N-terminal RGS domain identified in GRK2 exhibits little if any GAP activity yet binds readily to the activated state of G $\alpha_{q/11}$  subunits, for which it demonstrates considerable selectivity over other G $\alpha$  subfamilies (Siderovski et al., 1996; Carman et al., 1999; Sallese et al., 2000; Usui et al., 2000; Tesmer et al., 2005). Tesmer et al. (2005) have shown by X-ray crystallography that the GRK2 RGS domain binds G $\alpha_q$  in a mode unlike that of conventional (GAP-active) RGS proteins, not only explaining the lack of observable GAP activity but also affording a high-resolution structure of the key elements of G $\alpha_{q/11}$  effector antagonism that should prove useful for any future gene-therapy or “deliverable protein” therapeutic designs derived from the GRK2 N terminus.

### B. Gene- or Peptide-Based Strategies?

Antagonizing the oncogenic alleles of GNAQ and GNA11 at the mRNA transcript level, rather than at the protein level, may be an achievable pursuit given recent advances in RNA interference-based therapeutics (Tiemann and Rossi, 2009; Ashihara et al., 2010). There is precedence for a single siRNA oligonucleotide duplex affording effective knock-down of both G $\alpha_q$  and G $\alpha_{11}$  protein levels in cells (Barnes et al., 2005), given the high degree of nucleotide sequence relatedness between GNAQ and GNA11. For example, in an RNA interference screen for RGS protein specificity in modulating endogenous GPCR signaling, a single siRNA duplex targeted to both GNAQ and GNA11 was shown to be quite

effective in human cells in dampening GPCR signaling to intracellular calcium store mobilization (Laroche et al., 2010)—a well established signaling outcome downstream of activated G $\alpha_{q/11}$ -mediated by the stimulation of PLC $\beta$  and the resultant generation of the second messenger inositol-1,4,5-trisphosphate (Harden, 1992; Berridge, 2009). Sharpening the melanoma-selective nature of such siRNA-based therapy could possibly be achieved by exploiting the nucleotide-level changes in the oncogenic GNAQ/GNA11 alleles (versus their wild-type sequence) and/or using established physical approaches to liver-focused delivery such as transcatheter arterial chemoembolization (Sato, 2010; Schuster et al., 2010), given that hepatic metastatic disease is the critical, life-threatening feature of this disease.

Considering peptide-based strategies, the cyclic depsipeptide (1*R*)-1-[(3*S*,6*S*,9*S*,12*S*,18*R*,21*S*,22*R*)-21-acetamido-18-benzyl-3-[(1*R*)-1-methoxyethyl]-4,9,10,12,16,22-hexamethyl-15-methylene-2,5,8,11,14,17,-20-hepta-oxo-1,19-dioxo-4,7,10,13,16-pentaazacyclodocosan-6-yl]-2-methylpropyl rel-(2*S*,3*R*)-2-acetamido-3-hydroxy-4-methylpentanoate (YM-254890) might be useful but likely only for the lower frequency Arg-183 mutants of G $\alpha_q$  and G $\alpha_{11}$  seen in uveal melanoma. YM-254890 was originally identified from *Chromobacterium* spp. QS3666 culture broth (Taniguchi et al., 2003) as a secreted agent capable of blocking ADP-induced platelet aggregation [a physiological response to ADP dependent on signaling from purinergic GPCRs and G $\alpha_q$ -containing heterotrimers (Kunapuli et al., 2003)]. The structural determinants by which this cyclic depsipeptide selectively inhibited G $\alpha_q$ -mediated signaling were resolved via X-ray crystallography of a G $\alpha_q$ -GDP/G $\beta\gamma$  heterotrimer bound to YM-254890 (Nishimura et al., 2010). YM-254890 was found to bind a hydrophobic cleft between two interdomain linkers that connect the Ras-like and  $\alpha$ -helical domains of the G $\alpha_q$  subunit, thereby restricting interdomain movement presumed to be required for GDP/GTP exchange (Remmers et al., 1999). This mechanism of action as a GDP dissociation inhibitor explains earlier findings (Takasaki et al., 2004) that YM-254890 is unable to inhibit cellular signaling by G $\alpha_q$ (Q209L), yet can inhibit signaling by G $\alpha_q$ (R183C). As mentioned previously, the Q209L mutation creates a persistent GTP-bound, activated state and thus does not require on-going GDP/GTP exchange for persistent cellular signaling; in contrast, the fractional occupancy of the R183C mutant has been reported as only 40% GTP-loaded (Kleuss et al., 1994), presumably reflecting a remaining degree of GTP hydrolysis that, in the cellular context, requires either spontaneous nucleotide exchange or a return to G $\beta\gamma$  complexation and receptor-catalyzed exchange for continued function in driving downstream signaling. These exchange events by G $\alpha_q$ (R183C) can be blocked by YM-254890.

In considering peptide-based therapeutics inspired from G $\alpha_q$ -binding proteins, in addition to the GRK2/G $\alpha_q$  complex mentioned above, two more recent high-resolu-

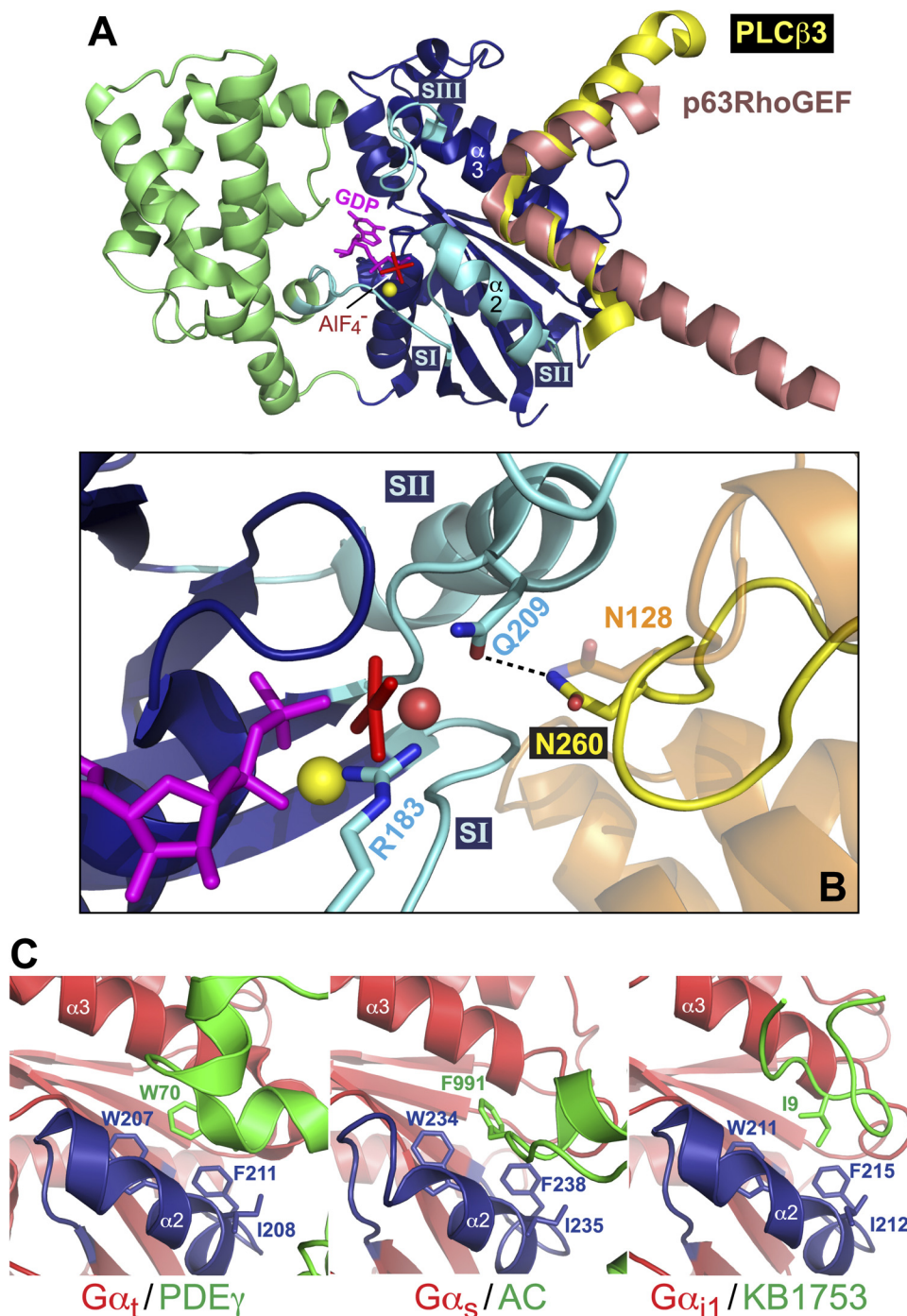


FIG. 10. Shared structural determinants of binding and GAP activity on activated  $G\alpha_q$ , as elucidated via recent high-resolution structures of  $G\alpha_q$ /PLC $\beta$ 3 and  $G\alpha_q$ /p63RhoGEF complexes. A, the two main effectors of  $G\alpha_q$ -mediated signaling, PLC $\beta$  (yellow) and p63RhoGEF (salmon), share a similar helix-turn-helix configuration in their binding sites for activated  $G\alpha_q$  and engage the same hydrophobic cleft circumscribed by the switch II (SII) ( $\alpha$ 2) and  $\alpha$ 3 helices of  $G\alpha$ . Structural coordinates were rendered in PyMol using PDB numbers 2RGN and 3OHM. Elements of  $G\alpha_q$  are colored similarly to the  $G\alpha$  illustrations of Fig. 2. SI, switch I. B, PLC $\beta$  isoforms are not only effectors but also GAPs for  $G\alpha_{q/11}$  subunits (section I.C.4). The crystal structure of the  $G\alpha_q$ /PLC $\beta$ 3 complex revealed a mechanism for accelerating  $G\alpha$  GTPase activity similar to that of the RGS proteins such as RGS4 (light orange; PDB number 1AGR): namely, helping to orient the critical hydrolytic glutamine residue (Gln-209 in  $G\alpha_q$ ) via contact with an asparagine residue (Asn-260 in PLC $\beta$ , Asn-128 in RGS4). This Gln-209 residue is mutated in  $G\alpha_q$  and  $G\alpha_{11}$  with high frequency in uveal melanoma, so it is unlikely that the GAP activity of PLC $\beta$  or a  $G\alpha_{q/11}$ -selective RGS protein would be beneficial in a gene-therapy strategy for uveal melanoma (section IV.A). Magnesium cation and water are illustrated as yellow and red spheres, respectively. C, consistent with the  $G\alpha_q$ /PLC $\beta$ 3 and  $G\alpha_q$ /p63RhoGEF structures, the  $\alpha$ 2- $\alpha$ 3 groove of activated  $G\alpha$  subunits is recognized by other effector molecules. In the GTP-bound state, switch II of  $G\alpha$  ( $\alpha$ 2 helix in blue) is oriented alongside the  $\alpha$ 3 helix (red), providing a hydrophobic groove that is used by diverse effector molecules (green). Left, transducin- $\alpha$  engages the inhibitory  $\gamma$  subunit of retinal phosphodiesterase (PDB number 1FQJ). Middle,  $G\alpha_s$  recognizes adenylyl cyclase (PDB number 1AZS). Right, the phage display peptide KB1753 binds to activated  $G\alpha_{i1}$  (PDB number 2G83). These three effector molecules each insert a hydrophobic side chain into the  $\alpha$ 2- $\alpha$ 3 groove (Trp-70, Phe-991, and Ile-9, respectively), suggesting this  $\alpha$ 2- $\alpha$ 3 groove position as a potential site for small molecule manipulation of the  $G\alpha$  subunit/effector interaction.



tion structures have been reported of active-state G $\alpha_q$  in complex with key binding partners/effector proteins: namely p63RhoGEF (Lutz et al., 2007) and, more recently, PLC $\beta$ 3 (Waldo et al., 2010). Both effector proteins bind selectively to activated G $\alpha_q$  through the use of a helix-turn-helix configuration of their respective secondary structures that engages a hydrophobic patch between switch II ( $\alpha$ 2-helix) and  $\alpha$ 3-helix of the G $\alpha$  subunit (Fig. 10); this engagement is a common structural theme seen in multiple other G $\alpha$  subunit/G $\alpha$ -effector pairings (e.g., Tesmer et al., 1997b; Slep et al., 2001; Chen et al., 2005; Johnston et al., 2006), including G $\alpha_{i1}$ -GDP- $\text{AlF}_4^-$  bound to KB-1753 (Fig. 10C), the effector-mimetic peptide originally identified in a phage-display screen for nucleotide-state-selective binding peptides specific for the G $\alpha_{i/o}$  family (for review, see Johnston et al., 2008). Strategies to identify analogous peptides that could serve as effector antagonists of activated G $\alpha_q$  and G $\alpha_{i1}$  could therefore include phage or mRNA display with either a semirandom peptide library as starting material (Ja et al., 2006; Johnston et al., 2008) or a peptide library that preserves core contact residues from the helix-turn-helix region of p63RhoGEF or PLC $\beta$ 3 (Lutz et al., 2007; Waldo et al., 2010). G $\alpha_{q/11}$ -binding peptides thus identified might be able to serve directly as leads for therapeutic development or else provide useful affinity probes for the creation of HTS assays of compound libraries for small molecules able to displace their binding, thereby identifying "effector antagonist" chemicals for further development as therapeutics. A similar strategy of HTS assay development was recently pursued with a short peptide from the RGS12 GoLoco motif that binds selectively to inactive-state G $\alpha_i$  subunits (Kimple et al., 2001); fluorescent dye-labeling of this GoLoco-peptide enabled a fluorescence polarization assay to be developed for the 1536-well form factor (Kimple et al., 2008) with excellent signal-to-noise and reproducibility characteristics ( $Z$  factor  $>0.8$ ). It is hoped that one of these outlined strategies will prove fruitful in developing a G $\alpha_{q/11}$ -targeted therapy to this particular cancer.

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