Adenylyl Cyclase 5 Regulation by $G\beta\gamma$ Involves Isoform-Specific Use of Multiple Interaction Sites

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ABSTRACT

Adenylyl cyclase (AC) converts ATP into cyclic AMP (cAMP), an important second messenger in cell signaling. Heterotrimeric G proteins and other regulators are important for control of AC activity. Depending on the AC isoform, $G\beta\gamma$ subunits can either conditionally stimulate or inhibit cAMP synthesis. We previously showed that the $G\alpha_s$ - $\beta\gamma$ heterotrimer binds to the N terminus (NT) of type 5 AC (AC5). We now show that $G\beta\gamma$ binds to the NT of a wide variety of AC isoforms. We hypothesized that $G\beta\gamma$ /AC5 interactions involving inactive heterotrimer and $G\beta\gamma$ stimulation of AC5 were separable events. Mutations of the $G\beta\gamma$ “hotspot” show that this site is necessary for AC5 stimulation but not for interactions with the first 198 aa of AC5NT, which is a G protein

scaffolding site. This contrasts with AC6, where the $G\beta\gamma$ hotspot is required for both interactions with AC6NT and for stimulation of AC6. Additionally, the SIGK hotspot peptide disrupts $G\beta\gamma$ regulation of AC isoforms 1, 2, and 6, but not AC5. $G\beta\gamma$ also binds the C1/C2 catalytic domains of AC5 and AC6. Finally, cellular interactions with full-length AC5 depend on multiple sites on $G\beta\gamma$. This suggests an isoform-specific mechanism in which bound $G\beta\gamma$ at the AC5NT is ideally situated for spatiotemporal control of AC5. We propose $G\beta\gamma$ regulation of AC involves multiple binding events, and the role of the AC NT for mechanisms of regulation by heterotrimeric G protein subunits is isoform-specific.

Introduction

Adenylyl cyclase (AC) converts ATP into cyclic AMP (cAMP), an important second messenger in cell signaling. Isoform specificity provides complexity to AC regulation, and allows for a wide variety of physiologic roles from AC/cAMP signaling (Sadana and Dessauer, 2009). Heterotrimeric G proteins are an important example of such AC regulation. All nine membrane-bound AC isoforms are activated by the GTP-bound “active” $G\alpha_s$ subunit. $G\beta\gamma$ subunits can enhance $G\alpha_s$ - or forskolin-stimulated AC 2, 4, 5, 6, and 7 activity but have no effect alone (Gao and Gilman, 1991; Tang and Gilman, 1991; Federman et al., 1992; Iñiguez-Lluhi et al., 1992; Taussig et al., 1993; Yoshimura et al., 1996; Bayewitch et al., 1998; Gao et al., 2007). However, $G\beta\gamma$ can also inhibit the activity of AC 1, 3, and 8 (Tang and Gilman, 1991; Steiner et al., 2005; Diel

et al., 2006). Thus, the effects of $G\beta\gamma$ on cAMP production are dependent on isoform specificity.

In the inactive $G\alpha$ - $\beta\gamma$ conformation, regions on the $G\alpha$ and $G\beta\gamma$ subunits required for effector regulation are concealed in a bound heterotrimer. After activation, such regions are exposed and can interact with downstream effector proteins such as AC (Sprang et al., 2007). Both $G\alpha_s$ and $G\beta\gamma$ subunits are needed to observe a full stimulatory AC6 response to the G_s -coupled receptor agonist isoproterenol (Gao et al., 2007). This suggests that $G\beta\gamma$ generated from activation of G_s enhances AC5/6 activity. This differs from the conditional $G\beta\gamma$ stimulation of AC2/4/7, in which the $G\beta\gamma$ subunit is provided via crosstalk by activation of G_i -coupled G protein-coupled receptors (GPCRs) to further increase AC activity generated from G_s -coupled GPCRs (Federman et al., 1992; Shen et al., 2012).

The structure of all AC isoforms includes an N terminus (NT) and two catalytic domains (C1, C2). Whereas the C1/C2 domains are well conserved across AC isoforms and form the catalytic pocket for cAMP production, the NT varies widely among AC isoforms in length, sequence, and regulatory binding sites. AC 5, 6, and 8 NT bind $G\beta\gamma$ (Crossthwaite et al., 2006; Gao et al., 2007). AC5NT associates with the guanine nucleotide exchange factor Ric8a (Wang et al., 2007),

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ABBREVIATIONS: AC, adenylyl cyclase; AKAP, A-kinase anchoring protein; BiFC, bifluorescence complementation; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GIRK, G protein-coupled inwardly-rectifying potassium channels; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; HEK293, human embryonic kidney 293 cells; NT, N terminus; YC, C-terminal half of YFP; YFP, yellow fluorescent protein; YN, N-terminal half of YFP.

whereas AC8NT binds the phosphatase PP2A (Crossthwaite et al., 2006), as well as facilitating AC8 stimulation by calmodulin (Simpson et al., 2006). AC NT binding sites for protein kinase C also vary by isoform (Lai et al., 1999; Chou et al., 2004; Crossthwaite et al., 2006; Simpson et al., 2006; Wang et al., 2007). The NT of AC 2, 5, 6, and 9 can interact with macromolecular signaling scaffolds such as A-kinase anchoring proteins (AKAPs) that facilitate spatiotemporal control of AC activity (Piggott et al., 2008; Efendiev et al., 2010).

A binding site for inactive G protein heterotrimer on the NT of AC5 (AC5NT) was previously identified but is not required for G $\beta\gamma$ stimulation of AC5. G $\beta\gamma$ binding to residues 66–137 of AC5NT is synergistically enhanced in the presence of GDP-G α_s , and can bind as a G protein heterotrimer to AC5NT (Sadana et al., 2009). Other G $\beta\gamma$ effectors, such as phospholipase C β (PLC β), G protein-coupled inwardly-rectifying potassium channels (GIRK), and receptor for activated C kinase 1 (RACK1), are also capable of scaffolding G protein heterotrimers (Dell et al., 2002; Yuan et al., 2007; Berlin et al., 2010). The binding of G α_s - $\beta\gamma$ at the AC5NT is independent of G $\beta\gamma$ stimulation of AC5, supporting a model in which inactive heterotrimer occupies the NT and a separate binding event results in G $\beta\gamma$ stimulation of AC5. Conversely for AC6, stimulation by G $\beta\gamma$ requires residues 77–151 of AC6NT (Gao et al., 2007). This is despite much regulatory overlap between AC5 and AC6, including stimulation by G $\beta\gamma$.

The G $\beta\gamma$ structure includes β -sheet WD40 repeats, or “blades,” and a “hotspot” area where the turns between blades intersect. The hotspot on G $\beta\gamma$ is required for interactions with either the α subunit or numerous effectors (Wall et al., 1995; Scott et al., 2001; Davis et al., 2005). We hypothesized that G $\beta\gamma$ requires the hotspot to stimulate AC5 but utilizes a different surface to interact with AC5NT. A similar scaffold/stimulation mechanism occurs for G $\beta\gamma$ and G α_i regulation of GIRK channels (Rubinstein et al., 2009; Berlin et al., 2010). Our data support this hypothesis for AC5, by mutagenesis of specific hotspot residues and by competition with the SIGK-disrupting peptide. Amino acid residues 23–27 of G β are also required for stimulation of AC5 and AC6. Despite G $\beta\gamma$ binding to the N termini of many AC isoforms, only interactions with AC5NT were not reliant on an intact G $\beta\gamma$ hotspot. Lastly, we show for the first time that G $\beta\gamma$ can bind to the C1 and C2 domains of AC5 and AC6 in a largely hotspot-dependent manner.

Materials and Methods

Plasmids and Viruses. Human AC5 and AC6 baculoviruses were constructed and expressed as described previously (Chen-Goodspeed et al., 2005). Construction and expression of rat AC1 and AC2 baculoviruses were described previously (Tang et al., 1991; Tang and Gilman, 1991). Baculoviruses for biotinylated G β_1 (both wild-type and mutants), G γ_2 , and G α_i were used to express biotinylated G $\beta_1\gamma_2$ variants as described (Bonacci et al., 2005; Davis et al., 2005).

Flag-tagged AC5 pcDNA was a generous gift from Dr. Michael Kapiloff (University of Miami, Miami, FL) and has been used previously (Sadana et al., 2009). YN-G β_1 was a generous gift from Catherine Berlot (Weis Center for Research, Danville, PA) and consists of G β_1 fused to the first 158 aa of yellow fluorescent protein (YN, N-terminal half of YFP). The YN-G β_1 -W99A pcDNA plasmid was generated by polymerase chain reaction mutagenesis. NT23–27 was generated by mutagenesis of the G β_1 aa 23–27 sequence KACAD to ANCAA and cloned into the YN-G β_1 or YN-G β_1 -W99A pcDNA template to produce either YN-G β_1 -NT23–27 or YN-G β_1 -NT23–27/W99A

double mutant pcDNA plasmid. YC- γ_7 was a generous gift from Catherine Berlot and consists of G γ_7 fused to aa 159–238 of yellow fluorescent protein (YFP; YC, C-terminal half of YFP). YC-AC5 consists of AC5 fused to aa 159–238 of YFP.

Antibodies. Antibodies used were rabbit anti-G β (Santa Cruz Biotechnology, Dallas, TX), mouse anti-GST (glutathione *S*-transferase; Santa Cruz Biotechnology), rabbit anti-H6 (Bethyl Laboratories Inc., Montgomery TX), rabbit anti-green fluorescent protein (Cell Signaling Technology, Danvers, MA), and anti-Flag (Sigma-Aldrich, St. Louis, MO). All antibodies diluted at 1:1000 in Tris-buffered saline/Tween 20 (TBST) for Western blots.

Protein Purifications and Sf9 Membrane Preparation. Proteins 5C1(670)H $_6$ (AC5 aa 364–670), H $_6$ 5C2 (AC5 aa 938-end), and G α_s H $_6$, were expressed in *Escherichia coli* and purified as described previously (Whisnant et al., 1996; Sunahara et al., 1997; Dessauer et al., 1998). GST-tagged proteins were expressed in *E. coli* and purified using glutathione agarose resin as described previously (Salim et al., 2003). Non-tagged or biotin-tagged G $\beta_1\gamma_2$ was coexpressed with G α_i H $_6$ in Sf9 cells and purified on nickel-NTA columns, followed by either overnight dialysis or ion exchange as described previously (Kozasa and Gilman, 1995). AC expression in Sf9 cells and the preparation of membranes was performed as previously described (Chen-Goodspeed et al., 2005).

Human Embryonic Kidney 293 Cell Transfections. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO $_2$. HEK293 (3–3.5 $\times$ 10 6 cells) were seeded 24 hours prior to transfection in 10-cm dishes. Medium was replaced the next day with fresh DMEM (no penicillin/streptomycin) and cells were transfected with the appropriate plasmids (10 μ g of DNA total per 10-cm plate) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C for 4–6 hours, the media was replaced, and membranes or lysates prepared approximately 40–48 hours after transfection.

Pull-Down Binding Assays. GST or GST-tagged AC NT (full-length or truncations) were incubated with purified G $\beta\gamma$ subunits in 50 μ l of binding buffer [20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM MgCl $_2$, 2 mM dithiothreitol (DTT), 100 mM NaCl, 0.1% C $_{12}$ E $_9$]. The proteins were incubated for 30 minutes at 4°C followed by addition of 100 μ l of 20% glutathione-agarose beads. After rotating for 2 hours at 4°C, the resin was washed three times with wash buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM MgCl $_2$, 2 mM DTT, 100 mM NaCl, 0.05% C $_{12}$ E $_9$). Bound proteins were eluted with 15 mM glutathione and analyzed by SDS-PAGE and Western blotting.

For biotin-tagged protein pulldowns, purified biotin-tagged G $\beta\gamma$ was incubated with His-tagged 5C1(670)H $_6$ in 50 μ l of binding buffer for 30 minutes at 4°C. After incubation, 100 μ l of 20% streptavidin-agarose beads was added to the samples, rotated for 2 hours at 4°C, and the resin was subsequently washed three times with wash buffer. Bound proteins were eluted from the streptavidin-agarose by direct addition of 1 $\times$ Laemmli buffer and analyzed by SDS-PAGE and Western blotting.

Flag-AC5 Immunoprecipitation. Human Flag-tagged AC5 was transfected in HEK293 cells (10-cm dish/immunoprecipitation). After 42 hours, HEK293 cells were rinsed with phosphate-buffered saline, resuspended in lysis buffer (50 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM MgCl $_2$, 1 mM DTT, 150 mM NaCl, 0.5% C $_{12}$ E $_9$, and protease inhibitors), and triturated using a 23-gauge syringe. Cellular debris was removed by centrifugation, and 30 μ l of anti-Flag agarose was added. Samples were rotated at 4°C for 2 hours, and then washed three times with lysis buffer with 0.05% C $_{12}$ E $_9$. Proteins were eluted from anti-Flag resin with SDS-PAGE sample buffer, and analyzed by SDS-PAGE and Western blotting.

G $\beta\gamma$ Binding Overlays. Bovine serum albumin controls, His-tagged AC5/6 C1 or C2 domains were run on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. After blocking with 5% milk for 1 hour, polyvinylidene fluoride membranes were incubated overnight with 10 μ g purified G $\beta\gamma$ in 3 ml of overlay wash buffer (Tris-buffered saline pH 7.4, 0.1% Tween-20, and 1 mM DTT) overnight at 4°C. Bound G $\beta\gamma$ was detected by Western blotting.

Adenylyl Cyclase Membrane Assays. Membrane assays were performed essentially as described previously (Dessauer, 2002). Sf9 membrane preparations were incubated for 8–10 minutes at 30°C with an AC mix containing [α^{32} P]ATP and appropriate activators, including forskolin or GTP γ S- G_{α_s} with or without the indicated concentrations of G $\beta\gamma$. Reactions were stopped with a solution of 2.5% SDS, 50 mM ATP, and 1.75 mM cAMP. Nucleotides in each reaction sample were then separated by column chromatography to isolate [32 P]cAMP product, using [3 H]cAMP to monitor column recovery rates by scintillation counting.

Bifluorescence Complementation Assay. HEK293 cells were transfected as indicated above in 12-well plates with YN- or YC-tagged AC5, G β , and G γ plasmids. Approximately 47 hours after transfection, cells were stained with DMEM containing DAPI (4',6-diamidino-2-phenylindole; 10 μ g/ml) for one hour at 37°C. Cells were then transferred in 150 μ l phosphate-buffered saline to black 96-well plates with clear, flat-bottom wells (Corning Inc., Corning, NY). YFP and DAPI signals were measured with a multiwell plate reader (Infinite 200; Tecan, Mannedorf, Switzerland) at room temperature. YFP intensity was measured at excitation wavelength 508 nm and emission wavelengths from 534–538 nm (2-nm step measurements). DAPI signal was measured with excitation and emission wavelengths at 358 and 461 nm, respectively. Peak YFP signals from 534–538-nm emissions were averaged and normalized to DAPI signal to account for potential differences in cell count. For imaging, HEK293 cells were transfected in 12-well plates on polylysine-coated coverslips. Bifluorescence complementation (BiFC) images were acquired using a TE 2000 microscope (Nikon, Tokyo, Japan) with a DG4 xenon light source and CoolSNAP camera (Roper Scientific, Trenton, NJ). YFP images were acquired approximately 48 hours after transfection (excitation 500/20 nm, emission 535/30 nm).

Results

G $\beta\gamma$ Binds to Various AC Isoform N Termini. G $\beta\gamma$ binding to the NT of AC has been observed previously for AC5 and AC6, either alone or as a G_{α_s} heterotrimer (Gao et al., 2007; Sadana et al., 2009). The NT of AC isoforms are varied in their size and sequence, and the respective AC isoforms also vary in the regulatory function of G $\beta\gamma$ (Fig. 1A). To determine if G $\beta\gamma$ binding is a conserved property of all AC NT, GST pulldowns were performed using GST-tagged AC NT and purified G $\beta\gamma$. As shown in Fig. 1B, G $\beta\gamma$ binds to the NT of all AC isoforms tested, including AC1, 2, 3, 5, 6, and 9. Previous studies have also identified an interaction between AC8 and G $\beta\gamma$ (Cross-thwaite et al., 2006).

G $\beta\gamma$ Scaffolding versus Stimulation of AC5, but Not AC6, Activity are Separable Events. Since the AC5NT serves as a heterotrimeric scaffolding site (Sadana et al., 2009), we predicted that G $\beta\gamma$ scaffolding at the AC5NT and activation of AC5 were separable events. To separate these events, we used alanine-substituted mutants of the G $\beta\gamma$ hotspot region required for effector interactions (Fig. 2A) (Ford et al., 1998; Yuan et al., 2007). Mutations within the G $\beta\gamma$ hotspot did not alter binding to AC5NT in GST-pulldown assays (Fig. 2B). However, when assayed for their ability to increase G_{α_s} -stimulated AC5 activity in vitro, G $\beta\gamma$ hotspot mutants were unable to stimulate AC5, with the exception of M101A, which was still significantly reduced compared with wild-type (Fig. 2C). Histidine 311 serves as a control as it is located largely outside the traditional hotspot of G $\beta\gamma$, located in the junction between blades 6 and 7 (Yuan et al., 2007). The diminished stimulation of AC5 by hotspot mutants compared with wild-type G $\beta\gamma$ was independent of the G $\beta\gamma$ scaffolding

Fig. 1. G $\beta\gamma$ binds to various AC N termini. (A) Schematic of AC N termini grouped by similar isoforms. Direct regulation by G $\beta\gamma$ is indicated; * indicates inferred regulation from cellular studies. (B) Pulldown of GST-tagged AC NTs with bound G $\beta\gamma$. The final concentration of GST or GST-tagged NTs was 2 μ M. Inputs and elutes from GST pulldowns were analyzed by Western blotting.

site within the AC5NT (aa 66–137) (Fig. 2D). Thus, AC5NT binding and stimulation of AC5 by G $\beta\gamma$ are indeed separable events, requiring different G $\beta\gamma$ surfaces.

As AC5 and AC6 are closely related AC isoforms, each containing a NT binding site for G $\beta\gamma$, we examined NT binding versus stimulation of AC6 by G $\beta\gamma$ hotspot mutants. Unlike AC5, hotspot mutants of G $\beta\gamma$ displayed reduced binding to the AC6NT (Fig. 3A), which paralleled the decreased stimulation of AC6 compared with wild-type G $\beta\gamma$ (Fig. 3B). Thus, G $\beta\gamma$ interactions with the N-terminus differ between AC5 and AC6.

Previous work had shown that G $\beta\gamma$ could conditionally stimulate AC5/6 in the presence of either G_{α_s} or forskolin (Gao et al., 2007). We show that the G $\beta\gamma$ hotspot is also required for AC5 and AC6 conditional stimulation by forskolin (Fig. 3C), suggesting that the requirement for the hotspot is attributable to direct interactions of G $\beta\gamma$ with AC, independent of G_{α_s} .

Since the G $\beta\gamma$ hotspot was necessary for binding to the AC6NT but not AC5NT, we screened the other AC NT with wild-type versus W99A hotspots G $\beta\gamma$ to determine the isoform specificity of hotspot-dependent G $\beta\gamma$ /ACNT binding. The hotspot mutation of G $\beta\gamma$ caused decreased binding of all non-AC5 NT, including AC 1, 2, 3, 6, 8 and 9, although the relative effect of W99A varied depending on the AC NT tested (Fig. 3, A and D). Thus, the relative necessity of the hotspot for AC NT binding by G $\beta\gamma$ is isoform-specific.

Fig. 2. G $\beta\gamma$ Hotspot mutants bind to AC5NT but are unable to stimulate AC5. (A) Schematic of the G $\beta\gamma$ structure, with indicated sites of the alanine-substitution hotspot and NT23–27 mutants that were used in this research. Schematic made using Visual Molecular Dynamics (VMD 1.9). (B) Pull-down of GST or GST-tagged AC5NT (2 μ M) incubated with 300 nM wild-type G $\beta\gamma$ (WT) or the indicated G $\beta\gamma$ hotspot mutants. Elutes from GST pull-downs were analyzed by Western blotting. (C) AC activity assay of Sf9 membranes expressing AC5 were stimulated with 50 nM G α_s with or without 300 nM G $\beta\gamma$. Statistics: Student's *t* test of experiment means comparing G $\beta\gamma$ WT group to the indicated mutant group; *n* = 4 with experiments performed in duplicate; **P* < 0.05; ***P* < 0.01. (D) AC activity assay with Sf9 membranes expressing AC5 with NT residues 66–137 deleted (Δ 66–137) were stimulated with 50 nM G α_s with or without 300 nM G $\beta\gamma$. Statistics: Student's *t* test of experiment means comparing the G $\beta\gamma$ WT group to the indicated hotspot mutant group; *n* = 3 with experiments performed in duplicate; **P* < 0.05; ***P* < 0.01.

Pharmacological Targeting of G $\beta\gamma$ Supports a Unique AC5 Regulatory Mechanism Compared with Other AC Isoforms. In addition to alanine-scanning mutants of G $\beta\gamma$, we also used a different method of investigating G $\beta\gamma$ -AC interactions. The small SIGK peptide has been used previously to disrupt select hotspot-dependent G $\beta\gamma$ interactions with effector proteins (Scott et al., 2001; Davis et al., 2005). Addition of SIGK prevented G $\beta\gamma$ regulation of several AC isoforms, including G $\beta\gamma$ inhibition of AC1 and stimulation of AC2 and AC6 (Fig. 4A). Addition of SIGK also had a small effect on AC 1, 2, and 6 activities in the absence of added G $\beta\gamma$, presumably owing to blockade of endogenous G $\beta\gamma$ present in Sf9 membranes (Fig. 4, A and C). However, SIGK was surprisingly unable to block AC5 stimulation by G $\beta\gamma$, even at higher concentrations of SIGK peptide (Fig. 4, A and B). The AC isoform differences from pharmacological targeting of the G $\beta\gamma$ hotspot support a unique AC5-G $\beta\gamma$ regulatory mechanism.

G $\beta\gamma$ Binds to AC5/6 Catalytic Domains. Although multiple G $\beta\gamma$ binding sites on AC5 were expected, only the AC5NT heterotrimer scaffolding site had previously been identified. We assumed on the basis of homology with AC6NT that a second site necessary for activation was probably present in AC5NT (see Fig. 1A). However, owing to expression issues in *E. coli*, we have never been able to test this region directly (aa residues 195–238). G $\beta\gamma$ interacts with multiple sites in AC2 to promote conditional stimulation, including the C1 and C2 catalytic domains (Diel et al., 2006, 2008; Boran et al., 2011). Therefore, we used several strategies to determine

if G $\beta\gamma$ interacted with the C1/C2 domains of AC5 and AC6. Streptavidin pulldowns of biotin-tagged purified G $\beta\gamma$ showed strong interactions with the H₆-tagged C1 domain of AC5 that was independent of the W99A mutation (Fig. 5A). However, owing to nonspecific binding issues, this method could not be used to determine if AC5–C2 bound to G $\beta\gamma$. To overcome this limitation, we performed a G $\beta\gamma$ -binding overlay assay in which the C1 and C2 domains were separated by SDS-PAGE, renatured, and incubated with wild-type or W99A purified G $\beta\gamma$ (Fig. 5, B and C). Although the detection of AC5–C1 interactions was weak, it did not depend on mutation of W99, as was observed by streptavidin pulldowns. A much stronger interaction was observed with AC5–C2 using this assay, probably owing to its greater capacity for renaturation (unpublished data). Thus, G $\beta\gamma$ is capable of interacting with all three cytoplasmic domains of AC5. Compared with wild-type G $\beta\gamma$, the W99A hotspot mutant showed a 2- to 3-fold decrease in binding affinity for the 5C2 domain (Fig. 5C). Likewise, G $\beta\gamma$ also bound to the AC6 C1 and C2 domains (Fig. 5D). The W99A-G $\beta\gamma$ mutant displayed impaired interactions with both AC6–C1 and AC6–C2, with an approximately 5- to 6-fold decrease in binding affinity for AC6–C2 compared with wild-type (Fig. 5E). Thus, G $\beta\gamma$ binds to the C1/C2 catalytic domains of both AC5 and AC6, including hotspot dependent interactions with the C2 domain.

AC5-G $\beta\gamma$ Interactions Depend on Multiple Sites within G $\beta\gamma$. Previously, Yuan et al. (2007) have shown that G $\beta\gamma$ can contact PLC β using the NT of G $\beta\gamma$ (residues 23–27) when the hotspot is otherwise occupied. Therefore we wanted

Fig. 3. $G\beta\gamma$ hotspot mutants interfere with AC6NT binding and stimulation of AC6. (A) Pull-down of GST or GST-tagged AC6NT (2 μ M) incubated with 300 nM wild-type $G\beta\gamma$ (WT) or the indicated $G\beta\gamma$ hotspot mutants. Elutes from GST pull-downs were analyzed by Western blotting. (B) AC activity assay with Sf9 membranes expressing AC6 were stimulated with 50 nM $G\alpha_s$ with or without 300 nM $G\beta\gamma$. Statistics: Student's *t* test of experiment means comparing the $G\beta\gamma$ WT group to the indicated hotspot mutant group; *n* = 4 with experiments performed in duplicate; **P* < 0.05; ***P* < 0.01. (C) AC activity assay with Sf9 membranes expressing AC5 or AC6 were stimulated with 50 μ M forskolin with or without 100 nM $G\beta\gamma$. Statistics: Student's *t* test of non-normalized experiment means of AC activity (nmol/min per milligram); *n* = 3 with experiments performed in duplicate; ***P* < 0.01; ****P* < 0.001. (D) Pull-down of GST-tagged AC NT (2 μ M) of the isoform indicated, incubated with 300 nM wild-type (WT) or hotspot mutant (W99A) $G\beta\gamma$. Elutes from GST pull-downs were analyzed by Western blotting.

to determine if $G\beta\gamma$ uses multiple interaction surfaces to regulate AC5 and/or AC6. Mutation of residues 23–27 of the $G\beta$ NT greatly impaired the ability of $G\beta\gamma$ to stimulate AC activity, with only marginal effects on binding to the AC5/6 NT (Fig. 6). Thus, multiple regions of $G\beta\gamma$ are required for regulation of AC5/6 activity.

Endogenous $G\alpha_s$ and $G\beta\gamma$ are readily detectable in immunoprecipitations of Flag-tagged AC5 from HEK293 cells (Sadana et al., 2009). To determine the surface(s) of $G\beta\gamma$ required, we analyzed the interactions of Flag-tagged AC5 with YN-tagged $G\beta$ (YN- $G\beta\gamma$) to differentiate them from endogenously expressed wild-type $G\beta\gamma$. The AC5- $G\beta\gamma$ binding observed was reduced with $G\beta\gamma$ -W99A or $G\beta\gamma$ -NT23–27, but mutation of both regions further reduced AC5- $G\beta\gamma$ association (Fig. 7, A and B). Similar to in vitro AC activity assays, both the hotspot and NT surfaces of $G\beta\gamma$ were required to interact with AC5 in cells. Immunoprecipitation results were consistent regardless of YN- $G\beta\gamma$ expression level, which ranged from 0.5- to 2.5-fold that of endogenous $G\beta\gamma$ compared by Western blot (Fig. 7C).

We further analyzed the requirement of both the $G\beta\gamma$ hotspot and NT sites in cellular interactions with AC5 using BiFC. $G\beta$ wild-type and mutants were tagged with the N-terminal half of YFP (YN) and AC5 was tagged with the YC (Fig. 8A). Interaction of YN- $G\beta\gamma$ and YC-AC5 brings the nonfluorescent fragments of YFP in close proximity to allow formation of a functional fluorescent protein (Fig. 8, B and C), as observed for the YN- $G\beta$ /YC- $G\gamma$ 7 control. The majority of the fluorescent signal for YN- $G\beta$ /YC- $G\gamma$ 7 and YN- $G\beta$ /YC-AC5 occurs at the plasma membrane (Fig. 8C). However, incorporation of either W99A or NT23–27 mutation in YN- $G\beta$ reduced interactions of YN- $G\beta\gamma$ with AC5, as indicated by lower BiFC fluorescence compared with wild-type YN- $G\beta\gamma$ (Fig. 8, B and C). A further reduction of BiFC was observed when both hotspot and NT regions of YN- $G\beta$ were mutated, similar to immunoprecipitation of AC5/ $G\beta\gamma$ complexes (Fig. 7). A low level of YFP fluorescence is detected over background in the

double $G\beta\gamma$ mutant (Fig. 8, B and C), consistent with 5NT scaffolding of $G\beta\gamma$ that is independent of the $G\beta$ hotspot or NT23–27 (Figs. 2B and 6A). Therefore, $G\beta\gamma$ regulation of AC5 utilizes multiple regions of both $G\beta\gamma$ and AC5 for scaffolding and enzyme stimulation.

Discussion

We have explored $G\beta\gamma$ interactions and regulation of AC isoforms, particularly the differences in scaffolding versus stimulation of AC5. We conclude that the $G\beta\gamma$ hotspot is necessary for AC5 stimulation but not solely required for interaction at the AC5NT. This contrasts with AC6, for which the $G\beta\gamma$ hotspot is required for all known AC6 interaction sites. For both AC5 and AC6, $G\beta\gamma$ interacts with all three intracellular domains of AC: NT, C1, and C2. The multiple interaction sites on AC5/6 mirror the multiple points of contact on $G\beta\gamma$, emphasizing the complexity of $G\beta\gamma$ regulation of ACs. The hotspot-independent interaction of $G\beta\gamma$ with AC5NT and insensitivity to SIGK inhibition highlights the mechanistic differences of AC5-versus-AC6 regulation by $G\beta\gamma$ and the potential for isoform-specific AC NT function with respect to $G\beta\gamma$.

Scaffolding Roles for the Adenylyl Cyclase N Terminus. All tested AC isoforms interact with $G\beta\gamma$ via the NT domain, despite the wide variance in NT sequence, size, and isoform-specific regulation by $G\beta\gamma$. The $G\beta\gamma$ binding site on AC5NT at aa 66–137 may represent a scaffolding site, a direct regulatory binding site, or both. The AC5NT 66–137 site is also not required for heterologous sensitization of AC5, despite being a process for which both $G\alpha_s$ and $G\beta\gamma$ are involved (Avidor-Reiss et al., 1996; Ejendal et al., 2012). AC6NT both interacts with the hotspot of $G\beta\gamma$ and is required for activation (Gao et al., 2007). Our pull-down binding assays may underestimate reversible interactions, such as those between $G\beta\gamma$ and AC6NT. The AC5NT probably contains two binding sites for $G\beta\gamma$; the first is a scaffolding site for inactive G

Fig. 4. SIGK peptide blocks G $\beta\gamma$ regulation of AC in an isoform-specific manner. (A) Sf9 membranes expressing the indicated AC isoform were stimulated with 50 nM G α_s with or without G $\beta\gamma$ (50 nM G $\beta\gamma$ for AC1 and AC2, 300 nM for AC5 and AC6). AC assays were in the presence or absence of 10 μ M SIGK peptide. A representative experiment is shown. Statistics: Paired *t* test of experiment means comparing the G $\beta\gamma$ -stimulated groups with and without SIGK; *n* = 3 with experiments performed in duplicate; **P* < 0.05; ***P* < 0.01; n.s., not significant. (B and C) SIGK inhibition curves with Sf9 membranes expressing AC5 (B) or AC6 (C). AC containing Sf9 membranes were stimulated with 50 nM G α_s with or without 300 nM G $\beta\gamma$ and the indicated concentrations of SIGK peptide (red) or a control peptide (SIGK L9A, black). A representative experiment is shown. Statistics: Paired *t* test of experiment means comparing the G $\beta\gamma$ -stimulated SIGK and L9A groups at each concentration indicated; *n* = 3 with experiments performed in duplicate; **P* < 0.05; ***P* < 0.01.

protein heterotrimer (Sadana et al., 2009). A distal second site, necessary for activation, may be present on AC5NT on the basis of homology with AC6. This is further supported by the fact that canine AC5, which is missing the first half of the AC6NT activation site, cannot be stimulated by G $\beta\gamma$ (Gao et al., 2007). Other AC isoforms may anchor G $\beta\gamma$ independently of any regulatory role. For example, G $\beta\gamma$ binds to AC9NT in a hotspot-dependent manner despite a lack of clarity as to whether G $\beta\gamma$ has a direct regulatory effect on human AC9 (Premont et al., 1996; Hacker et al., 1998). The distal C terminus of GIRK1 also anchors G $\beta\gamma$ yet is not a required interaction for GIRK1 regulation; it may be important for localized recruitment of G $\beta\gamma$ (Kahanovitch et al., 2014). The purpose of G $\beta\gamma$ binding to AC NT without a direct effect on AC activity is not clear at this time, but we propose that such isoform-specific scaffolding can localize G $\beta\gamma$ and/or G α subunits for dynamic signaling events.

It is noteworthy that AC5NT/G $\beta\gamma$ binding was not hotspot-dependent. The AC5NT may localize inactive G α_s - $\beta\gamma$ near an appropriate GPCR, allowing for rapid coupling and signal transduction. This is analogous to AKAPs, whose primary purpose is to act as a regulatory scaffold for protein kinase A and other signaling molecules. Notably, AKAP79/150 interacts with upstream β -AR receptors, as well as AC5/6 at their NT (Bauman et al., 2006; Efendiev et al., 2010). A larger complex facilitated by AKAP79/150 may contain all necessary

components for signaling from a G α_s -coupled GPCR to protein kinase A and downstream targets via AC5 generation of cAMP.

AC/G $\beta\gamma$ Interaction at Multiple Sites. We show that AC5 and AC6 have multiple interaction sites with G $\beta\gamma$. This is analogous to AC2, the AC isoform most thoroughly mapped for G $\beta\gamma$ binding. In addition to our observed binding of G $\beta\gamma$ to AC2NT, three binding sites have been mapped to the C1 domain and another two sites on the C2 domain of AC2 (Weng et al., 1996; Diel et al., 2006, 2008; Boran et al., 2011). AC5/6 show 65% homology to the site located within the C1a domain of AC2 (aa 339–360), whereas sites located within the C1b and C2 domains of AC2 are poorly conserved or not present in AC5/6 (Boran et al., 2011).

The multiple G $\beta\gamma$ binding sites on AC are consistent with the numerous interaction sites on G $\beta\gamma$. We show that both the G β NT residues 23–27 and the hotspot are necessary for AC5/6 stimulation by G $\beta\gamma$. Inhibition of G $\beta\gamma$ regulation by the G $\beta\gamma$ -disrupting peptide SIGK confirms hotspot interactions with AC isoforms 1, 2, and 6, consistent with previous studies detailing AC2 interactions with blades 1, 2, 4, 5, and 7 of the G $\beta\gamma$ hotspot (Ford et al., 1998; Panchenko et al., 1998). This is comparable to small peptide competition of other effectors that use various contacts within the hotspot, namely PLC β 2 and PI3K (Scott et al., 2001). Surprisingly, AC5 was resistant to competition with the SIGK peptide at concentrations up to

Fig. 5. $G\beta\gamma$ interacts with AC5/6 catalytic domains. (A) Streptavidin pulldown of 300 nM biotin-tagged $G\beta\gamma$ wild-type (WT) or mutant incubated with 500 nM His-tagged AC5-C1. Pulldowns analyzed by SDS-PAGE and Western blot. (B and D) $G\beta\gamma$ overlay assay using AC5-C1 and AC6-C2 (B) or AC6-C1 and AC6-C2 (D) immobilized on polyvinylidene fluoride membrane and probed with $G\beta\gamma$ WT or W99A. (C and E) Quantitation of relative WT or W99A $G\beta\gamma$ binding to AC5-C2 (C) or AC6-C2 (E) domains ($n = 3$).

30 μ M. There is also precedence for unsuccessful peptide competition for regulatory $G\beta\gamma$ interactions, namely voltage-gated calcium channels (Scott et al., 2001). However, to our knowledge this is the first noted contrast between different isoforms of a given effector, emphasizing a clear difference in $G\beta\gamma$ regulation of AC5. Additional contacts in AC5 probably

exist to support these AC/ $G\beta\gamma$ regulatory distinctions. The SIGK peptide interacts with the inner core region of $G\beta$, spanning six of seven blades of the WD40 barrel-like structure at the hotspot (Bonacci et al., 2006). Interactions with $G\beta$ NT residues 23–27 may help to overcome competition by SIGK. Moreover, interactions with AC5NT and C1 domain are the

Fig. 6. Mutation of $G\beta$ NT inhibits stimulation of AC5/6 but not binding to AC5/6 NT. Pulldown of GST-tagged AC5NT (A) or AC6NT (C) with purified $G\beta\gamma$ wild-type (WT) or $G\beta$ (NT23–27) γ mutant. Final concentrations were 2 μ M GST-ACNT and 300 nM $G\beta\gamma$. Elutes from GST pulldowns were analyzed by Western blotting. (B and D) AC activity assays with Sf9 membranes expressing AC5 (B) or AC6 (D) were stimulated with 50 nM $G\alpha_s$ and indicated concentration of $G\beta\gamma$. Statistics: Student's t test comparing non-normalized experimental means of AC activity (nmol/min per milligram) from $G\beta\gamma$ WT group to NT23–27 mutant group at the concentration indicated; $n = 4$ for AC5; $n = 3$ for AC6, with experiments performed in duplicate; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 7. Multiple sites on G $\beta\gamma$ are involved in binding to AC5 in HEK293 cells. (A) Immunoprecipitation of Flag-tagged AC5 and YN-G $\beta\gamma$ wild-type (WT) or mutants. HEK293 cells were transfected with the indicated plasmids and subjected to immunoprecipitation using anti-Flag agarose resin. Wild-type G γ 2 was coexpressed with all YN-G β constructs. Associated proteins were analyzed by Western blotting. (B) Quantitation of Flag-AC5/YN-G $\beta\gamma$ IP-Westerns. Statistics: immunoprecipitation band intensity of YN-G β was corrected for total YN-G β expression level in lysate and a paired t test was performed, comparing YN-G $\beta\gamma$ WT to the indicated YN-G $\beta\gamma$ mutant ($n = 4$); $*P < 0.05$. YN-G $\beta\gamma$ was set to 100%. (C) Western blotting of lysate samples from (A) was performed using an anti-G β antibody to compare expression of YN-G $\beta\gamma$ to endogenous G $\beta\gamma$ 42 hours after transfection.

least reliant on G $\beta\gamma$ hotspot interactions. G $\beta\gamma$ residues 23–27 are used for regulation of PLC β (Bonacci et al., 2005), and for interaction with AGS8 when the hotspot is otherwise occupied (Yuan et al., 2007). It has also been suggested that PI3K interacts with the G β NT at residues 31–45 (Dbouk et al., 2012). However, G β NT residues 23–27 are also critical for AC6 regulation by G $\beta\gamma$ and thus cannot solely explain the

differential sensitivity to SIGK. Alternatively, AC5 may interact with blade 6 of G $\beta\gamma$, a region that does not contact SIGK, to provide G $\beta\gamma$ regulation that is independent of SIGK competitive inhibition.

The mechanisms of G $\beta_1\gamma_2$ regulation on AC examined herein probably translate to other β and γ isoforms. G $\beta\gamma$ heterodimers containing G β_{1-4} all likewise inhibit AC1 and

Fig. 8. Multiple sites on G $\beta\gamma$ are required for cellular AC5 interactions as measured by bifluorescence complementation. (A) Schematic of YN-G $\beta\gamma$ and YC-AC5 constructs. (B) Quantitation of BiFC. As indicated, AC5, G β , and G γ subunits were transiently transfected in HEK293 cells. Wild-type (WT) G γ 2 was coexpressed with all YN-G β constructs with the exception for the indicated YC-G γ 7 control. YFP fluorescence was measured 48 hours after transfection and normalized to DAPI fluorescence. A representative experiment is shown ($n = 4$, each experiment performed in triplicate). Statistics: one-way analysis of variance of experimental means ($n = 4$), comparing the YN-G $\beta\gamma$ WT + YC-AC5 group and the three YN-G $\beta\gamma$ mutant + YC-AC5 groups; $**P < 0.01$. (C) YFP images of HEK293 cells expressing the indicated YN/YC-tagged G $\beta\gamma$ /AC5 proteins.

stimulate AC2, albeit with slight differences in potency (Iníguez-Lluhi et al., 1992). In addition, $G\beta_2$ was previously identified as a binding partner for AC8NT (Crossthwaite et al., 2006); we show that $G\beta_1$ also binds AC8NT (Fig. 3D). $G\beta_5$, the isoform associated with regulators of G protein–signaling proteins rather than traditional γ subunits, probably does not interact with AC isoforms by a similar mechanism. Previous work on $G\beta_5$ /RGS9-2 inhibition of AC5 showed that $G\beta_5$ /RGS9-2 interacts with the AC5 C1/C2 domains but not AC5NT (Xie et al., 2012).

AC5/ $G\beta\gamma$ Regulatory Mechanism. An analogous mechanism to the $G\alpha_s$ -dependent conditional stimulation of AC by $G\beta\gamma$ is displayed by the bacterial invasion protein BepA (Pulliainen et al., 2012). BepA directly contacts the C2 domain of AC2 but only enhances activity in the presence of forskolin or $G\alpha_s$. Conditional regulators such as BepA or $G\beta\gamma$ may bind sites on the C1/C2 domains but are unable to induce an active state of AC without the presence of a strong activator. AC 2, 5, and 6 all display a high degree of synergy between forskolin and $G\alpha_s$ and are therefore likewise poised for conditional stimulation. The NT domain may also participate in conditional regulation, forming direct contacts with the C1 domain and, to a lesser extent, the C2 domains of AC5 to increase catalytic activity (Sadana et al., 2009). $G\beta\gamma$ binding to the distal NT of AC5/6 may facilitate these interactions and the closure of the C1/C2 catalytic site. Whatever the mechanism, the transmembrane domains may facilitate this process, as $G\beta\gamma$ is incapable of stimulating the purified NT/C1/C2 domains in vitro (Sadana et al., 2009). In addition, $G\beta\gamma$ association with the plasma membrane via γ subunit prenylation was required for $G\beta\gamma$ stimulation of AC (Gao et al., 2007). We propose a model in which inactive G protein heterotrimer is scaffolded to AC5NT, whereas activated G protein subunits interact with the AC5/6 catalytic domains and the distal NT site of AC6 (and possibly AC5) to directly enhance AC5/6 activity.

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Authorship Contributions

Participated in research design: Brand, Sadana, Dessauer.

Conducted experiments: Brand, Sadana.

Contributed new reagents or analytic tools: Malik, Smrcka.

Performed data analysis: Brand, Dessauer.

Wrote or contributed to the writing of the manuscript: Brand, Dessauer.

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