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 $G_{\alpha\gamma}-G_{\beta\gamma}$ 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 ACSNT, 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 ACSNT 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" $G_{\alpha}$ subunit. $G_{\beta\gamma}$ subunits can enhance $G_{\alpha\gamma}$- 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; Diei et al., 2006). Thus, the effects of $G_{\beta\gamma}$ on cAMP production are dependent on isoform specificity.

In the inactive $G_{\alpha\gamma}$ 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 $G_{\alpha\gamma}$ and $G_{\beta\gamma}$ subunits are needed to observe a full stimulatory AC6 response to the $G_{\alpha}$-coupled receptor agonist isoproterenol (Gao et al., 2007). This suggests that $G_{\beta\gamma}$ generated from activation of $G_{\alpha}$ 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 $G_{\alpha}$-coupled G protein–coupled receptors (GPCRs) to further increase AC activity generated from $G_{\alpha}$-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),...
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 Gβγ stimulation of AC5. Gβγ binding to residues 66–137 of AC5NT is synergistically enhanced in the presence of GDP-Ga_i, and can bind as a G protein heterotrimer to AC5NT (Sadana et al., 2009). Other Gβγ effectors, such as phospholipase C β (PLCβ), 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 Ga_i-βγ at the AC5NT is independent of Gβγ stimulation of AC5, supporting a model in which inactive heterotrimer occupies the NT and a separate binding event results in Gβγ stimulation of AC5. Conversely for AC6, stimulation by Gβγ requires residues 77–151 of AC6NT (Gao et al., 2007). This is despite much regulatory overlap between AC5 and AC6, including stimulation by Gβγ.

The Gβγ structure includes β-sheet WD40 repeats, or “blades,” and a “hotspot” area where the turns between blades intersect. The hotspot on Gβγ 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 Gβγ requires the hotspot to stimulate AC5 but utilizes a different surface to interact with AC5NT. A similar scaffold/stimulation mechanism occurs for Gβγ and Ga_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 Gβγ are also required for stimulation of AC5 and AC6. Despite Gβγ binding to the N termini of many AC isoforms, only interactions with AC5NT were not reliant on an intact Gβγ hotspot. Lastly, we show for the first time that Gβγ 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 Gβi (both wild-type and mutants), Gγ2, and Ga_i were used to express biotinylated Gβiγ2 variants as described (Bonacci et al., 2005; Davis et al., 2005).

Flag-tagged AC5 pcDNA was a generous gift from Dr. Michael Kaploff (University of Miami, Miami, FL) and has been used previously (Sadana et al., 2009). YN-Gβ1 was a generous gift from Catherine Berlot (Weis Center for Research, Danville, PA) and consists of Gβ1 fused to the first 158 aa of yellow fluorescent protein (YN, N-terminal half of YFP). The YN-Gβ1-W99A pcDNA plasmid was generated by polymerase chain reaction mutagenesis. NT23–27 was generated by mutagenesis of the Gβ1 aa 23–27 sequence KACAD to ANCAA and cloned into the YN-Gβ1 or YN-Gβ1-W99A pcDNA template to produce either YN-Gβ1-NT23–27 or YN-Gβ1-NT23–27/W99A double mutant pcDNA plasmid. YC-γ1 was a generous gift from Catherine Berlot and consists of Ga_i 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-Gβ (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)H6 (AC5 aa 364–670), H65C2 (AC2 aa 938-end), and Ga_iH6 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 Gβiγ2 was coexpressed with Ga_iH6 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% CO2. HEK293 (3–5 × 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 μg 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 Gβγ subunits in 50 μl of binding buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM MgCl2, 2 mM dithiothreitol (DTT), 100 mM NaCl, 0.1% C12E8). The proteins were incubated for 30 minutes at 4°C followed by addition of 100 μl 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 MgCl2, 2 mM DTT, 100 mM NaCl, 0.05% C12E8). Bound proteins were eluted with 15 mM glutathione and analyzed by SDS-PAGE. For biotin-tagged protein pulldowns, purified biotin-tagged Gβγ was incubated with His-tagged 5C1(670)H6 in 50 μl of binding buffer for 30 minutes at 4°C. After incubation, 100 μl 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× 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 48 hours, HEK293 cells were rinsed with phosphate-buffered saline, resuspended in lysis buffer (50 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM MgCl2, 1 mM DTT, 150 mM NaCl, 0.5% C12E8, and protease inhibitors), and triturated using a 23-gauge syringe. Cellular debris was removed by centrifugation, and 30 μl 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% C12E8. Proteins were eluted from anti-Flag resin with SDS-PAGE sample buffer, and analyzed by SDS-PAGE and Western blotting.

Gβγ Binding Overlays. Bovine serum albumin controls, His-tagged AC5/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 μg purified Gβγ 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 Gβγ was detected by Western blotting.
Adenyl Cyclase Membrane Assays. Membrane assays were performed essentially as described previously (Dessauer, 2002). SI9 membrane preparations were incubated for 8–10 minutes at 30°C with an AC mix containing [α-32P]ATP and appropriate activators, including forskolin or GTPγS-Gαs, with or without the indicated concentrations of Gβγ. 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 [32P]cAMP product, using [3H]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, Gαs, and Gγ plasmids. Approximately 47 hours after transfection, cells were stained with DMEM containing DAPI (4',6-diamidino-2-phenylindole; 10 μg/ml) for one hour at 37°C. Cells were then transferred in 150 μl 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

Gβγ Binds to Various AC Isoform N Termini. Gβγ binding to the NT of AC has been observed previously for AC5 and AC6, either alone or as a Gα 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 Gβγ (Fig. 1A). To determine if Gβγ binding is a conserved property of all AC NT, GST pulldowns were performed using GST-tagged AC NT and purified Gβγ. As shown in Fig. 1B, Gβγ 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 Gβγ (Crosswhaite et al., 2006).

Gβγ 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 Gβγ scaffolding at the AC5NT and activation of AC5 were separable events. To separate these events, we used alanine-substituted mutants of the Gβγ hotspot region required for effector interactions (Fig. 2A) (Ford et al., 1998; Yuan et al., 2007). Mutations within the Gβγ hotspot did not alter binding to AC5NT in GST-pulldown assays (Fig. 2B). However, when assayed for their ability to increase Gαs-stimulated AC5 activity in vitro, Gβγ 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 Gβγ, 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 Gβγ was independent of the Gβγ scaffolding site within the AC5NT (aa 66–137) (Fig. 2D). Thus, AC5NT binding and stimulation of AC5 by Gβγ are indeed separable events, requiring different Gβγ surfaces.

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

Previous work had shown that Gβγ could conditionally stimulate AC5/6 in the presence of either Gαs or forskolin (Gao et al., 2007). We show that the Gβγ 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 Gβγ with AC, independent of Gαs.

Since the Gβγ hotspot was necessary for binding to the AC6NT but not AC5NT, we screened the other AC NT with wild-type versus W99A hotspots Gβγ to determine the isomform specificity of hotspot-dependent Gβγ/ACNT binding. The hotspot mutation of Gβγ 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 Gβγ is isoform-specific.
Pharmacological Targeting of Gβγ Supports a Unique AC5 Regulatory Mechanism Compared with Other AC Isoforms. In addition to alanine-scanning mutants of Gβγ, we also used a different method of investigating Gβγ-AC interactions. The small SIGK peptide has been used previously to disrupt select hotspot-dependent Gβγ interactions with effector proteins (Scott et al., 2001; Davis et al., 2005). Addition of SIGK prevented Gβγ regulation of several AC isoforms, including Gβγ inhibition of AC1 and stimulation of AC2 and AC6 (Fig. 4A). Addition of SIGK also had a small effect on AC1, 2, and 6 activities in the absence of added Gβγ, presumably owing to blockade of endogenous Gβγ present in Sf9 membranes (Fig. 4, A and C). However, SIGK was surprisingly unable to block AC5 stimulation by Gβγ, even at higher concentrations of SIGK peptide (Fig. 4, A and B). The AC isoform differences from pharmacological targeting of the Gβγ hotspot support a unique AC5-Gβγ regulatory mechanism.

Gβγ Binds to AC5/6 Catalytic Domains. Although multiple Gβγ 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). Gβγ 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 Gβγ interacted with the C1/C2 domains of AC5 and AC6. Streptavidin pulldowns of biotin-tagged purified Gβγ showed strong interactions with the H4-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 Gβγ. To overcome this limitation, we performed a Gβγ-binding overlay assay in which the C1 and C2 domains were separated by SDS-PAGE, re-natured, and incubated with wild-type or W99A purified Gβγ (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, Gβγ is capable of interacting with all three cytoplasmic domains of AC5. Compared with wild-type Gβγ, the W99A hotspot mutant showed a 2- to 3-fold decrease in binding affinity for the 5C2 domain (Fig. 5C). Likewise, Gβγ also bound to the AC6 C1 and C2 domains (Fig. 5D). The W99A-Gβγ 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, Gβγ binds to the C1/C2 catalytic domains of both AC5 and AC6, including hotspot dependent interactions with the C2 domain.

AC5-Gβγ Interactions Depend on Multiple Sites within Gβγ. Previously, Yuan et al. (2007) have shown that Gβγ can contact PLCβ6 using the NT of Gβγ (residues 23–27) when the hotspot is otherwise occupied. Therefore we wanted...
to determine if Gβγ uses multiple interaction surfaces to regulate AC5 and/or AC6. Mutation of residues 23–27 of the Gβ NT greatly impaired the ability of Gβγ to stimulate AC activity, with only marginal effects on binding to the AC5/6 NT (Fig. 6). Thus, multiple regions of Gβγ are required for regulation of AC5/6 activity.

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

We further analyzed the requirement of both the Gβγ hotspot and NT sites in cellular interactions with AC5 using BiFC. Gβγ 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-Gβγ 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-Gβ/YC-Gγ7 control. The majority of the fluorescent signal for YN-GβγYC-Gγ7 and YN-GβγYC-AC5 occurs at the plasma membrane (Fig. 8C). However, incorporation of either W99A or NT23–27 mutation in YN-Gβ reduced interactions of YN-Gβγ with AC5, as indicated by lower BiFC fluorescence compared with wild-type YN-Gβγ (Fig. 8, B and C). A further reduction of BiFC was observed when both hotspot and NT regions of YN-Gβ were mutated, similar to immunoprecipitation of AC5/Gβγ complexes (Fig. 7). A low level of YFP fluorescence is detected over background in the double Gβγ mutant (Fig. 8, B and C), consistent with 5NT scaffolding of Gβγ that is independent of the Gβ hotspot or NT23–27 (Figs. 2B and 6A). Therefore, Gβγ regulation of AC5 utilizes multiple regions of both Gβγ and AC5 for scaffolding and enzyme stimulation.

**Discussion**

We have explored Gβγ interactions and regulation of AC isoforms, particularly the differences in scaffolding versus stimulation of AC5. We conclude that the Gβγ hotspot is necessary for AC5 stimulation but not solely required for interaction at the AC5NT. This contrasts with AC6, for which the Gβγ hotspot is required for all known AC6 interaction sites. For both AC5 and AC6, Gβγ 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 Gβγ, emphasizing the complexity of Gβγ regulation of ACs. The hotspot-independent interaction of Gβγ with AC5NT and insensitivity to SIGK inhibition highlights the mechanistic differences of AC5-versus-AC6 regulation by Gβγ and the potential for isoform-specific AC NT function with respect to Gβγ.

**Scaffolding Roles for the Adenylyl Cyclase N Terminus.** All tested AC isoforms interact with Gβγ via the NT domain, despite the wide variance in NT sequence, size, and isoform-specific regulation by Gβγ. The Gβγ 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 Gαs and Gβγ are involved (Avidor-Reiss et al., 1996; Ejendal et al., 2012). AC6NT both interacts with the hotspot of Gβγ and is required for activation (Gao et al., 2007). Our pulldown binding assays may underestimate reversible interactions, such as those between Gβγ and AC6NT. The AC5NT probably contains two binding sites for Gβγ; the first is a scaffolding site for inactive Gβγ.
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 Gbg (Gao et al., 2007). Other AC isoforms may anchor Gbg independently of any regulatory role. For example, Gbg binds to AC9NT in a hotspot-dependent manner despite a lack of clarity as to whether Gbg has a direct regulatory effect on human AC9 (Premont et al., 1996; Hacker et al., 1998). The distal C terminus of GIRK1 also anchors Gbg yet is not a required interaction for GIRK1 regulation; it may be important for localized recruitment of Gbg (Kahanovitch et al., 2014). The purpose of Gbg 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 Gbg and/or Ga subunits for dynamic signaling events.

It is noteworthy that AC5NT/Gbg binding was not hotspot-dependent. The AC5NT may localize inactive Gaβγ 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 Gaα-coupled GPCR to protein kinase A and downstream targets via AC5 generation of cAMP.

**AC/Gbg Interaction at Multiple Sites.** We show that AC5 and AC6 have multiple interaction sites with Gbg. This is analogous to AC2, the AC isoform most thoroughly mapped for Gbg binding. In addition to our observed binding of Gbg 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 Gbg binding sites on AC are consistent with the numerous interaction sites on Gbg. We show that both the Gbg NT residues 23–27 and the hotspot are necessary for AC5/6 stimulation by Gbg. Inhibition of Gbg regulation by the Gbg-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 Gbg 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 PLCβ2 and PI3K (Scott et al., 2001). Surprisingly, AC5 was resistant to competition with the SIGK peptide at concentrations up to

![Fig. 4. SIGK peptide blocks Gbg regulation of AC in an isoform-specific manner. (A) SF9 membranes expressing the indicated AC isoform were stimulated with 50 nM Gaα with or without Gbg (50 nM Gbg for AC1 and AC2, 300 nM for AC5 and AC6). AC assays were in the presence or absence of 10 μM SIGK peptide. A representative experiment is shown. Statistics: Paired t test of experiment means comparing the Gbg-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 Gaα, with or without 300 nM Gbg 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 Gbg-stimulated SIGK and L9A groups at each concentration indicated; n = 3 with experiments performed in duplicate; *P < 0.05; **P < 0.01.](molpharm.aspetjournals.org)
There is also precedence for unsuccessful peptide competition for regulatory Gβγ 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 Gβγ regulation of AC5. Additional contacts in AC5 probably exist to support these AC/Gβγ regulatory distinctions. The SIGK peptide interacts with the inner core region of Gβ, spanning six of seven blades of the WD40 barrel-like structure at the hotspot (Bonacci et al., 2006). Interactions with Gβ NT residues 23–27 may help to overcome competition by SIGK. Moreover, interactions with AC5NT and C1 domain are the

30 μM. There is also precedence for unsuccessful peptide competition for regulatory Gβγ 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 Gβγ regulation of AC5. Additional contacts in AC5 probably exist to support these AC/Gβγ regulatory distinctions. The SIGK peptide interacts with the inner core region of Gβ, spanning six of seven blades of the WD40 barrel-like structure at the hotspot (Bonacci et al., 2006). Interactions with Gβ NT residues 23–27 may help to overcome competition by SIGK. Moreover, interactions with AC5NT and C1 domain are the

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

**Fig. 6.** Mutation of Gβ NT inhibits stimulation of AC5/6 but not binding to AC5/6 NT. Pulldown of GST-tagged AC5NT (A) or AC6NT (C) with purified Gβγ wild-type (WT) or Gβ(NT23–27)γ mutant. Final concentrations were 2 μM GST-ACNT and 300 nM Gβγ. 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 Ga and indicated concentration of Gβγ. Statistics: Student’s t test comparing non-normalized experimental means of AC activity (nmol/min per milligram) from Gβγ 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.
least reliant on Gβγ hotspot interactions. Gβγ residues 23–27 are used for regulation of PLCβ (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 Gβ NT at residues 31–45 (Dbouk et al., 2012). However, Gβ NT residues 23–27 are also critical for AC6 regulation by Gβγ and thus cannot solely explain the differential sensitivity to SIGK. Alternatively, AC5 may interact with blade 6 of Gβγ, a region that does not contact SIGK, to provide Gβγ regulation that is independent of SIGK competitive inhibition.

The mechanisms of Gβ1γγ regulation on AC examined herein probably translate to other β and γ isoforms. Gβγ heterodimers containing Gβ1–4 all likewise inhibit AC1 and
stimulate AC2, albeit with slight differences in potency (Iniguez-Lluhi et al., 1992). In addition, Gβ2 was previously identified as a binding partner for AC8NT (Crosthwaiate et al., 2006); we show that Gβ2 also binds AC8NT (Fig. 3D). Gβ2 binds to a site common to AC5 and AC6, which is therefore likely to be a site for Gβγ-mediated regulation. Previous work on Gβγ/RGS9-2 inhibition of AC5 showed that Gβ2/RGS9-2 interacts with the AC5/C2 domains but not AC5NT (Xie et al., 2012).

AC5/Gβγ Regulatory Mechanism. An analogous mechanism to the Gα2-dependent conditional stimulation of AC by Gβγ 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 Gαs. Conditional regulators such as BepA or Gαγ 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 syntegrity between forskolin and Gαs, and they 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). Gβγ 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 Gβγ is incapable of stabilizing the purified NT/C1/C2 domains in vitro (Sadana et al., 2009). In addition, Gβγ association with the plasma membrane via γ subunit prenylation was required for Gβγ 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

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References


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