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Title Page

Adenylyl Cyclase 5 Regulation by Gβγ Involves Isoform Specific Use of Multiple Interaction Sites

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Running Title Page:

Complexity of Gβγ Regulation of AC

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Abbreviations:

AC: Adenylyl Cyclase

AKAP: A-Kinase Anchoring Protein

BiFC: Bifluorescence Complementation

C1: Catalytic Domain 1

C2: Catalytic Domain 2

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle Medium

GFP: Green Fluorescent Protein

GPCR: G-Protein Coupled Receptor

GST: Glutathione S-Transferase

HEK: Human Embryonic Kidney

NT: N-Terminus

PKA: Protein Kinase A

PVDF: Polyvinylidene Fluoride

SIGK: Gβγ hotspot-competing peptide

YFP: Yellow Fluorescent Protein

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Abstract

Adenylyl cyclase (AC) converts ATP into cyclic AMP (cAMP), an important 2nd messenger in cell signaling. Heterotrimeric G proteins and other regulators are important for control of AC activity. Depending on the AC isoform, Gβγ subunits can either conditionally stimulate or inhibit cAMP synthesis. We previously showed that the Gαs-βγ heterotrimer binds to the N-terminus (NT) of type 5 AC (AC5). We now show that Gβγ binds to the NT of a wide variety of AC isoforms. We hypothesized that Gβγ/AC5 interactions involving inactive heterotrimer and Gβγ stimulation of AC5 were separable events. Mutations of the Gβγ "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 Gβγ "hotspot" is required for both interactions with AC6NT and for stimulation of AC6. Additionally, the SIGK hotspot peptide disrupts Gβγ regulation of AC isoforms 1, 2, and 6, but not AC5. Gβγ also binds the C1/C2 catalytic domains of AC5 and AC6. Finally, cellular interactions with full-length AC5 depend upon multiple sites on Gβγ. This suggests an isoform-specific mechanism where bound Gβγ at the AC5NT is ideally situated for spatiotemporal control of AC5. We propose Gβγ 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.

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Introduction

Adenylyl cyclase (AC) converts ATP into cyclic AMP (cAMP), an important 2nd messenger in cell signaling. Isoform specificity provides complexity to AC regulation, and allows for a wide variety of physiological 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 GTP-bound "active" Gαs subunit. Gβγ subunits can enhance Gαs- or forskolin-stimulated AC 2, 4, 5, 6, and 7 activity but have no effect alone (Bayewitch et al., 1998; Federman et al., 1992; Gao and Gilman, 1991; Gao et al., 2007; Iniguez-Lluhi et al., 1992; Tang and Gilman, 1991; Taussig et al., 1993; Yoshimura et al., 1996). However, Gβγ can also inhibit the activity of AC 1, 3, and 8 (Diel et al., 2006; Steiner et al., 2005; Tang and Gilman, 1991). Thus, the effects of Gβγ on cAMP production are dependent on isoform specificity.

In the inactive Gα-βγ conformation, regions on the Gα and Gβγ 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 Gαs and Gβγ subunits are needed to observe a full stimulatory AC6 response to the Gs-coupled receptor agonist isoproterenol (Gao et al., 2007). This suggests that Gβγ generated from activation of Gs enhances AC5/6 activity. This differs from the conditional Gβγ stimulation of AC2/4/7, where the Gβγ subunit is provided via crosstalk by activation of Gi-coupled GPCRs to further increase AC activity generated from Gs-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

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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 Gβγ (Crossthwaite et al., 2006; Gao et al., 2007), AC5NT associates with the guanine nucleotide exchange factor Ric8a (Wang et al., 2007), while 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 PKC also vary by isoform (Chou et al., 2004; Crossthwaite et al., 2006; Lai et al., 1999; 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 (Efendiev et al., 2010; Piggott et al., 2008).

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-Gαs, and can bind as a G protein heterotrimer to AC5NT (Sadana et al., 2009). Other Gβγ effectors, such as PLCβ, GIRK channels, and RACK1, are also capable of scaffolding G protein heterotrimers (Berlin et al., 2010; Dell et al., 2002; Yuan et al., 2007). The binding of Gαs-βγ at the AC5NT is independent from Gβγ stimulation of AC5, supporting a model where 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 motif repeats, or “blades”, and a “hotspot” area where the turns between blades intersect. The “hotspot” on Gβγ is required for interactions with either the alpha subunit or numerous effectors (Davis et al., 2005; Scott et al., 2001; Wall et al., 1995). We hypothesized that Gβγ requires the “hotspot” to stimulate AC5, but utilizes a

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different surface to interact with AC5NT. A similar scaffold/stimulation mechanism occurs for Gβγ and Gαi regulation of GIRK channels (Berlin et al., 2010; Rubinstein et al., 2009). 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 Gβ 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.

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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 and Gilman, 1991; Tang et al., 1991). Baculoviruses for biotinylated Gβ1 (both wild-type and mutants), Gγ2, and Gαi were used to express b-Gβ1γ2 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, 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 YFP (YN). The YN-Gβ1-W99A pcDNA plasmid was generated by PCR 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-γ7 was a generous gift from Catherine Berlot and consists of Gγ7 fused to aa 159-238 of YFP (YC). YC-AC5 consists of AC5 fused to aa 159-238 of YFP.

Antibodies

Antibodies used were rabbit anti-Gβ (Santa Cruz), mouse anti-GST (Santa Cruz), rabbit anti-H6 (Bethyl), rabbit anti-GFP (Cell Signaling), and anti-Flag (Sigma). All antibodies diluted at 1:1000 in TBS-T for Western blots.

Protein Purifications and Sf9 Membrane Preparation

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Proteins 5C1(670)H_6 (AC5 aa 364-670), $H_6$5C2 (AC5 aa 938-end), and GαsH_6, were expressed in *Escherichia coli* and purified as described previously (Dessauer et al., 1998; Sunahara et al., 1997; Whisnant et al., 1996). GST-tagged proteins were expressed in *Escherichia coli* and purified using glutathione agarose resin as described previously (Salim et al., 2003). Non-tagged or biotin-tagged G$\beta_1\gamma_2$ was coexpressed with G$\alpha_i H_6$ 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).

HEK293 Cell Transfections

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. HEK293 (3-3.5 X 10^6 cells) were seeded 24 hours prior to transfection in 10 cm dish. Medium was replaced the next day with fresh DMEM (no penicillin/streptomycin) and cells were transfected with the appropriate plasmids (10 µg DNA total per 10 cm plate) using Lipofectamine 2000 (Invitrogen). 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 µl of binding buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 100 mM NaCl, 0.1% $C_{12}E_9$). The proteins were incubated for 30 min at 4°C followed by addition of 100 µl of 20% glutathione-agarose beads. After rotating for 2 hours at 4°C, the resin

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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 Gβγ was incubated with His-tagged 5C1(670)H_6 in 50 µl of binding buffer for 30 min 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 1X 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/IP). 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 µl of anti-Flag agarose was added. Samples were rotated at 4°C for 2 h, 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.

Gβγ Binding Overlays

BSA 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, PVDF membranes were incubated overnight with 10 µg purified Gβγ in 3 ml of overlay wash buffer

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(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.

Adenylyl Cyclase Membrane Assays

Membrane assays were performed essentially as described previously (Dessauer, 2002). Sf9 membrane preparations were incubated for 8-10 min at 30°C with an AC mix containing [α^{32}P]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 [^{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, Gβ, and Gγ plasmids. Approximately 47 hours after transfection, cells were stained with DMEM containing DAPI (10 µg/ml) for one hour at 37°C. Cells were then transferred in 150 µl PBS to black 96-well plates with clear, flat well bottoms (Greiner). YFP and DAPI signals were measured with a multi-well plate reader (Infinite M200, Tecan) 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 nm 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 poly-lysine coated coverslips.

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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).

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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 Gs 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 (Crossthwaite 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. In order to separate these events, we utilized 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, except for M101A which was still significantly reduced compared to 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 to wild-type Gβγ was independent of the Gβγ scaffolding site within the AC5NT (aa 66-137) (Fig.

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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 when compared to 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 due 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 hotspot Gβγ to determine the isoform 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. 3A, 3D). 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 to 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 (Davis et al., 2005; Scott et al.,

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2001). 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 AC 1, 2, and 6 activities in the absence of added Gβγ, presumably due to blockade of endogenous Gβγ present in Sf9 membranes (Fig. 4A, 4C). However, SIGK was surprisingly unable to block AC5 stimulation by Gβγ, even at higher concentrations of SIGK peptide (Fig. 4A, 4B). 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 based upon homology with AC6NT that a second site necessary for activation was likely present in AC5NT (see Fig. 1A). However, due 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 (Boran et al., 2011; Diel et al., 2008; Diel et al., 2006). 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 H_6-tagged C1 domain of AC5 that was independent of the W99A mutation (Fig. 5A). However, due 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 where the C1 and C2 domains were separated by SDS-PAGE, renatured, and incubated with WT or W99A purified Gβγ (Fig. 5B, 5C). Although the detection of AC5-C1 interactions was weak, it did not depend on mutation of W99, similar to what was observed by

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streptavidin pulldowns. A much stronger interaction was observed with AC5-C2 using this assay, likely due to its greater capacity for renaturation (data not shown). Thus, Gβγ is capable of interacting with all three cytoplasmic domains of AC5. Compared to WT Gβγ, the W99A hotspot mutant showed a 2-3 fold decrease in binding affinity for the 5C2 domain (Fig. 5C). Similarly, 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 5-6 fold decrease in binding affinity for AC6-C2 compared to WT (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 have shown that Gβγ can contact PLCβ using the NT of Gβγ (residues 23-27) when the hotspot is otherwise occupied (Yuan et al., 2007). 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 which surface(s) of Gβγ is required, we analyzed the interactions of Flag-tagged AC5 with YN-tagged Gβ (YN-Gβγ) to differentiate 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. 7A, 7B). Similar to *in vitro* AC activity assays, both the hotspot and NT surfaces of Gβγ

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were required to interact with AC5 in cells. Immunoprecipitation results were consistent regardless of YN-Gβγ expression level, which ranged from 0.5-fold to 2.5-fold that of endogenous Gβγ when 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 bifluorescence complementation (BiFC). Gβ wild-type and mutants were tagged with the N-terminal half of YFP (YN) while AC5 was tagged with the C-terminal half of YFP (YC) (Fig. 8A). Interaction of YN-Gβγ and YC-AC5 brings the non-fluorescent fragments of YFP in close proximity to allow formation of a functional fluorescent protein (Fig 8B, 8C), as observed for the YN-Gβ/YC-Gγ7 control. The majority of the fluorescent signal for YN-Gβ/YC-Gγ7 and YN-Gβ/YC-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 to wild-type YN-Gβγ (Fig. 8B, 8C). 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. 8B, 8C), consistent with 5NT scaffolding of Gβγ that is independent of the Gβ hotspot or NT23-27 (Fig. 2B and 6A). Therefore, Gβγ regulation of AC5 utilizes multiple regions of both Gβγ and AC5 for scaffolding and enzyme stimulation.

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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, where 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 mirrors 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 likely contains two binding sites for Gβγ; the first is a scaffolding site for inactive G protein heterotrimer (Sadana et al., 2009). A distal second site, necessary for activation, may be present on AC5NT based upon homology with AC6. This is further supported

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by the fact that canine AC5, which is missing the first half of the AC6NT activation site, cannot be stimulated by Gβγ (Gao et al., 2007). Other AC isoforms may anchor Gβγ independent of any regulatory role. For example, Gβγ binds to AC9NT in a hotspot-dependent manner despite a lack of clarity as to whether Gβγ has a direct regulatory effect on human AC9 (Hacker et al., 1998; Premont et al., 1996). The distal C-terminus of GIRK1 also anchors Gβγ, yet is not a required interaction for GIRK1 regulation; it may be important for localized recruitment of Gβγ (Kahanovitch et al., 2014). The purpose of Gβγ 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 Gβγ and/or Gα subunits for dynamic signaling events.

It is noteworthy that AC5NT/Gβγ binding was not hotspot-dependent. The AC5NT may localize inactive Gαs-βγ near an appropriate GPCR, allowing for rapid coupling and signal transduction. This is analogous to A-kinase anchoring proteins (AKAPs) whose primary purpose is as a regulatory scaffold for PKA 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 Gαs-coupled GPCR to PKA and downstream targets via AC5 generation of cAMP.

AC/Gβγ Interaction at Multiple Sites

We show that AC5 and AC6 have multiple interaction sites with Gβγ. This is analogous to AC2, the AC isoform most thoroughly mapped for Gβγ binding. In addition to our observed binding of Gβγ to AC2NT, three binding sites have been mapped to the C1 domain and another two sites on the C2 domain of AC2 (Boran et al., 2011; Diel et al., 2008; Diel et al., 2006; Weng

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et al., 1996). AC5/6 show 65% homology to the site located within the C1a domain of AC2 (aa 339-360), while 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 Gβγ binding sites on AC are consistent with the numerous interaction sites on Gβγ. We show that both the Gβ NT residues 23-27 and the hotspot are necessary for AC5/6 stimulation by Gβγ. Inhibition of Gβγ regulation by the Gβγ 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 Gβγ hotspot (Ford et al., 1998; Panchenko et al., 1998). This is comparable to small peptide competition of other effectors that utilize 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 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 likely exist to support these AC/Gβγ regulatory distinctions. The SIGK peptide interacts with the inner core region of Gβ, spanning 6 of 7 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 least reliant on Gβγ hotspot interactions. Gβγ residues 23-27 are utilized for regulation of PLCβ (Bonacci et al., 2005), and for interaction with AGS8 when the hotspot is otherwise occupied (Yuan et al., 2007). PI3K has also been suggested to interact 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.

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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 Gβ1γ2 regulation on AC examined herein likely translate to other β and γ isoforms. Gβγ heterodimers containing Gβ1-4 all similarly 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 (Crossthwaite et al., 2006); we show that Gβ1 also binds AC8NT (Fig. 3D). Gβ5, the isoform associated with RGS proteins rather than traditional γ subunits, likely does not interact with AC isoforms by a similar mechanism. Previous work on Gβ5/RGS9-2 inhibition of AC5 showed that Gβ5/RGS9-2 interacts with the AC5 C1/C2 domains but not AC5NT (Xie et al., 2012).

AC5/Gβγ Regulatory Mechanism

An analogous mechanism to the Gαs-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 Gβγ 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 Gαs, and are therefore similarly 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

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stimulating 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 where 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 reagents or analytic tools: Malik, Smrcka

Performed data analysis: Brand, Dessauer

Wrote or contributed to writing of manuscript: Brand, Dessauer

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Footnotes

a) This work was supported by the National Institutes of Health Institute of General Medical Sciences [GM60419, GM81772].

b) Portions of this work were previously presented in published abstract form: Cameron S. Brand, Rachna Sadana, Sundeep Malik, Alan V. Smrcka, and Carmen W. Dessauer. Two Distinct Sites on Gβγ are Required for Binding to the N-Terminus Versus the Activation Site on Adenylyl Cyclase. Experimental Biology, April 9-13, 2011, Washington, D.C.

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Figure Legends

Figure 1: Gβγ Binds to Various AC N-Termini. A, Schematic of AC N-Termini grouped by similar isoforms. Direct regulation by Gβγ is indicated; * indicates inferred regulation from cellular studies. **B,** Pulldown of GST-tagged AC NT's with bound Gβγ. The final concentration of GST or GST-tagged NT's was 2 μM. Inputs and elutes from GST pulldowns were analyzed by Western blotting.

Figure 2: Gβγ Hotspot Mutants Bind to AC5NT But Are Unable to Stimulate AC5. A, Schematic of the Gβγ structure, with indicated sites of the alanine-substitution hotspot and NT23-27 mutants that were used in this paper. Schematic made using Visual Molecular Dynamics (VMD 1.9). **B,** Pulldown of GST or GST-tagged AC5NT (2 μM) incubated with 300 nM wild-type Gβγ (WT) or the indicated Gβγ hotspot mutants. Elutes from GST pulldowns were analyzed by Western blotting. **C,** AC activity assay of Sf9 membranes expressing AC5 were stimulated with 50 nM Gαs +/- 300 nM Gβγ. Statistics: student's t-test of experiment means comparing Gβγ 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 (Δ66-137) were stimulated with 50 nM Gαs +/- 300 nM Gβγ. Statistics: student's t-test of experiment means comparing the Gβγ WT group to the indicated hotspot mutant group, n=3 with experiments performed in duplicate, *$p < 0.05$, **$p < 0.01$.

Figure 3: Gβγ Hotspot Mutants Interfere with AC6NT Binding and Stimulation of AC6. A, Pulldown of GST or GST-tagged AC6NT (2 μM) incubated with 300 nM wild-type Gβγ (WT) or the indicated Gβγ hotspot mutants. Elutes from GST pulldowns were analyzed by Western

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blotting. **B,** AC activity assay with Sf9 membranes expressing AC6 were stimulated with 50 nM Gαs +/- 300 nM Gβγ. Statistics: student's t-test of experiment means comparing the Gβγ 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 50μM forskolin +/- 100 nM Gβγ. Statistics: student's t-test of non-normalized experiment means of AC activity (nmol/min/mg), n=3 with experiments performed in duplicate, **p < 0.01, ***p < 0.001. **D**, Pulldown of GST-tagged AC NT (2 μM) of the isoform indicated, incubated with 300 nM wild-type (WT) or hotspot mutant (W99A) Gβγ. Elutes from GST pulldowns were analyzed by Western blotting.

Figure 4: SIGK Peptide Blocks Gβγ Regulation of AC in an Isoform-Specific Manner. A, Sf9 membranes expressing the indicated AC isoform were stimulated with 50 nM Gαs +/- Gβγ (50 nM Gβγ 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 Gβγ-stimulated groups with and without SIGK, n=3 with experiments performed in duplicate, *p < 0.05, **p < 0.01. **B, C,** SIGK inhibition curves with Sf9 membranes expressing AC5 (**B**) or AC6 (**C**). AC containing Sf9 membranes were stimulated with 50 nM Gαs +/- 300 nM Gβγ 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 Gβγ-stimulated SIGK and L9A groups at each concentration indicated, n=3 with experiments performed in duplicate, *p < 0.05, **p < 0.01.

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Figure 5: Gβγ Interacts with AC5/6 Catalytic Domains. A, Streptavidin pulldown of 300 nM biotin-tagged Gβγ wild-type 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 PVDF 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).

Figure 6: Mutation of Gβ NT Inhibits Stimulation of AC5/6, but Not Binding to AC5/6 NT. A and B, Pulldown of GST-tagged AC5NT **(A)** or AC6NT **(B)** with purified Gβγ wild-type 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. **C, D,** AC activity assay with Sf9 membranes expressing AC5 (C) or AC6 (D) were stimulated with 50 nM Gαs and indicated concentration of Gβγ. Statistics: student's t-test comparing non-normalized experimental means of AC activity (nmol/min/mg) 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, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 7: Multiple Sites on Gβγ Are Involved in Binding to AC5 in HEK293 Cells. A, Immunoprecipitation of Flag-tagged AC5 and YN-Gβγ wild-type or mutants. HEK293 cells were transfected with the indicated plasmids and subjected to immunoprecipitation using anti-Flag agarose resin. Wild-type Gγ2 was co-expressed with all YN-Gβ constructs. Associated proteins were analyzed by Western blotting. **B,** Quantitation of Flag-AC5/YN-Gβγ IP-Westerns. Statistics: IP band intensity of YN-Gβ was corrected for total YN-Gβ expression level in lysate

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and a paired t-test was performed, comparing YN-Gβγ WT to the indicated YN-Gβγ mutant (n=4), *p < 0.05. For panel 7B, YN-Gβγ was set to 100%. **C,** Western blotting of lysate samples from panel A was performed using an anti-Gβ antibody to compare expression of YN-Gβγ to endogenous Gβγ 42 hours after transfection.

Figure 8: Multiple Sites on Gβγ Are Required for Cellular AC5 Interactions as Measured by Bifluorescence Complementation. A, Schematic of YN-Gβγ and YC-AC5 constructs. **B,** Quantitation of BiFC. As indicated, AC5, Gβ, and Gγ subunits were transiently transfected in HEK293 cells. Wild-type Gγ2 was co-expressed with all YN-Gβ constructs except for the indicated YC-Gγ7 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 ANOVA of experimental means (n=4), comparing the YN-Gβγ WT + YC-AC5 group and the three YN-Gβγ mutant + YC-AC5 groups, **p < 0.01. **C,** YFP images of HEK293 cells expressing the indicated YN/YC-tagged Gβγ/AC5 proteins.

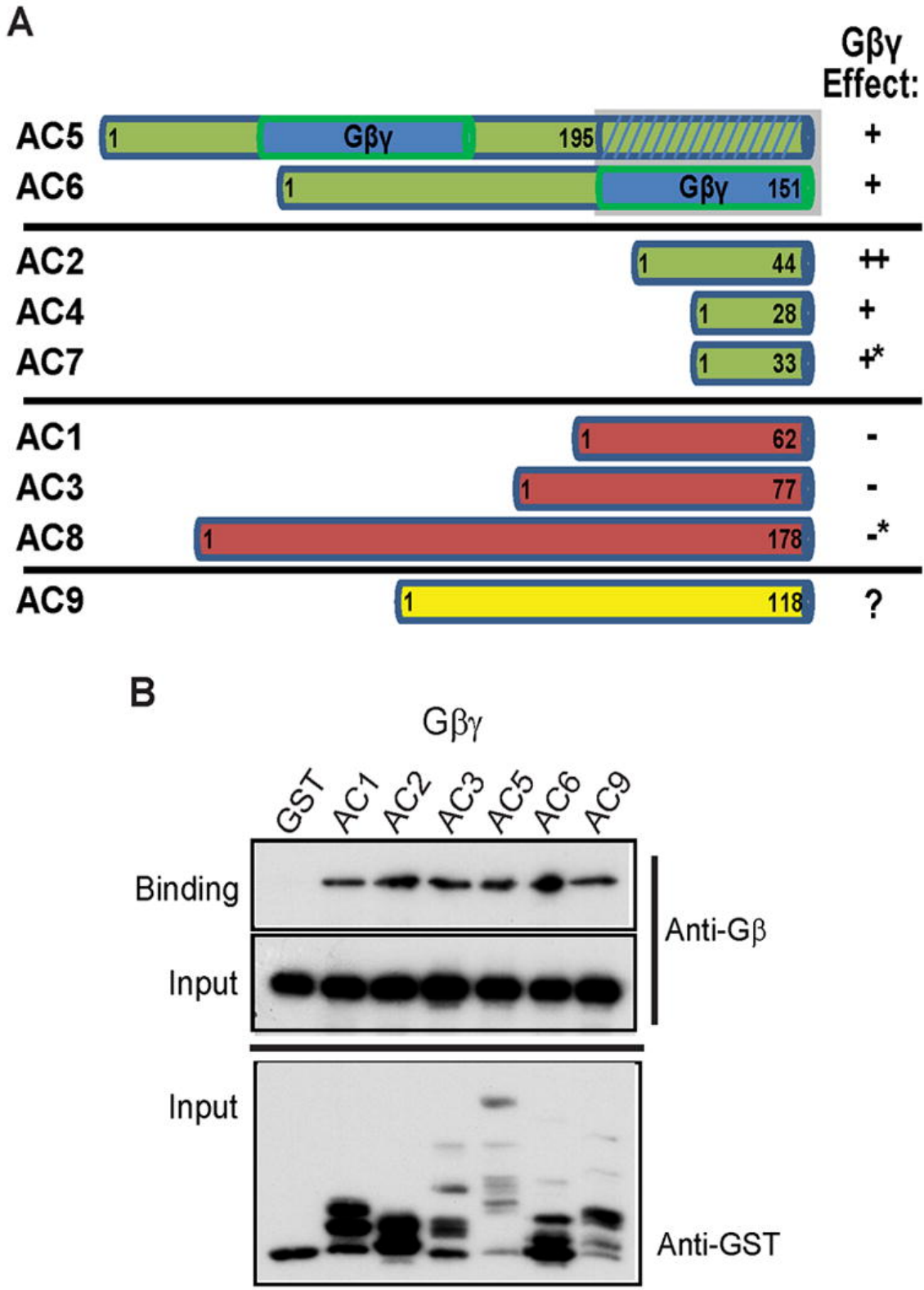
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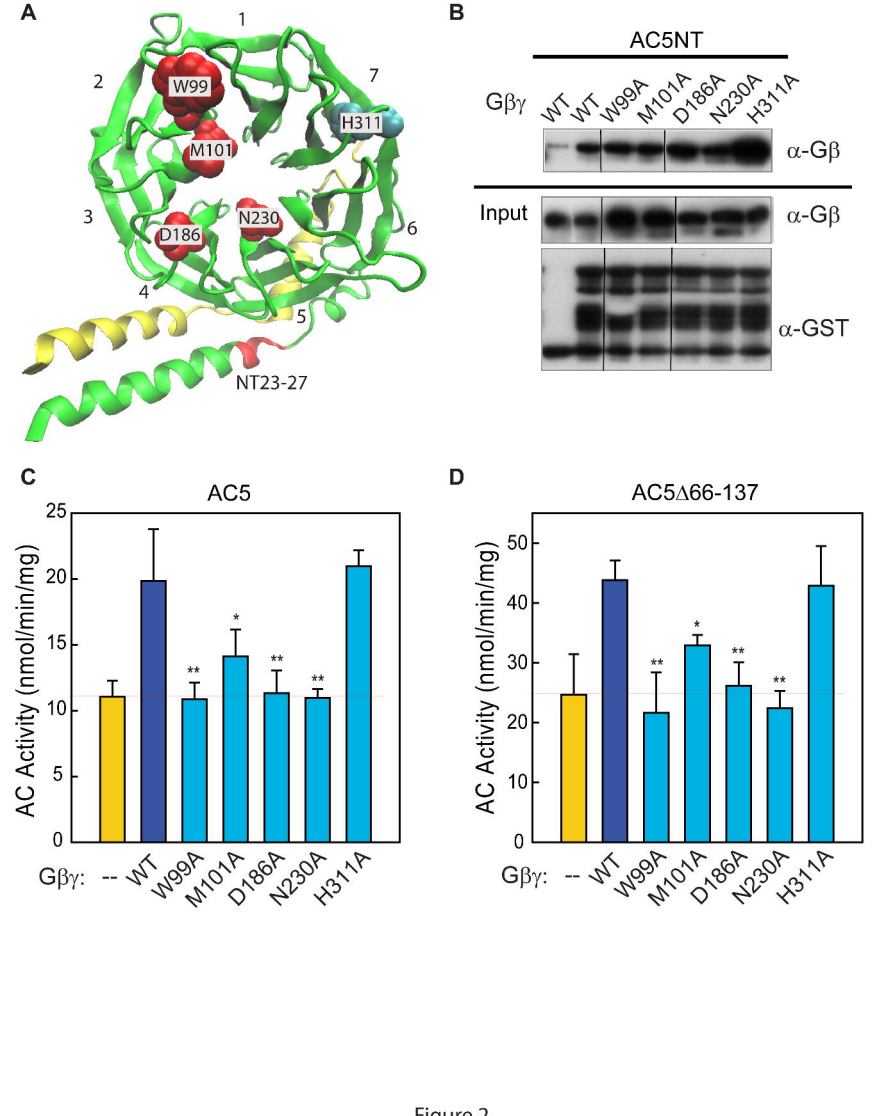
Figure 1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10
0
Gβγ: -- WT W99A M101A D186A N230A H311A

Figure 2

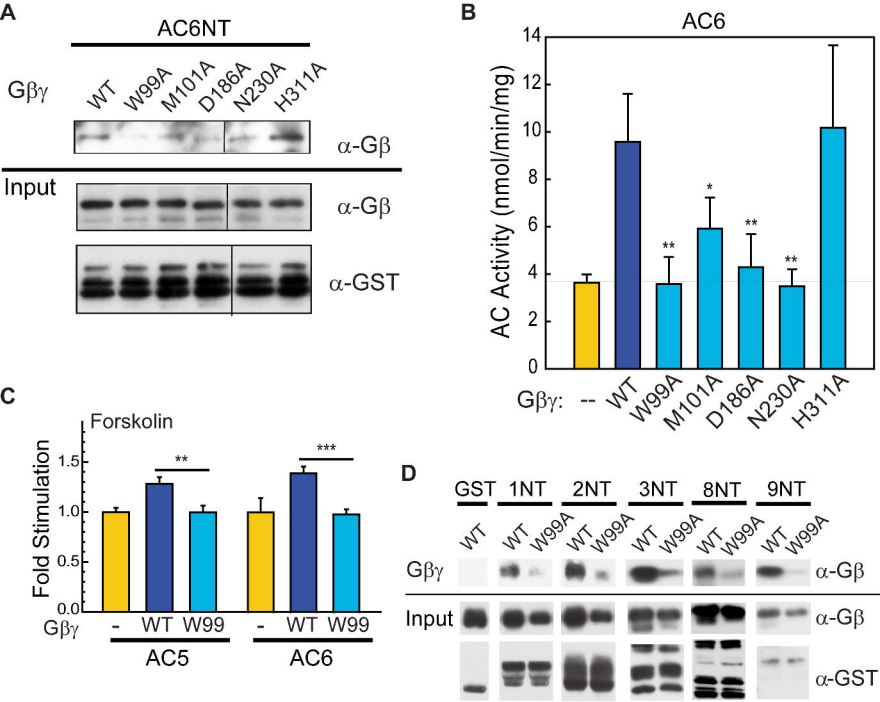
A
AC6NT
Gβγ
WT
W99A
M101A
D186A
N230A
H311A
α-Gβ
Input
α-Gβ
α-GST
B
AC6
AC Activity (nmol/min/mg)
14
12
10
8
6
4
2
0
*
**
**
**
**
Gβγ: -- WT W99A M101A D186A N230A H311A
C
Forskolin
Fold Stimulation
1.5
1.0
0.5
0.0
**
***
Gβγ - WT W99 - WT W99
AC5
AC6
D
GST
1NT
2NT
3NT
8NT
9NT
WT
WT W99A WT W99A WT W99A WT W99A WT W99A
Gβγ
α-Gβ
Input
α-Gβ
α-GST

Figure 3

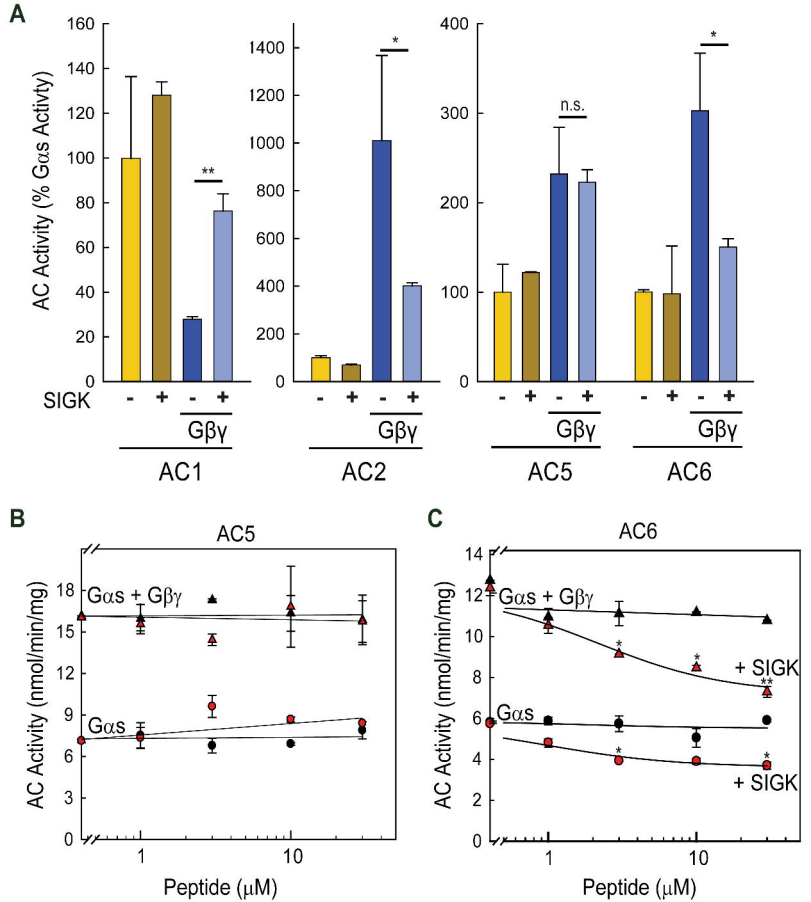
A
AC Activity (% Gαs Activty)
SIGK
Gβγ
AC1
AC2
AC5
AC6
n.s.
B
AC5
Gαs + Gβγ
Gαs
AC Activity (nmol/min/mg)
Peptide (μM)
C
AC6
Gαs + Gβγ
+ SIGK
Gαs
+ SIGK
AC Activity (nmol/min/mg)
Peptide (μM)

Figure 4

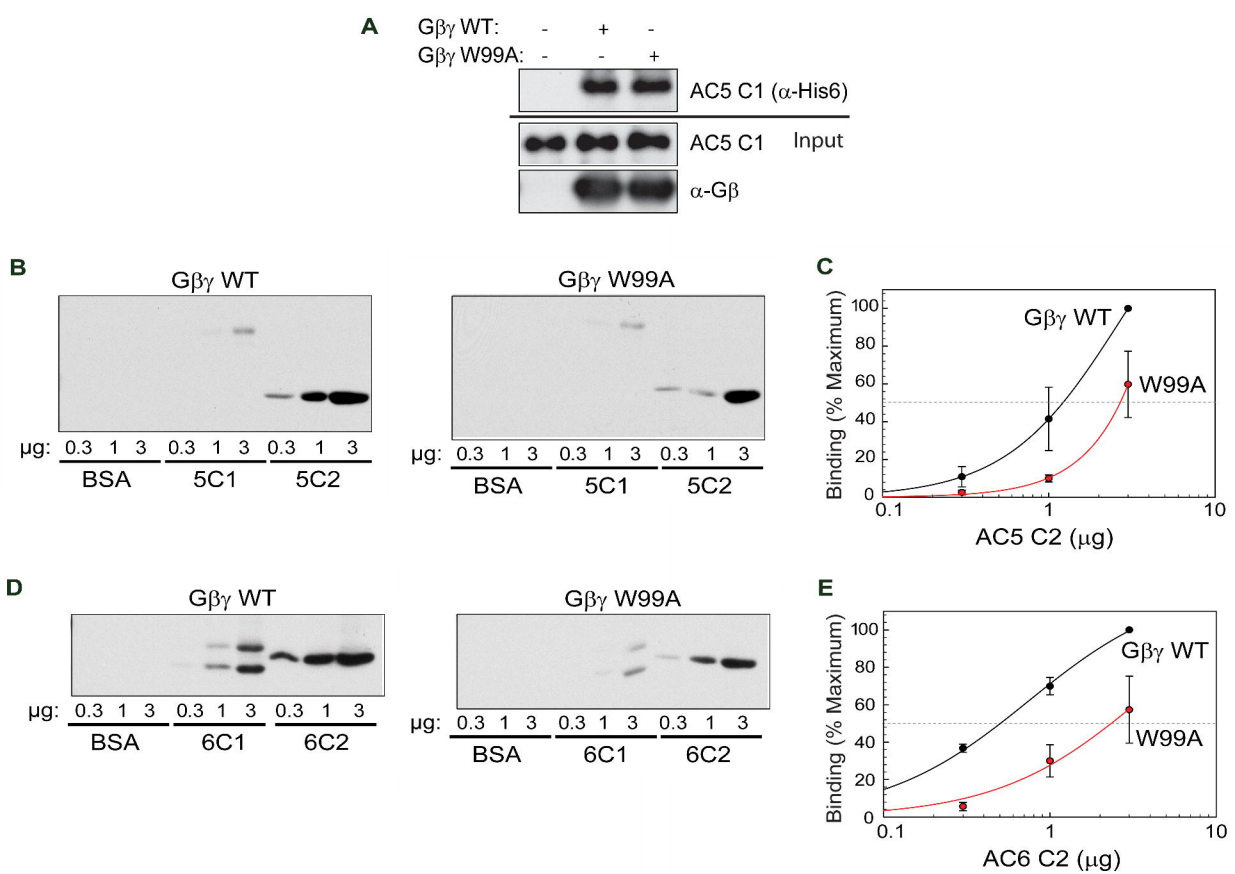
A
Gβγ WT: - + -
Gβγ W99A: - - +
AC5 C1 (α-His6)
AC5 C1
Input
α-Gβ
B
Gβγ WT
Gβγ W99A
μg: 0.3 1 3 0.3 1 3 0.3 1 3
BSA
5C1
5C2
C
Gβγ WT
W99A
Binding (% Maximum)
AC5 C2 (μg)
D
Gβγ WT
Gβγ W99A
μg: 0.3 1 3 0.3 1 3 0.3 1 3
BSA
6C1
6C2
E
Gβγ WT
W99A
Binding (% Maximum)
AC6 C2 (μg)

Figure 5

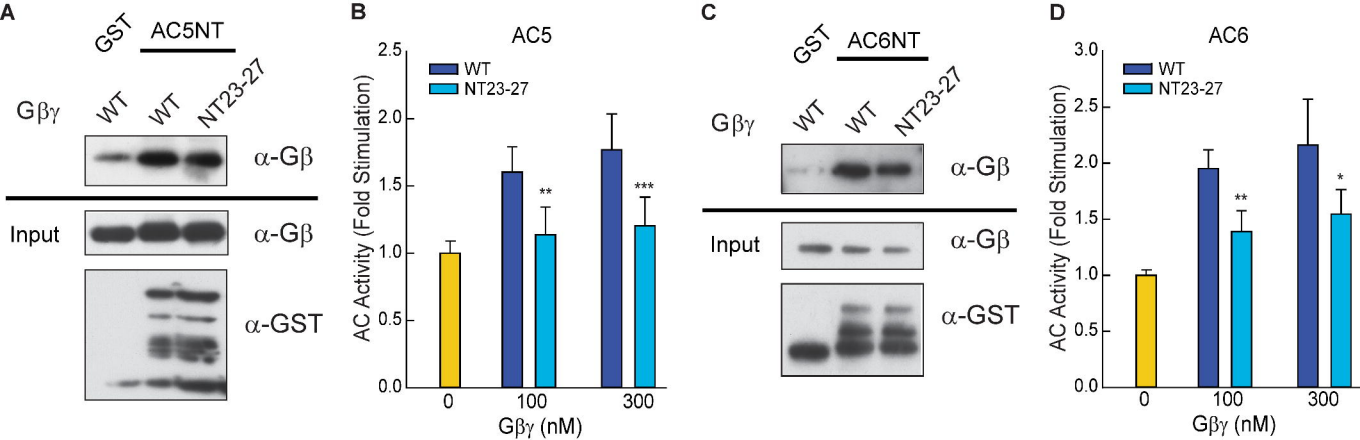
A
GST
AC5NT
Gβγ
WT
WT
NT23-27
α-Gβ
Input
α-Gβ
α-GST
B
AC5
WT
NT23-27
AC Activity (Fold Stimulation)
2.5
2.0
1.5
1.0
0.5
0.0
**
***
0
100
300
Gβγ (nM)
C
GST
AC6NT
Gβγ
WT
WT
NT23-27
α-Gβ
Input
α-Gβ
α-GST
D
AC6
WT
NT23-27
AC Activity (Fold Stimulation)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
0
100
300
Gβγ (nM)

Figure 6

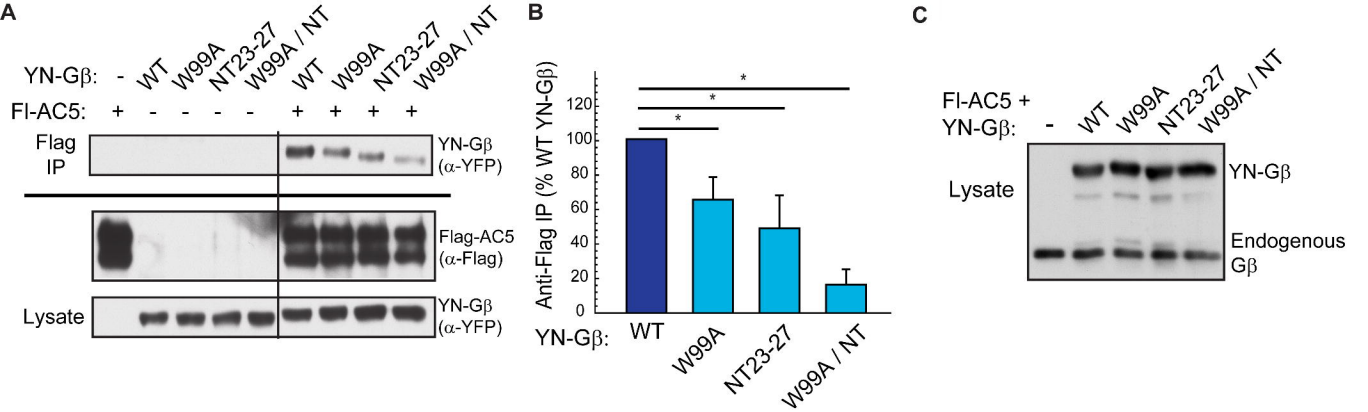
A
YN-Gβ: - WT W99A NT23-27 W99A / NT WT W99A NT23-27 W99A / NT
Fl-AC5: + - - - - + + + +
Flag IP
YN-Gβ (α-YFP)
Flag-AC5 (α-Flag)
Lysate
YN-Gβ (α-YFP)
B
Anti-Flag IP (% WT YN-Gβ)
120
100
80
60
40
20
0
*
*
*
YN-Gβ: WT W99A NT23-27 W99A / NT
C
Fl-AC5 + YN-Gβ: - WT W99A NT23-27 W99A / NT
Lysate
YN-Gβ
Endogenous Gβ

Figure 7

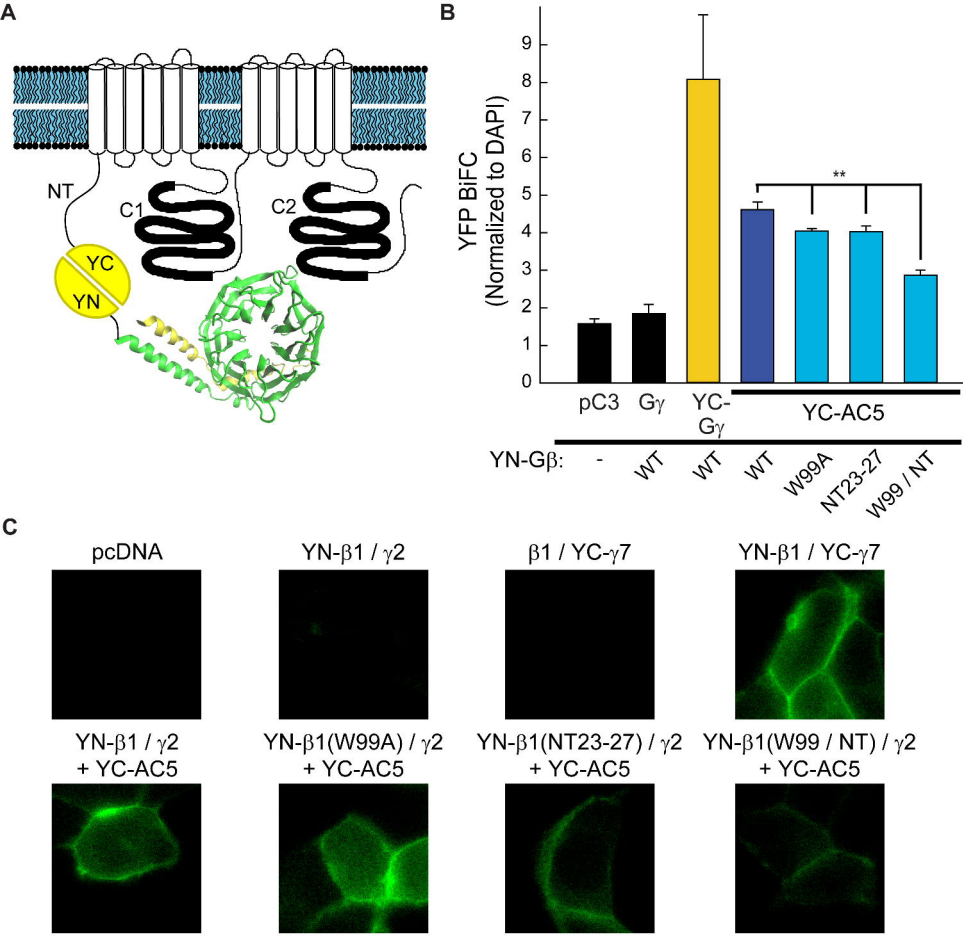
A
NT
C1
C2
YC
YN
B
YFP BiFC
(Normalized to DAPI)
0
1
2
3
4
5
6
7
8
9
**
pC3
Gγ
YC-
Gγ
YC-AC5
YN-Gβ:
-
WT
WT
WT
W99A
NT23-27
W99 / NT
C
pcDNA
YN-β1 / γ2
β1 / YC-γ7
YN-β1 / YC-γ7
YN-β1 / γ2
+ YC-AC5
YN-β1(W99A) / γ2
+ YC-AC5
YN-β1(NT23-27) / γ2
+ YC-AC5
YN-β1(W99 / NT) / γ2
+ YC-AC5

Figure 8