N-terminus of Type 5 Adenylyl Cyclase Scaffolds Gs Heterotrimer

Rachna Sadana, Nathan Dascal and Carmen W. Dessauer

Department of Integrative Biology and Pharmacology
University of Texas Health Science Center at Houston
Houston, Texas 77030 USA (R.S., C.W.D)

Department of Physiology and Pharmacology,
Sackler School of Medicine,
Tel Aviv University, Tel Aviv, 69978, Israel (N.D.)
Running Title: AC5 forms a stable complex with Gs heterotrimer

Corresponding author:
Carmen W. Dessauer, Ph.D.
Department of Integrative Biology and Pharmacology
University of Texas Health Science Center, Medical School
6431 Fannin St, Houston, TX 77030, USA
Tel: 713 500 6308, Fax: 713 500 7444
Email: Carmen.W.Dessauer@uth.tmc.edu

Number of text pages: 16
Number of tables: 1
Number of figures: 5
Number of references: 49
Number of words in Abstract: 247
Number of words in Introduction: 579
Number of words in Discussion: 1,310

Abbreviations
AC: Adenylyl cyclase; NT: N-terminus; AKAP: A-kinase anchoring protein; FRET: Fluorescence resonance energy transfer; YFP: Yellow fluorescent protein; CFP: Cyan fluorescent protein; Cer: Cerulean
ABSTRACT

According to accepted doctrine, agonist-bound G-protein coupled receptors catalyze the exchange of GDP for GTP and facilitate the dissociation of Ga and Gβγ, which in turn regulate their respective effectors. More recently, the existence of preformed signaling complexes which may include receptors, heterotrimeric G-proteins, and/or effectors is gaining acceptance. We show herein the existence of a pre-formed complex of inactive heterotrimer (Gαs·βγ) and the effector type 5 adenylyl cyclase (AC5), localized by the N-terminus of AC5. GST-fusions of AC5 N-terminus (5NT) bind to purified G-protein subunits (GDP-Gαs and Gβγ) with an apparent affinity of 270 ± 21 nM and 190 ± 7 nM respectively. GDP-bound Gαs and Gβγ did not complete, but rather facilitated their interaction with 5NT, consistent with the isolation of a ternary complex (5NT, Gαs and Gβγ) by gel filtration. The AC5/Gβγ interaction was also demonstrated by immunoprecipitation and FRET and the binding site of heterotrimer Gαs·βγ mapped to aa 60-129 of 5NT. Deletion of this region in full length AC5 resulted in significant reduction of FRET between Gβγ and AC. 5NT also interacts with the catalytic core of AC, mainly via the C1 domain to enhance Gαs- and forskolin-stimulated activity of C1/C2 domains. The N-terminus also serves to constrain Gai-mediated inhibition of AC5, which is relieved in the presence of Gβγ. These results reveal that 5NT plays a key regulatory role by interacting with the catalytic core and scaffolding inactive heterotrimeric G-proteins, forming a preassembled complex that is potentially braced for GPCR activation.
Cyclic AMP (cAMP) is a universal second messenger produced by a family of adenylyl cyclase (AC) enzymes. Nine membrane-bound AC isoforms have been identified and characterized. The topology of mammalian ACs consists of a variable N-terminus (NT) and two large cytoplasmic domains separated by two membrane spanning domains (6 TM’s each). The two cytoplasmic domains (C1 and C2) are roughly 40% identical and together form the enzyme’s catalytic core at their interface (Sadana and Dessauer, 2009). Many intracellular regulators of AC activity target the catalytic domains, including kinases, RGS proteins, and heterotrimeric G proteins. For example, Gαs binds to the C2 domain to increase affinity between the domains and thus increase activity, whereas Gαi binds to the C1 domain to inhibit a subset of AC isoforms, including AC 1, 5, and 6.

Increasing evidence suggests that the NT also plays an important role in regulating various isoforms of AC. For example, the NT can be the target of phosphorylation by protein kinases (Chou et al., 2004; Lai et al., 1999; Lai et al., 1997; Lin et al., 2002), anchor additional regulators of AC activity, including phosphatases (Crossthwaite et al., 2006), calmodulin (Simpson et al., 2006), or Ric8a (Wang et al., 2007); or recruit AC to larger multi-protein complexes involving AKAPs, as the case for AC2 and Yotiao (Piggott et al., 2008) or AC5 and mAKAP (Kapiloff et al., 2009). Finally, we recently showed that the NT of AC5 and AC6 can anchor Gβγ, facilitating Gβγ enhancement of isoproterenol stimulated activity of AC6 (Gao et al., 2007).

Although the nine membrane-bound AC isoforms each have unique and complex regulatory patterns, they are all stimulated by heterotrimeric G proteins via Gs-coupled receptors (Sadana and Dessauer, 2009). In the classical signaling paradigm of G-protein activation, an agonist-bound G protein coupled receptor (GPCR) promotes GDP-to-GTP exchange on Ga,
converting the inactive Gα·βγ complex into an active GTP-Gα, which then dissociates from Gβγ to allow for activation of AC (Gilman, 1987). More recently, a broadened concept of “preformed complexes” that include GPCR dimers, G protein heterotrimerics, effectors, and possibly other regulators, is gaining acceptance (Gales et al., 2005; Rebois et al., 2006). The evidence in support of this idea comes from biochemical and FRET/BRET studies on the effector proteins AC, inward rectifying K+ channels (GIRK) (Rebois et al., 2006), and PLCβ (Yuan et al., 2007). New models suggest a rearrangement and only a partial separation of GTP-Gα from Gβγ, such that the effector binding sites at the interface between Gα and Gβγ become exposed (Bünemann et al., 2003; Frank et al., 2005). Despite growing acceptance of this model, many key issues remain unresolved. We suggest that it is the effectors themselves that may help to hold G protein subunits in close proximity. We show herein that the N-terminus of AC5 (5NT) anchors “inactive” heterotrimeric G-proteins (GDP-Gαs·βγ). The heterotrimeric G protein binding site was mapped to amino acid region 60-129 of 5NT. Deletion of this region in full length AC resulted in a significant reduction of FRET between AC5 and Gβγ. Despite reduced FRET, mutant AC5 was stimulated by exogenously added GTPγS-Gαs and Gβγ suggestive of a separate activation site for Gβγ on AC5. We also show that AC5 NT interacts with its catalytic domains (5C1/5C2) to enhance the Gαs or forskolin stimulated activity of C1/C2. We propose that the N-terminus of AC5 is as a key regulatory domain that brings the inactive heterotrimeric G-proteins and catalytic core in close proximity for efficient GPCR activation.
MATERIALS AND METHODS

Materials. Antibodies used were rabbit-anti-\(G_\alpha\) (Calbiochem), rabbit-anti-\(G_\beta\) (Santa Cruz), mouse-anti-GST (Santa Cruz), rabbit-anti-H6 (Bethyl), rabbit-anti-AC5 (Santa Cruz and Core therapeutics), and anti-flag agarose (Sigma).

Plasmids Construction and Generation of Recombinant Viruses. Human AC5 N-terminus (5NT) was fused with GST (GST-5NT) in pGEX-CS vector (Gao et al., 2007). 6NT was generated by PCR, fusing human AC6 aa 1-143 to GST in pGEX-4T vector. Truncations of 5NT fused with GST (\(\Delta60, \Delta144, 1-147, 60-147, 1-129\) and 60-129) were generated by PCR. AC5\(\Delta66-137\) was created using PCR, deleting aa residues 66-137 within full length hAC5-pcDNA3. N-terminally tagged YFP-AC5 was created in multiple steps. YFP tagged pcDNA3 was created by PCR, generating Kpn1 and BamH1 sites at the 5’ and 3’ ends of YFP, respectively. Human AC5 was subcloned in two steps into the BamHI and XbaI sites of YFP-pcDNA3. YFP-AC5\(\Delta66-137\) was created by subcloning the SacII-NotI fragment from AC5\(\Delta66-137\) pcDNA3 into YFP-AC5 pcDNA3. Sequences were confirmed by nucleotide sequencing and restriction digests. N-terminal flag-tagged human AC5 and cerulean tagged G\(\beta1\) were generous gifts from Drs. Michael Kapiloff and Moritz Bünemann, respectively (Bünemann et al., 2003). Recombinant baculoviruses for full length AC5 and AC5\(\Delta66-137\) were created and expressed as previously described (Chen-Goodspeed et al., 2005).

Tissue Culture and Transfection. Human Embryonic Kidney cells (HEK293) and COS7 cells were maintained in Dulbecco’s modified Eagle’s media supplemented with 10% (v/v) fetal bovine serum and 50 \(\mu\)g/ml penicillin and streptomycin. For transient expression of proteins, cells were plated 24 hours prior to transfection. Transfections were performed using
Lipofectamine-2000 (Invitrogen) or Jet PEI (Genesee Scientific) as per manufacturer’s protocol. After 48 hrs of transfection, cells were used for the desired experiment.

**Protein Purification.** Proteins 5C1(670)H₆, H₆5C2, Gα₃-H₆, and myristoylated Gai-H₆ were expressed in *Escherichia coli* and purified as previously described (Dessauer et al., 1998; Sunahara et al., 1997; Whisnant et al., 1996). GST-tagged proteins were purified using glutathione agarose resin (Salim et al., 2003) . Non-tagged Gβγ₂ was co-expressed with Gai-H₆ in Sf9 cells and purified on Ni-NTA followed by ion-exchange as previously described (Kozasa and Gilman, 1995).

**GST Pull-Down Assays.** GST or GST-5NT (full length or truncations) were incubated with G-protein subunits in 50 µl binding buffer (20 mM Hapes, pH 8.0, 1 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 50 mM NaCl, 0.2% C₁₂E₉ and 2 µM GDP). For assays utilizing the cytoplasmic domains (5C1 or 5C2), the binding buffer included 0.05% C₁₂E₉ and no GDP. 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 hrs, the resin was washed three times with binding buffer containing 150 mM NaCl and 0.05% C₁₂E₉. For incubations containing 5C1 and 5C2 the NaCl was increased to 250 mM. Bound proteins were eluted with 15 mM glutathione, boiled with Laemmlli buffer and analyzed by SDS-PAGE and western blotting.

**Immunoprecipitation of AC5.** Human flag-tagged AC5 was transfected in HEK293 cells (10 cm dish/IP). After 48 hrs HEK293 cells were rinsed with PBS, resuspended in lysis buffer (20 mM Hapes, pH 8.0, 1 mM EDTA, 1 mM MgCl₂, 1 mM DTT, 150 mM NaCl, 0.5% C₁₂E₁₀, 1 µM GDP, and protease inhibitors), and homogenized 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 that contained only 0.05% C₁₂E₁₀.
Proteins were eluted with SDS-PAGE sample buffer, and analyzed by Western blotting.

**Gel Filtration Chromatography.** Proteins (10 µM, 100 µl sample volume) were applied on tandem superdex 75/200 columns (GE Healthcare) and resolved in gel filtration buffer (20 mM Hepes pH 8.0, 1mM EDTA, 5 mM MgCl₂, 1 mM DTT, 100 mM NaCl, 0.05% C₁₂E₉ and 10 µM GDP) at 0.3 ml/min, 4°C. Fractions (0.4 ml) were collected and samples were analyzed by SDS-PAGE and immunoblotting.

**Preparation of Membranes from HEK293 and Sf9 cells.** HEK293 cells were transfected with 10 µg of total DNA (per 10 cm plate) that included 2.5 µg of plasmid encoding AC5 or AC5Δ66-137. Membranes were prepared after 48 hrs as described (Piggott et al., 2008). Membranes from Sf9 cells expressing AC5 or AC5Δ66-137 were prepared after 48 hrs of infection with the respective baculovirus as described in (Dessauer et al., 2002).

**Assay of Adenylyl Cyclase Activity.** AC activity was measured in membranes as previously described (Dessauer, 2002). In assays containing purified 5C1 and 5C2 catalytic domain proteins, limiting concentrations of the C1 domain protein were assayed with 1 µM of 5C2 to promote interaction between the C1 and C2 proteins as previously described (Whisnant et al., 1996). GST or GST-5NT was preincubated with 5C1/5C2 prior to addition of activators.

**Fluorescence Resonance Energy Transfer (FRET).** Cell preparation: COS7 cells were plated on poly-L-lysine (0.01 mg/ml) coated coverslips in 6-well dishes at ~10-15% confluency. The next day cells were transfected with 2 µg of total DNA, which included 0.4 µg of YFP-AC5 or YFP-AC5Δ66-137, 0.9 µg Cerulean-Gβ1, and 0.7 µg of Gγ2. Prior to imaging, media was exchanged with tyrodes buffer (10 mM HEPES, pH 7.4, 145 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose).

Fluorescence Resonance Energy Transfer (FRET): Fluorescence images were acquired
after 48 hrs of transfection using Nikon TE 2000 microscope with a DG4 xenon light source and two coolsnap cameras. For FRET determinations, three images were acquired sequentially (exposure time, 200 ms) using the following filter sets: Donor (CFP; ex, 436/20 nm; em, 465/30 nm), FRET (CFP/YFP; ex, 436/20 nm; em, 535/30 nm) and Acceptor (YFP; ex, 500/20 nm; em 535/30 nm). Corrected, sensitized FRET (FRET\textsuperscript{c}) was calculated using the equation FRET\textsuperscript{c} = I_{\text{FRET}} - (a \times I_{\text{CFP}}) - (b \times I_{\text{YFP}}), where I_{\text{FRET}}, I_{\text{CFP}}, and I_{\text{YFP}} correspond to background-subtracted images of cells expressing CFP and YFP acquired through the FRET, CFP, and YFP channels, respectively. The values a and b are the bleedthrough values of CFP and YFP in FRET channel respectively. Calibrations of bleed-through were performed in cells expressing only CFP or YFP-tagged proteins and were calculated as 0.53 and 0.04 for CFP and YFP, respectively. In cells expressing both CFP and YFP-tagged proteins, normalized FRET values were calculated according to the following two methods (Vanderklish et al., 2000; Xia and Liu, 2001)

1. \( N^{\text{FRET}} = \frac{\text{FRET}^{\text{c}}}{\sqrt{I_D \times I_A}} \)

2. \( F^{\text{C/D}} = \frac{\text{FRET}^{\text{c}}}{I_D} \)

where FRET\textsuperscript{c} is the mean corrected FRET as calculated above, and I\textsubscript{D} and I\textsubscript{A} are the mean intensities of the donor (CFP or Cerulean), and acceptor (YFP) fluorescence. Pseudocolor FRET\textsuperscript{C/D} images were obtained using the Slidebook software (Nikon) and are displayed with deep blue indicating low values and bright red indicating high values of FRET.

**Statistical Analysis.** Each experiment was repeated at least three times in duplicate or triplicate. Comparison between different experiments groups was determined with the non-paired Student's \( t \) test. \( p < 0.05 \) is indicated with an asterisk in the figures. For fluorescence images, figures show representative images from 18-20 different cells from 4 different experiments.
RESULTS

AC5 N-terminus Anchors Heterotrimeric G-Proteins. We previously published that G\(\beta_1\gamma_2\) bound to the NT of human AC5 (5NT) (Gao et al., 2007). G\(\beta\gamma\) has no effect on AC activity alone, but increases the activity of AC5 and AC6 approximately 1.5 - 2 fold in the presence of G\(\alpha_s\) or forskolin. To further examine the nature of this interaction, we sought to compete G\(\beta\gamma\) binding to 5NT by addition of an excess of GDP-bound G\(\alpha_s\), which should mask the G\(\beta\gamma\) effector surface. Numerous crystallographic and mapping studies have identified an overlapping surface on G\(\beta\gamma\) that is used for both binding to alpha subunits and various effectors (Wall et al., 1995). However not only did GDP-G\(\alpha_s\) fail to compete with binding of G\(\beta\gamma\) to GST-5NT, it actually enhanced binding over two fold (Fig. 1A). In addition, the alpha subunit also bound to 5NT in the absence of G\(\beta\gamma\) (Fig. 1A,B). G\(\alpha_s\) binding to 5NT was enhanced by G\(\beta\gamma\) when present in the GDP-bound, aluminum fluoride-bound, and to GTP\(\gamma\)S-bound state (Fig 1A, B and S1). At the concentrations of G proteins used in these binding assays (1 \(\mu\)M), a complex of G\(\alpha\beta\gamma\) would be expected since the affinity of GDP-G\(\alpha_s\) for G\(\beta\gamma\) is 27 nM under the conditions used in our binding assays (Sarvazyan et al., 2002). Therefore, separate sites on G\(\alpha_s\) and G\(\beta\gamma\) are likely utilized to bind 5NT compared to the G\(\alpha\)-\(\beta\gamma\) interaction surface or effectors binding sites on G\(\alpha_s\) and G\(\beta\gamma\).

Apparent Affinity Measurements for G protein Binding to AC 5NT. To further characterize interactions with 5NT, we determined the apparent affinities for binding G\(\beta\gamma\) or G\(\alpha\). Increasing amounts of GDP-bound G\(\alpha\) or G\(\beta\gamma\) were added to GST-5NT (125-150 nM) and GST-pull down assays were performed (Fig. 1B). Binding to GST alone was tested at the highest level for each protein (3 \(\mu\)M). Binding data was quantitated from three independent experiments to determine apparent affinities (Fig 1B). G\(\beta\gamma\) (190 ± 7 nM) and GDP-G\(\alpha_s\) (270 ± 21 nM) binding to 5NT.
was saturable and displayed fairly high affinity binding. GTPγS- and GDP-bound Gαs displayed similar binding to 5NT and was comparable to the affinity of GTPγS-bound Gαs for the activation site on the C2 domain of AC5 (400 nM) (Sunahara et al., 1997). GDP-bound myristoylated Gαi1 also bound to 5NT but with a much reduced apparent affinity (~1.5 µM) compared to GDP-bound Gαs.

**Isolation of AC5/Gαsβγ Complex.** To show that it is indeed the heterotrimer that is bound to 5NT, we used gel filtration chromatography to evaluate the size and composition of potential complexes between GDP-Gαs, Gβγ, and 5NT (Fig 1C). An approximately 90 kDa complex of Gαsβγ can be isolated by gel filtration which shifts in the presence of 5NT to a complex of 140-145 kDa. This is consistent with a 1:1:1 complex consisting of 5NT/Gαsβγ. Smaller complexes of 5NT with either Gαs or Gβγ are also evident. Note that GST-tagged 5NT runs mainly as a monomer of 50 kDa but is also found at lower levels as a dimeric species, likely due to the tendency of GST to dimerize. Thus our results suggest that inactive heterotrimeric Gs can bind to AC5, challenging the dogma that heterotrimeric G proteins only interact with effectors in their “activated” GTP-bound state.

Gαsβγ can also be found in complex with full-length AC5. Endogenous Gαs and Gβγ are readily detectable in immunoprecipitations of flag-tagged AC5 from HEK293 cells (Fig 1D). This is consistent with a model in which heterotrimeric G proteins do not release from the effector and are poised to allow for regulation of AC by both subunits.

**G-Protein Subunits Bind to aa 60-129 of 5NT.** The binding sites for Gαs and Gβγ were further mapped on 5NT. GST fusion proteins of N- or C-terminal truncations of 5NT (Fig. 2A) were tested for binding GDP-bound Gαs or Gβγ. All fragments of 5NT supported binding to both Gαs and Gβγ except the region of 144-195 (Δ144). Both G protein subunits could be mapped to a
minimal region of 69 amino acids (aa 60-129), consistent with their binding as a closely associated heterotrimeric unit (Fig. 2B). These results were confirmed by gel-filtration chromatography where 5NT-60-129 clearly formed a complex with G\(\alpha_s\cdot\beta\gamma\) heterotrimer but \(\Delta144\) did not (Fig. 2C and S2).

We previously identified aa 77-151 in AC 6NT as necessary for G\(\beta\gamma\) regulation (Gao et al., 2007). In general, the N-terminal domains of different AC isoforms have little similarity; however there are small stretches of homology between aa 60-129 of 5NT and 77-151 of 6NT. We now show that both GDP-G\(\alpha_s\) and G\(\beta\gamma\) directly interact with 6NT as well (Fig. S3).

**5NT Interacts with the Catalytic Core to Enhance Gs or Forskolin Stimulated Activity.** The question remained as to how the NT may be regulating functional properties of AC. Previous reports have suggested that AC could act as a GAP or possibly GEF for G\(\alpha_s\) (Scholich et al., 1999), however, purified 5NT displays neither of these activities for G\(\alpha_s\) or G\(\alpha_i\) (Fig. S4). Rather, the NT pulls down a complex of 5C1/5C2/G\(\alpha_s\) in the presence or absence of forskolin and binds directly to the C1 domain of AC5 (Fig. 3A, B and S5), and to a lesser degree the C2 domain (Fig 3C). This interaction is isoform specific, as there is no interaction between the N-terminus of AC2 and the C1 domain of AC5 (Fig 3A). We have further mapped the binding site of the C1 and C2 domains on 5NT to aa 60-144, similar to that of the G protein subunits, however C1 does not compete with G\(\alpha_s\) or G\(\beta\gamma\) for 5NT binding (Fig. 3D, E). Computer modeling of 5NT suggests that this region is highly helical and thus different faces of this helix may be required for interactions with the C1 domain and heterotrimeric G proteins.

Most intracellular regulators of AC activity bind to the C1 and C2 domains of AC to exert their stimulatory or inhibitory affects. Thus it is possible that the NT can also affect the enzymatic state of AC5. 5NT increased the activity of the isolated C1 and C2 domains of AC5.
by 1.75 and 2 fold when stimulated by Gαs or forskolin, respectively, but displayed no effect on basal C1/C2 activity (S6). Consistent with our mapping studies, full length 5NT and 5NTΔ60, but not 5NTΔ144, could enhance the Gαs- or forskolin-stimulated activity of the AC5 C1/C2 domains in a dose-dependent manner (EC50 ~2 μM, Fig. 3F, G).

**AC5Δ66-137 Is Conditionally Stimulated By Exogenously Added Gβγ.** In order to determine if the Gαs·βγ binding site on NT is necessary for conditional stimulation of AC5 by Gβγ, we deleted the G protein binding site (Δ66-137) in the context of the full-length AC5 enzyme (Fig. 4A). AC5Δ66-137 can be activated by Gαs and/or forskolin when assayed in membranes from HEK293, COS7, and Sf9 cells (Fig. 4B, 4D, and 5A). However, AC5Δ66-137 displayed a small but highly reproducible 38% right shift of the Gαs dose-response curve (P<0.05, n=6). AC5Δ66-137 was also conditionally stimulated by exogenously added Gβγ, suggesting that AC5 activation by Gβγ does not require its binding to the N-terminus (Fig. 4D).

Previous studies have suggested that deletion of residues 1-86 of AC6 increases Gai inhibition (Kao et al., 2004). We examined the inhibition of AC5Δ66-137 by GTPγS-Gai in the presence and absence of Gβγ. Consistent with previous studies, deletion of 66-137 increased Gai efficacy and the IC50 compared to wild type AC5. This is likely due to the loss of 5NT interactions with the C1 domain, which binds GTPγS-Gai (Fig. 4E; (Dessauer et al., 1998; Kao et al., 2004). Upon addition of Gβγ, the percent inhibition of wild-type AC5 by Gai increased and was nearly identical to AC5Δ66-137 in the presence of Gβγ. Thus, Gβγ binding appears to reverse the constraints of the N-terminus on Gai inhibition, although the overall activity is still increased by Gβγ.

**Fluorescence Resonance Energy Transfer (FRET) between AC5 and Gβγ.** To explore the trafficking of AC5 to the PM, we constructed plasmids that place yellow fluorescent protein
(YFP) at the N-terminus of AC5 or AC5Δ66-137. Tagged proteins were tested for plasma membrane localization and activity. Upon expression in COS7 cells, YFP-tagged ACs displayed activity equal to their non-tagged versions (Fig. 5A). In addition, both YFP-AC5 and YFP-AC5Δ66-137 were found to be largely localized to the PM when expressed in HEK293 or COS7 cells at low levels (Fig. 5B). Higher levels of expression generally produced a particulate cytoplasmic fluorescence that was excluded from the nucleus. Therefore, we have carefully titrated AC expression levels that give rise to a largely PM expression pattern for all fluorescence and activity assays. Although previous reports have suggested that AC5 may be present on the nuclear envelope in cardiac myocytes (Belcheva et al., 1995; Boivin et al., 2006; Yamamoto et al., 1998), we detected no such localization upon expression in COS7 cells.

To analyze G protein interactions with AC5 in living cells, we used a FRET-based approach employing YFP-tagged AC5 and N-terminal Cerulean tagged Gβ1 (Cer-Gβ1). Cer-Gβ1 has previously been characterized for its plasma membrane localization and interactions with Gγ2 and Gαi (Bünemann et al., 2003). There is a strong inverse distance relationship between FRET and the distance separation between chromophores, such that FRET between the donor molecule CFP and acceptor molecule YFP only occurs if the two proteins are in close proximity (<100 Å). Significant FRET was observed at the plasma membrane between YFP-AC5 and Cer-Gβ1γ2 compared to myristoylated tagged CFP and YFP-AC5 (negative control; Fig 5B, and C). In addition, no significant FRET was observed between Cer-Gβ1γ2 and the transmembrane protein YFP-Na,K-ATPase (data not shown). Numerous mathematical methods are used to quantify FRET. We compared the two most commonly used methods FC/D (Vanderklish et al., 2000) and NFRET (Xia and Liu, 2001). Using either method, a significant decrease in FRET was observed for YFP-AC5Δ66-137 and Cer-Gβ1γ2 as compared to YFP-
AC5 (Table 1). Despite a reduction in FRET, the deletion mutant was still stimulated by exogenously added Giβγ in membrane AC assays, consistent with a separate activation site for Giβγ on AC5.
DISCUSSION

In this report, we show by various biochemical techniques that 1) preformed complexes occur between AC5 and heterotrimeric G-proteins (GDP-Gαs-βγ), 2) these complexes are mediated by the N-terminus of AC5, and 3) the N-terminus also interacts with the catalytic core to augment Gαs-stimulatory and Gαi-inhibitory activity.

N-Terminus and G-Protein Interactions. In our previous studies, we proposed a model for Gβγ activation of AC5/6 that involved the release of Gβγ upon receptor-stimulated activation of Gαs, where both subunits were required for full activation by agonist. We suggested that it is the Gβγ released upon activation of Gαs that stimulates AC, serving to enhance regulation by the alpha-s subunit (Gao et al., 2007). Herein we show that, GDP bound Gαs also directly binds to 5NT and that GDP-Gαs and Gβγ do not compete for binding on 5NT rather enhance binding to 5NT. Gel filtration studies confirmed the binding of G-protein subunits as a heterotrimer to 5NT and endogenous Gαs and Gβγ co-purified with AC5 from immunoprecipitates of HEK293 cells.

Existence of G-proteins and AC as a stable complex was first proposed by Levitzki in 1988, (Bar-Sinai et al., 1992; Levitzki, 1988; Levitzki and Klein, 2002) based upon copurification of AC and G-proteins from turkey erythrocyte membranes that was independent of the activation state of the G-proteins. There is now additional evidence that other effectors namely GIRK channel (Kir 3.1) (Peleg et al., 2002), phospholipase Cβ (PLCβ) (Yuan et al., 2007), and RACK1 (Dell et al., 2002) exist as stable complexes with G-protein heterotrimers. In the case of PLCβ, the activator of G protein signaling AGS8 forms a complex with both Gαi-βγ and PLCβ. Gβγ activates PLCβ through the non-dissociated heterotrimer complex using an alternate interaction site, not the “hot spot” on Gβγ normally associated with effector interactions (Smrcka, 2008; Yuan et al., 2007). For the GIRK channel, it is the N-terminus of GIRK1 that
anchors the inactive heterotrimer Gαi·βγ. Experimental data from several groups suggest a model where GPCR activation of the heterotrimer bound to GIRK1 triggers partial or full separation of Gαi from Gβγ, causing Gβγ to occupy a separate activation site on GIRK1 to open the channel (Clancy et al., 2005; Huang et al., 1995; Ivanina et al., 2003; Peleg et al., 2002; Rishal et al., 2005; Riven et al., 2006; Rubinstein et al., 2009).

In an inactive Gα·βγ complex, the effector surface of Gα, and Gβγ are masked. It is only upon activation of the alpha subunit and subunit dissociation that effector interaction sites are exposed. In our case, it is unlikely that the normal effector face of Gαs is responsible for interactions with 5NT, as it is likely masked by Gβγ binding. This is supported by the fact that addition of excess 5NT does not sequester Gαs to reduce Gαs-stimulated AC5 activity (Fig S7).

**Mechanism of Gβγ Activation of AC5.** The binding site for Gαs·βγ on 5NT was mapped to 69 aa region (60-129). Upon deletion of the Gαs·βγ binding site on AC5 (AC5Δ66-137), FRET between AC5Δ66-137 and Gβγ was significantly reduced compared to wild type AC5. AC5Δ66-137 was fully functional in terms of proper localization (by fractionation and YFP fluorescence), Gαs stimulation, and surprisingly conditional Gβγ stimulation. These results indicate that the activation site of Gβγ on AC reside somewhere other than aa 66-137 of 5NT. The C1/C2 domains were not stimulated by Gβγ in absence or presence of 5NT (Fig. S8), suggesting that the three cytoplasmic domains of AC5 are not sufficient for conditional stimulation by Gβγ. This is consistent with the fact that the cytoplasmic domains alone are not sufficient for conditional stimulation of AC2 by Gβγ (Dessauer and Gilman, 1996; Weitmann et al., 2001). Thus, it is likely that the AC5 membrane domains must properly orient the cytoplasmic domains for Gβγ activation.

We have previously mapped the Gβγ binding and activation site on AC6 to the NT.
residues 77-151 (Gao et al., 2007) and now show that both GDP-Gαs and Gβγ directly bind to 6NT (Fig. S3). As opposed to AC5, deletion of the Gβγ binding site on AC6-NT abolished stimulation by exogenously added Gβγ. The NT of all 9 isoforms of AC are highly variable even among the closely related isoforms, AC5 and AC6. So the possibility of somewhat different mechanisms of activation by Gβγ is not completely unexpected. In fact AC5 and AC6 also display differences in their stimulation by Gαs, inhibition by Gαi, and phosphorylation by protein kinase C (Chen-Goodspeed et al., 2005; Harry et al., 1997; Lai et al., 1999).

**Role of N-Terminus in Modulating Activity.** The divergence of the N-terminus provides additional regulatory specificity among the 9 isoforms of AC. Numerous physiological regulators bind to the NT of ACs, but in most cases the mechanism for regulation of AC activity is unclear. Previous binding assays using *in vitro* translated proteins suggested that AC6 NT contacts the C1 domain to modulate Gαi-mediated inhibition (Kao et al., 2004). We have now shown that there is a direct interaction between AC5 NT and C1/C2 domains which increases catalytic activity, and similar to AC6, limits the inhibition by Gαi. Gβγ relieves the constraints of the N-terminus on Gαi, although it is unclear if this is due to direct binding to 5NT or to an allosteric effect of the Gβγ activation site. However, Gβγ binding to 5NT does not compete with 5NT-C1 binding, thus it is unlikely to be a simple competition between interaction sites.

Post-translational modifications or direct binding to AC NT by other factors may also regulate activity by altering the interaction between NT and C1/C2 domains. For example, phosphorylation of AC6 NT by PKC δ and ε inhibits AC6 activity (Lai et al., 1999; Lai et al., 1997; Lin et al., 2002); whereas AC8 NT forms part of the calmodulin binding site that stimulates AC8 activity, although the precise mechanism for either regulation is still unclear (Simpson et al., 2006). Alternatively, the NT of ACs may simply serve as a scaffold to facilitate
interactions between regulators and the catalytic domains. For example, AC5 NT also interacts with the G protein exchange factor RiC8a to suppress AC activity (Wang et al., 2007). The NT of AC2 binds to the AKAP scaffolding protein, Yotiao, facilitating inhibition by a yet unknown regulator (Piggott et al., 2008). Finally, both AC5 and AC6 NT bind Gβγ to conditionally stimulate the enzyme, although they differ in their mechanism as discussed above (Gao et al., 2007). The possibility for NT regulation of ACs allows for even more diverse modulation of these complex enzymes.

Possible Physiological Consequences

The question arises as to what is the functional role of heterotrimer binding to the N-terminus of AC5. FRET measurements and immunoprecipitations confirm the stable interaction of Gβγ to AC5 in cells. Prior reports from BRET studies indicate that Gβγ traffics together with AC2 (and also with GIRK) to the plasma membrane (Rebois et al., 2006). The deletion of the NT Gβγ binding site had no effect on trafficking of AC5 to the PM, and we observe no FRET between AC5 and Gβγ on intracellular sites within the cell. Thus it does not appear that heterotrimer binding to the N-terminus is required for proper trafficking of AC5. Another possible effect could be on AC5 activity, as is the case of GIRK channel. Heterotrimer binding to a scaffold formed by NT and CT of GIRK1 lowers the basal activity of GIRK1 and predisposes the channel to GPCR mediated activation (Rubinstein et al., 2009). Our model of heterotrimeric G-protein scaffolding by AC5 resembles the activation of GIRK channels by Gβγ, since both effectors bind G protein heterotrimers on their NT yet the Gβγ activation site is distinct from the NT at a secondary location on each effector. We hypothesize that the NT of AC5 brings catalytic core and regulators (heterotrimeric G-protein subunits) in close proximity to prepare for potential GPCR mediated activation of AC.
Acknowledgements

We thank Kathryn Hassell for excellent technical support, Dr. Jim Tomlinson for the generous gift of AC5/AC6 antibody, Dr. Moritz Bünemann for cerulean tagged Gβ, and Dr. Michael Kapiloff for flag-tagged human AC5.
REFERENCES


Footnotes

* This research was supported by the National Institute for Health [GM060419 and GM68493], the American Heart Association [09GRNT2200034] and the US Israel Binational Science Foundation [01-122].
Figure legends

**Figure 1.** The N-terminus of AC5 Anchors Heterotrimeric G-Proteins. (A), GST or GST-tagged 5NT (2 µM final) was incubated with G-protein subunits, Gαs (GDP or GTPγS bound) and/or Gβ1γ2 as indicated and GST pull-down assay was performed. Western blot analysis indicated that 5NT binds GDP-Gαs and Gβγ independently and together their binding is enhanced (upper panel). Protein input for Gαs, Gβγ, and GST-tagged proteins is shown below. Quantitation of binding for GDP-Gαs and Gβγ is shown (n=3, P<0.05). (B), Dose response of 5NT binding to Gβγ, GDP-Gαi, GDP-Gαs, GTPγS-Gαs, or GDP-Gαs in the presence of 100 nM Gβγ was performed by GST pull-down of 5NT (0.125 µM) and varying concentration of G-protein subunits. Quantitation of binding from 3 independent experiments is shown (P<0.05). (C), Gel filtration analysis of complex formation between 5NT and Gαs·βγ. Proteins (10 µM of each) were applied on tandem Superdex 75/200 columns in buffer containing 0.05% C12E9 and 10 µM GDP and analyzed by SDS-PAGE and immunoblotting. Upper panel: complex of 5NT/Gαs/Gβγ, middle panel: 5NT alone, lower panel: Gαs/Gβγ. The 5NT/Gαs/Gβγ complex is boxed while smaller complexes containing 5NT with Gαs or Gβγ are marked with an asterisk. (D), IP of AC5-Gαs-Gβγ complex. Flag-tagged human AC5 (Fl-AC5) or pCDNA3 vector was transfected in HEK293 cells (10 cm dish/IP), immunoprecipitated with anti-Flag agarose, and subjected to western blotting with anti-Gαs, anti-Gβ, or anti-flag. The input represents 5% of the total used in the IP.

**Figure 2.** Mapping the G Protein Binding Site on 5NT. (A), Schematic diagram of full length AC5 and GST-tagged NT fragments. (B), Purified 5NT fragments (2 µM) were incubated with 1 µM G-protein subunits (GDP-Gαs or Gβ1γ2) and GST pull-down assay was performed (n=3). Immunoblots for protein input are shown. (C), Gel filtration analysis of 5NTΔ144 and Gαs·βγ.
Proteins (10 μM) were resolved on tandem Superdex 75/200 columns and analyzed by SDS-PAGE and immunoblotting. Upper panel: 5NTΔ144/Gαs/Gβ1γ2, lower panel: 5NTΔ144 alone (n=2).

**Figure 3. Interaction of 5NT with the Catalytic Core.** (A), 5NT pulls down 5C1/5C2/GTPγS-Gαs/forskolin complex. 5C1, 5C2, and GTPγS-Gαs (1 μM each) were incubated in presence of 100 μM forskolin for 30 min on ice prior to addition of GST, GST-5NT, or 2NT (2 μM). GST pull-down assay was performed as described in the Methods section. Western blot analysis of input and eluted proteins is shown. (B and C), 5C1 (B) and 5C2 (C) were subjected to a GST pull-down assay with 5NT, Δ60 or Δ144 (2 μM). Western blot analysis of bound proteins and input is shown. (D and E), Competition reactions between 5C1 (0 – 3 μM) and 200 nM Gβγ (D) or 200 nM Gαs-GDP (E) for binding to GST-5NT using GST pull-downs. (F), 5NT enhances the Gαs or forskolin stimulated activity of 5C1/5C2. Purified AC5 catalytic domains, 5C1 (70 nM) and 5C2 (1 μM), were preincubated with GST or GST-tagged 5NT (5 μM) for 10 min prior to stimulation with either 400 nM GTPγS-GαS or 100 μM forskolin. (G), Dose dependent enhancement of 5C1/5C2 activity by 5NT. AC activity assay was performed as described in D with varying concentrations of GST, 5NT, 5NTΔ60, or 5NTΔ144.

**Figure 4. Regulation of AC5Δ66-137 by Exogenously Added GTPγS-Gαs, Gβγ, and GTPγS-Gαi.** (A), Diagram of AC5 and AC5Δ66-137. (B), Dose response of GTPγS-Gαs stimulated AC5 and AC5Δ66-137 activity. Membranes from HEK293 cells expressing vector, AC5, or AC5Δ66-137 were stimulated with the indicated concentrations GTPγS-Gαs. (C), Characterization of AC5 and AC5Δ66-137 expression by western blotting in Sf9 membranes.
(D), Stimulation of Sf9 membranes expressing AC5 or AC5Δ66-137 by 30nM GTPγS-Gas in the presence or absence of 100 nM Gβ1γ2 and the indicated concentrations of GTPγS-Gai. Lower panel, each curve was normalized to the AC activity in the absence of Gai. (E), Model of Gai regulation by 5NT. GTPγS-Gas binds to 5C2 to stimulate activity while GTPγS-Gas binds to 5C1 to inhibit AC5. 5NT also interacts with 5C1 to limit Gai inhibition. Addition of Gβγ increases activity and relieves the influence of 5NT on Gai inhibition. Note, that although Gβγ is shown bound to 5NT, it clearly must have additional unknown activation sites.

**Figure 5. Cellular Interaction of YFP-AC5 and Gβγ in COS7 cells by FRET.** (A), Characterization of YFP-tagged proteins. Membranes from COS7 cells expressing vector, YFP-AC5, or YFPAC5Δ66-137 were stimulated with 1 μM GTPγS-Gas. (B), FRET analysis of AC5 and Gβ1γ2 in COS7 cells. Fluorescence microscopy images of COS7 cells transiently transfected with the indicated proteins were recorded using three different channels (First, Donor; CFPex/CFPem, Second, FRET; CFPex/YFPem and third, Acceptor; YFPex/YFPem). A representative cell is shown for each combination of proteins (upper panel: Myr-CFP and YFP-AC5; Middle panel: Cer-Gβ1, Gγ2, and YFP-AC5; Lower panel: Cer-Gβ1, Gγ2, and YFP-AC5Δ66-137. Pseudocolor FRET C/D images were obtained using Slidebook software (Nikon) (C), Quantitative analysis of FRET by F C/D method (n=4 utilizing images from 18-20 cells, P<0.01).
### TABLE 1

Quantitation of FRET Measurements by $\text{FRET}^C/D$ and $\text{FRET}^C/\sqrt{D*A}$ Method

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$\text{FRET}^C/\sqrt{D*A}$ Average (± S.E.M)</th>
<th>$\text{FRET}^C/D$ Average (± S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP-Myr</td>
<td>0.38 ± 0.09</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>Cer-G$\beta\gamma$ YFP-AC5</td>
<td>2.13 ± 0.20</td>
<td>1.61 ± 0.26</td>
</tr>
<tr>
<td>Cer-G$\beta\gamma$ YFP-AC5Δ66-137</td>
<td>0.88 ± 0.24</td>
<td>0.64 ± 0.13</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A

B

C

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3
Figure 4
Figure 5
Supplementary Figure Legends:

Figure S1. Gβγ Enhances GDP-Gαs Binding to 5NT in the Presence and Absence of Aluminum Fluoride. GST or GST-tagged 5NT (2 µM final) was incubated with G-protein subunits, GDP-Gαs and/or Gβ1γ2 (1 µM). GST pull-down assay was performed in the presence or absence of AlF4 (n=2). When present, 30 µM aluminum and 10 mM fluoride was present in all incubation and wash buffers.

Figure S2. Gel Filtration Analysis of Complex Formation between 5NT 60-129 and Gαs·βγ. Proteins (10 µM of each) were applied on a superdex 200 column in buffer containing 0.1% C12E10, 75 mM NaCl, and 10 µM GDP and fractions (0.3 ml) were analyzed by SDS-PAGE and immunoblotting. Upper panel: complex of 5NT 60-129/Gαs/Gβγ, middle panel: 5NT 60-129 alone, lower panel: Gαs/Gβγ. The 5NT60-129/Gαs/Gβγ complex is boxed while smaller complexes containing 5NT with Gαs or Gβγ are marked with an asterisk (n=2).

Figure S3. Gβγ and GDP-Gαs Bind to 6NT. GST proteins (2 µM final) were incubated with G-protein subunits, GDP-Gαs or Gβγ and subjected to GST pull-down assay as described in Fig. S1 (n=5).

Figure S4. The N-terminus of AC5 Does Not Serve As GAP or GEF. (A), GTPγS filter binding assay was performed as described in (Graziano and Gilman, 1989). Briefly, G-protein subunits, GDP-Gαs·βγ (1 µM) in presence or absence of GST or GST-tagged 5NT (10 µM final) were incubated with 100 uM [35S]GTPγS (~ 2000 cpm/pmol). Bound [35S]GTPγS was detected by binding to nitrocellulose filters in a 96 well format. (B) GTPase assay was performed as
described in (Graziano and Gilman, 1989) with slight modification. Briefly, Gαs (12.5 pmol) was incubated with GST or GST-tagged 5NT (10 µM final). The reaction was started by addition of [γ-32P]GTP (~60000 cpm/pmol). An aliquot was taken at indicated time points and 32Pi was separated using activated charcoal and measured by scintillation counting.

**Figure S5. 5NT and 5NT60-129 Pull Down a 5C1/5C2/GTPγS-Gαs Complex in the Presence or Absence of Forskolin.** 5C1, 5C2, and GTPγS-Gαs (1 µM each) were incubated in presence or absence of 100 µM forskolin for 30 min on ice prior to addition of GST, GST-5NT, or 5NT 60-129 (2 µM). GST pull-down assay was performed as described in the Methods section. Western blot analysis of input and eluted proteins is shown (n=2).

**Figure S6. 5NT Has No Effect on C1/C2 Basal AC Activity.** Purified AC5 catalytic domains, 5C1 (70 nM) and 5C2 (1 µM), were preincubated with GST or GST-tagged 5NT deletions (5 µM) for 10 min prior to start of the assay (n=3).

**Figure S7. Purified 5NT Does Not Alter Full-Length AC5 Activity.** Sf9 membranes expressing AC5 or AC5Δ66-137 were assayed for basal activity, Gαs-stimulated activity (50 nM) or forskolin-stimulated activity (50 µM) in the presence 5 µM GST, 5NT, or 5NT deletions (n=2).

**Figure S8. Gβγ Does Not Enhance 5C1/5C2/5NT Activity. (A)** Purified AC5 catalytic domains, 5C1 (70 nM) and 5C2 (1 µM), were preincubated with GST or GST-tagged 5NT in the presence or absence of Gβ1γ2 as indicated for 10 min prior to stimulation with 400 nM GTPγS-
$\text{Gas (n=3)}$.

**References:**
