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# Regions in the G Protein γ Subunit Important for Interaction with Receptors and Effectors*

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Running Title: Interaction of G Protein γ Subunit with Receptors and Effectors

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**Abbreviations**

[1]The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; Sf9 cells, Spodoptera frugiperda cells (ATCC no. CRL 1711); PLC-β; phosphatidylinositol-specific phospholipase C-β isoform; PtdIns 3-kinase, phosphatidylinositol (4,5) bisphosphate 3-kinase, CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; Genapol C-100, polyoxyethylene (10) dodecyl ether. $^{125}$I-ABA, $N^6$-(4-amino-3-$^{125}$iodo-benzyl)adenosine; DTT, dithiotreitol, EDTA, ethylenediamine tetraacetic acid, GTP-γ-S, Guanosine 5'-(3-O-thio) triphosphate.

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## Abstract

Gβγ dimers containing the $\gamma_{11}$ or $\gamma_1$ subunits are often less potent and effective in their ability to regulate effectors as compared to dimers containing the $\gamma_2$ subunit. To explore the regions of the γ subunit which affect the activity of the βγ dimer, we constructed 8 chimeric γ subunits from the $\gamma_1$ and $\gamma_2$ subunits. Two chimeras were made in which the N-terminal regions of $\gamma_1$ and $\gamma_2$ were exchanged and two in which the C-terminal regions were transposed. Another set of chimeras was made in which the *CAAX* motifs of the chimeras were altered to direct modification with different prenyl groups. All 8 γ chimeras were expressed in Sf9 cells with the $\beta_1$ subunit, Gβγ dimers purified, and assayed *in vitro* for their ability to bind to the $G\alpha_{i1}$ subunit, to couple $G\alpha_{i1}$ to the A1 adenosine receptor, to stimulate phospholipase C-β and to regulate type I or type II adenyl cyclases. Dimers containing the C-terminal sequence of the $\gamma_2$ subunit modified with the geranylgeranyl lipid had the highest affinity for $G_{i1}$ α (range 0.5-1.2 nM) and were most effective at coupling the $G_{i1}$ α subunit to receptor. These dimers were most effective at stimulating PLC-β and inhibiting type I adenyl cyclase. In contrast, βγ dimers containing the N-terminal sequence of the $\gamma_2$ subunit and a geranylgeranyl group are most effective at activating type II adenyl cyclase. The results indicate both the N and C terminal regions of the γ subunit impart specificity to receptor and effector interactions.

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Among the best characterized signal transduction systems are the pathways used by receptors coupled to heterotrimeric G proteins[1]. The G protein βγ subunit plays an important role in these pathways. It is required for the interaction between the receptor and the α subunit to initiate GDP/GTP exchange and also stabilizes the basal state of the receptor-α-GDP-βγ complex which forms the high affinity, ligand binding conformation of receptors (Cabrera-Vera *et al.*, 2003). Once released from the activated α subunit, the βγ subunit can regulate over 20 effectors including PLC-β, adenylyl cyclase, ion channels (Cabrera-Vera *et al.*, 2003;Hildebrandt, 1997), PtdIns 3-kinase (Kerchner *et al.*, 2004) and guanine nucleotide exchangers for small GTP binding proteins (Welch *et al.*, 2002). The free βγ dimer can also participate in regulatory events by binding to cytoplasmic proteins such as the β-adrenergic receptor kinase or phosducin (Cabrera-Vera *et al.*, 2003). Importantly, the multiple isoforms of the βγ dimer can differentially regulate effectors (Cabrera-Vera *et al.*, 2003;Hildebrandt, 1997), making it necessary to understand the regions of these subunits which interact with their targets.

To date, seven β and twelve γ subunits have been identified in mammalian systems (Downes and Gautam, 1999); thus the βγ dimers which can be assembled from the known subunits yield a large number of potentially unique complexes which differ in their interactions with receptors and effectors (Cabrera-Vera *et al.*, 2003;Hildebrandt, 1997). While the $\beta_1$-$\beta_4$ subunits are more than 85% identical in amino acid sequence, the sequence identity of the γ subunits ranges from 10-70 % (Downes and Gautam, 1999). Interestingly, the diversity of either the β or the γ subunit can impart specificity to the interactions of the dimer with receptors (Richardson and Robishaw, 1999;McIntire *et al.*, 2001;Hou *et al.*, 2000) or effectors (Cabrera-Vera *et al.*, 2003) and the type of prenyl group on the γ subunit is clearly important in the interactions with both receptors (Yasuda *et al.*, 1996;Kisselev *et al.*, 1995) and effectors (Myung *et al.*, 1999). Multiple experiments suggest that the γ subunit is divided into three functionally different regions. The central region of all γ subunits is thought to be involved in the interactions between the β and γ subunits. For example, mutagenesis data indicate that a 14 amino acid sequence (amino acids 36-49 on $\gamma_1$ or 33-46 on $\gamma_2$) is critical for the interaction with the

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$\beta_1$ subunit (Lee *et al.*, 1995) and the crystal structure of the $\beta\gamma$ dimer confirms that 17 of the 27 residues in the $\gamma$ subunit which interact with the $\beta$ subunit are centrally located (Sondek *et al.*, 1996). The N- and C-terminal regions of the $\gamma$ subunits are the least conserved (Downes and Gautam, 1999;Sondek *et al.*, 1996), and several lines of evidence suggest that these regions are important for the interaction with receptors (Yasuda *et al.*, 1996;Hou *et al.*, 2000) and effectors (Cabrera-Vera *et al.*, 2003;Hildebrandt, 1997;Yasuda *et al.*, 1996;Yasuda *et al.*, 1998;Akgoz *et al.*, 2002).

The $\gamma$ subunits segregate into five groups with $\gamma_1$, $\gamma_{11}$ and $\gamma_8$ comprising subfamily I, $\gamma_2$, $\gamma_3$, $\gamma_4$ and $\gamma_9$ subfamily II, $\gamma_7$ and $\gamma_{12}$ subfamily III, $\gamma_5$ and $\gamma_{10}$ subfamily IV and $\gamma_{13}$ forming its own family (Downes and Gautam, 1999). Experiments show that $\beta\gamma$ dimers containing the $\gamma_1$ or the $\gamma_{11}$ subunit are less potent in activating PLC-$\beta$ and type II adenyl cyclase than dimers containing the $\gamma_2$ subunit (Myung *et al.*, 1999). Whereas the expression of $\gamma_1$ is restricted to the visual system, $\gamma_{11}$ and $\gamma_8$ are widely expressed (Morishita *et al.*, 1998;Downes and Gautam, 1999). These three $\gamma$ subunits of subfamily I are modified with the farnesyl lipid (Downes and Gautam, 1999) and the distribution of charged amino acids in the N and C terminus is quite different from the other nine $\gamma$ subunits.

To examine how the N- and C-terminal domains of the $\gamma$ subunit and its prenyl group affect the interaction with $\alpha$ subunits, receptors and effectors, we constructed 8 chimeric $\gamma$ subunits from the $\gamma_1$ and the $\gamma_2$ subunits. These two $\gamma$ subunits were used for the chimeras as the N and C termini of these proteins are representative of the major differences between the $\gamma$ subunits in subfamily I and the other nine $\gamma$ subunits. Two chimeras were made in which the N-terminal amino acids of $\gamma_1$ and $\gamma_2$ were exchanged. Two other chimeras were made in which the C-terminal 23 amino acids of $\gamma_1$ and $\gamma_2$ were exchanged. Finally, another set of chimeras was made in which the *CAAX* motifs in the four chimeras were altered to direct modification with different prenyl groups. All 8 $\gamma$ chimeras were expressed in Sf9 cells with the $\beta_1$ subunit and purified. Each dimer was tested using five *in vitro* assays chosen to probe major facets of $\beta\gamma$ activity; the ability to bind to the $G\alpha_{i1}$ subunit, to couple $G\alpha_{i1}$ to the A1 adenosine receptor, to stimulate PLC-$\beta$ or to regulate type I and II adenylyl cyclases. The *in vitro* results indicate the $\gamma$ subunit's C terminus and its

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prenyl group are important for coupling $\alpha$ subunits to the A1 adenosine receptor, for the activation of PLC-$\beta$ and for the inhibition of type I adenylyl cyclase. In contrast, the $\gamma$ subunit's N terminus is very important for the activation of type II adenylyl cyclase.

## Materials and Methods

*Strategy for Construction of the Chimeric $\gamma$ Subunits* — The $\gamma_1$ and $\gamma_2$ subunits have a conserved set of three residues about 20 amino acids from the N terminus (QLK) and the C terminus (DPL). The strategy for making the chimeras was based on creating new cDNAs for the $\gamma_1$ and $\gamma_2$ subunits which had restriction sites engineered into the QLK and DPL sequences and is similar to those published (Jian *et al.*, 2001). These sites are highlighted in Figure 1A. Also note the similarities in the N and C terminal regions of the $\gamma$1 and $\gamma$11 subunits. Thus, while the chimeras were made with regions from $\gamma$1 because the cDNA for $\gamma$1 contained a convenient restriction site in the DPL sequence, the regions were selected to be representative of $\gamma$11 (see Figure 1). An *Afl*II site was used to create the QLK sequence and a *Bam*HI site to code for the DPL sequence in each molecule. The nomenclature used to describe these chimeric $\gamma$ subunits is shown in Figure 1B. The protein was divided into three regions by the QLK and DPL sites: designated as AAA for $\gamma_1$ and BBB for $\gamma_2$. If the C-terminal prenyl group was switched from the native modification, the amino acids in the mutant *CAAX* motif are indicated (Yasuda *et al.*, 1996). For example, $\gamma_{2\text{-L71S}}$ indicates the native $\gamma_2$ subunit modified to change only the normal C-20 geranylgeranyl to the C-15 farnesyl. A designation of $\gamma_{BBA}$ indicates a chimeric $\gamma$ subunit where the C terminus of the $\gamma_1$ subunit (molecule A) is added to the N-terminal and middle regions of the $\gamma_2$ subunit (molecule B) at the conserved sequence. The prenyl group of this chimera would be farnesyl. A designation of $\gamma_{BBA\text{-S74L}}$ would indicate the same chimeric $\gamma$ subunit mutated to code for addition of geranylgeranyl at the C terminus.

The $\gamma_1$ cDNA was excised from a pEV plasmid (*Hind*III/*Bam*HI) and subcloned into the pGem7zF+ plasmid utilizing the same restriction sites; it was then excised with *Hind*III/*Asp*700 for subcloning into the pAlter-1 mutagenesis plasmid at the *Hind*III and

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*Sma*I sites. The $\gamma_2$ cDNA in pGem4z was excised by digestion with *Xba*I and *Sma*I and was subcloned into pAlter-1 using the same restriction sites. To construct the chimeras, identical restriction sites were created in the QLK and DPL sequences in both the $\gamma_1$ and $\gamma_2$ cDNAs; an *Afl*II site was engineered to code for the QLK sequence and a *Bam*HI site was engineered to code for the DPL sequence. As the *Bam*HI restriction site already existed in the $\gamma_1$ cDNA in the site corresponding to the DPL residues, a *Bam*HI site was added to the $\gamma_2$ cDNA through site-directed mutagenesis by altering one base in the $\gamma_2$ sequence (GAA to GAG, encoding Glu) using the Morph™ Site-Specific Plasmid DNA Mutagenesis Kit (5Prime -> 3Prime, Boulder, CO) taking care to conserve the protein sequence. The *Afl*II restriction site for the QLK sequence was engineered into both the $\gamma_1$ and $\gamma_2$ subunits using the same mutagenesis kit. The resulting $\gamma_1$ and $\gamma_2$ cDNAs in the pAlter-1 plasmid containing the new *Afl*II and *Bam*HI sites were used to make the C- or N-terminal chimeric $\gamma$ subunits.

*Construction of N- and C-terminal $\gamma$ Chimeras and Production of Recombinant Baculoviruses* — The $\gamma_1$ and $\gamma_2$ C-terminal chimeras ($\gamma_{AAB}$ and $\gamma_{BBA}$) were constructed by digesting the $\gamma_1$ and $\gamma_2$ cDNAs in the pAlter-1 plasmid with *Bam*HI and *Nhe*I. The four resulting DNA fragments contain the C termini of $\gamma_1$ and $\gamma_2$ and cDNAs encoding for the intact N terminus and middle regions of the $\gamma_1$ and $\gamma_2$ proteins. The C-terminal cDNA fragments were gel purified and ligated back into either the cDNA encoding the N terminus and middle regions of the $\gamma_1$ or $\gamma_2$ as desired. This protocol generated the constructs containing the $\gamma_{AAB}$ and $\gamma_{BBA}$ chimeras. To exchange the N termini of the $\gamma_1$ and $\gamma_2$ molecules, the respective cDNAs in the pAlter-1 vector were digested with *Afl*II and *Nhe*I, the N-terminal cDNA fragments gel purified and ligated back into either the cDNA encoding the middle regions and C terminus of $\gamma_1$ or $\gamma_2$ as desired. This protocol generated the constructs containing $\gamma_{BAA}$ and $\gamma_{ABB}$ chimeras. It was desirable to subclone the $\gamma$ cDNAs into the *Xba*I site in the baculovirus transfer vector, pVL1393. Thus, the four $\gamma$ chimeras in the pAlter-1 plasmid were subcloned into the linear pCNTR plasmid which added the *Xba*I restriction sites to the end of each chimera. Each of the four chimeras was subcloned into the pVL1393 baculovirus transfer vector by excising them from pCNTR with *Xba*I and ligating the fragments into pVL1393 at its *Xba*I sites. The 4 $\gamma$

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chimeras containing altered *CAAX* sequences, $\gamma_{AAB\text{-}L71S}$ (farnesyl), $\gamma_{BBA\text{-}S74L}$ (geranylgeranyl), $\gamma_{BAA\text{-}S74L}$ (geranylgeranyl) and $\gamma_{ABB\text{-}L71S}$ (farnesyl), were constructed using PCR on the cDNAs in pVL1393 as described (Lindorfer *et al.*, 1996). Each of the eight pVL1393 transfer vectors containing the cDNA for a chimeric $\gamma$ subunit was sequenced to ensure fidelity. Recombinant baculoviruses encoding for the eight $\gamma$ chimeras were produced by co-transfecting each recombinant plasmid DNA with linear wild type BaculoGold® viral DNA into Sf9 cells and purified by one round of plaque purification (Graber *et al.*, 1994). The recombinant baculoviruses encoding the $\alpha_s$, $\alpha_{i1}$, $\beta_1$, $\gamma_1$, $\gamma_{1\text{-}S74L}$, $\gamma_2$ and $\gamma_{2\text{-}L71S}$ subunits have been described (Lindorfer *et al.*, 1996;Graber *et al.*, 1994).

*Expression and Purification of G protein $\alpha$ and $\beta\gamma$ Subunits* — G protein $\alpha$ subunits were overexpressed in bacteria (Sarvazyan *et al.*, 1998) and baculovirus-infected Sf9 insect cells (Graber *et al.*, 1994). Bacterially expressed, myristoylated $\alpha$ i1 was purified from *Escherichia coli* (BL21/DE3) following the method of Mumby and Linder (Mumby and Linder, 1994). The amount of $\alpha$ i1 protein was determined using the Bradford assay. The $G_{i1}$ $\alpha$ subunits expressed in Sf9 insect cells were purified to homogeneity as described (Graber *et al.*, 1994) and the $G_s$ $\alpha$ subunit used in the adenylyl cyclase assays was prepared from a 0.1% (w/v) CHAPS extract of crude cell lysates (McIntire *et al.*, 2001). The specific activity of GTP binding of these preparations was ~11-15 nmol/mg of $\alpha$ subunit as measured by [$^{35}$S]-GTP-$\gamma$-S binding (Sarvazyan *et al.*, 1998;Graber *et al.*, 1994). Bovine brain $\beta\gamma$ dimer was isolated from brain cortex synaptosomal membranes as described (Sarvazyan *et al.*, 1998). The membranes were a gift from Dr. T. Ueda, University of Michigan. Recombinant $\beta\gamma$ subunits were prepared from baculovirus infected Sf9 insect cells. Sf9 cells were coinfected with the appropriate recombinant baculoviruses encoding the $\beta_1$ subunit and native or chimeric $\gamma$ subunits in Sf9 cells at a multiplicity of infection of 3 and harvested 48 hr after infection. All $\beta\gamma$ subunits were extracted from frozen cell pellets with 0.1% Genapol C-100 and the detergent-extracted $\beta\gamma$ dimers purified on a DEAE column followed by affinity chromatography on a $G_{i1}$-$\alpha$-agarose column (Graber *et al.*, 1996). The $\beta\gamma$ combinations used in this study are properly folded as they were purified on $\alpha$ subunit affinity column, have a high affinity for

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the $\alpha$ subunit (see Figure 2) and were released following activation with $AlF_4^-$. The purified $\beta\gamma$ dimers were resolved on 12% polyacrylamide gels, stained with silver and the concentration of the purified $\beta\gamma$ dimers estimated using ovalbumin standards. All preparations of the dimers were highly pure, examples of their purity have been published (McIntire *et al.*, 2001). The experiments shown in *Results* were performed using at least two different preparations of each $\beta\gamma$ dimer with consistent results.

*Analysis of the Post-Translational Processing of the $\gamma$ Subunit by Mass Spectrometry* — To confirm that the proper chimeric $\gamma$ subunits were being expressed and that their C termini were properly modified, the molecular masses of the $\gamma$ subunits in the 12 $\beta\gamma$ dimers used in this study were determined using matrix-assisted laser desorption ionization (MALDI) mass spectrometry as described (Lindorfer *et al.*, 1996). The molecular mass of each chimeric $\gamma$ subunit was estimated by the University of Wisconsin GCG program. The results summarized in Table I indicate that each chimeric $\gamma$ subunit was faithfully expressed and had a fully processed C terminus. For example, the $\gamma_{BAA}$ protein in the sample was comprised of one molecular weight species with a molecular mass of 7,818 Da. This result is consistent with the predicted amino acid sequence of the chimera and processing of the molecule by removal of the three C-terminal amino acids (-VIS), addition of a farnesyl lipid to the C-terminal cysteine, addition of a carboxylmethyl group to the C terminus, removal of the N-terminal methionine and acetylation of the resulting N-terminal alanine. The observed prenyl modification of the $\gamma$ subunits in this study is consistent with the electrospray mass spectrometric analysis of previous samples which show that over 90% of the purified protein is properly modified (Lindorfer *et al.*, 1996). Finally, the data in Table I, combined with that in Figures 2-5, show that use of the conserved QLK and DPL sites which are located in the two helical regions of the $\gamma$ subunit (Sondek *et al.*, 1996) to construct the chimeras appears to generate fully functional molecules. These chimeras combine with $\beta$ subunits, purify efficiently over an $\alpha$ subunit affinity column and interact well with receptors and effectors (see Figures 2-5).

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*Expression of A1 Adenosine Receptors, Phospholipase C-β, Type I and II Adenylyl Cyclase* — A recombinant baculovirus encoding the A1 adenosine receptor was used to over-express the receptor in Sf9 insect cells and membranes were purified as described (Yasuda *et al.*, 1996). Sf9 insect cell membranes overexpressing recombinant type I or type II adenylyl cyclase were prepared as described (McIntire *et al.*, 2001). Recombinant turkey PLC-β was overexpressed in Sf9 cells and purified as described (Myung *et al.*, 1999).

*Assay of the Activity of βγ Dimers* — Different facets of the activity of each βγ dimer were measured in a set of *in vitro* assays chosen to probe the known protein-protein interaction domains of the dimer. Each of these assays was selected for its ability to highlight a known function of the βγ dimer and because the outcome was directly dependent on βγ activity. The affinity of the βγ dimer for the α subunit reflects interactions of the top and sides of the β subunit with the switch II and C terminal regions of the α subunit (Cabrera-Vera *et al.*, 2003). The prenyl group on the C terminal region of the γ subunit is also important for binding the α subunit (Iniguez-Lluhi *et al.*, 1992). The affinity of the βγ dimers for Gαi1 was measured in solution with pure proteins by flow cytometry, using fluorescein-labeled bacterially-expressed myristoylated αi1 and biotinylated bovine brain βγ as previously described (Sarvazyan et al., 1998). Briefly, 1 nM of biotinylated brain βγ was prebound to strepavidin-coated polystyrene beads (SVP-60-5, Spherotech Inc., Libertyville, IL) and incubated with 1 nM fluorescein-labeled, myristoylated αi1 together with varying amounts of recombinant βγ in 20 mM Hepes, pH 8, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 1.2 mM $MgCl_2$, 0.1% Lubrol and 10 μM GDP. After a 30 min incubation at room temperature, samples were analyzed with a Becton Dickinson FACScan capturing events on the forward scatter, side scatter, and fluorescein (FL-1) channels. Histograms of FL-1 fluorescence from singlet bead populations were obtained and mean channel numbers calculated using LYSIS II software (BD, San Jose, CA).

The ability of each particular β1γx dimer to support coupling of the $G_{i1}$ α subunit to the A1 adenosine receptor was used to monitor the interactions of the N terminal, central and C

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terminal regions of the 10 γ subunit chimeras with the receptor. In this assay, the receptor is expressed in an Sf9 cell membrane in the absence of G proteins causing it to be in its low affinity, agonist binding conformation (Yasuda *et al.*, 1996). Reconstitution of the G protein heterotrimer into the membrane re-establishes the high affinity, agonist binding conformation of the receptor as measured by a radioligand binding assay using an agonist ligand (see Fig 2A and (McIntire *et al.*, 2002)). Alternatively, if an excess of α subunit and graded amounts of a βγ dimer are reconstituted into the membrane, the amount of the receptor returned to the high affinity, agonist binding state as measured using a low concentration of the agonist ligand reflects the affinity of the receptor - α subunit complex for the βγ dimer (McIntire *et al.*, 2002). As only the γ subunit was different in the experiments shown in Figure 2B, the assay provides a measure of the ability of the dimers containing the different chimeric γ subunits to interact with the receptor and α subunit. This value is expressed as $EC_{50}$ of receptor coupling in Fig. 2B. The ability of the three regions of the γ subunit to inteact with PLC-β was measured by reconstituting dimers containing the chimeric γ subunits and pure PLC-β into synthetic lipid vesicles (Myung *et al.*, 1999). Sf9 cells infected with recombinant baculoviruses for adenyl cyclases can be used to monitor the direct interaction of the βγ dimer with type I and type II adenyl cyclase (McIntire *et al.*, 2001;Taussig *et al.*, 1994).

To measure type I or II adenylyl cyclase activity, Sf9 membranes over-expressing the desired enzyme (10 μg membrane protein/assay tube) were reconstituted with the purified GTP-γ–S-activated $G_s$ α subunit and varying concentrations of pure βγ dimers containing either a native or chimeric γ subunit and held on ice for 30 min (McIntire *et al.*, 2001). Each assay was incubated at 30 °C for 7 min and the reaction terminated with 1 ml 0.11 N HCl. The samples were prepared for the assay of cAMP and the assay performed as described (McIntire *et al.*, 2001). The activated $G_s$ α subunit was used as the co-activator of type I and type II cyclase in these experiments because: (a) the βγ dimer does not increase the activity of type II cyclase in the absence of $G_s$ α or in the presence of forskolin (Taussig *et al.*, 1994); (b) the βγ dimer only modestly inhibits type I cyclase if it is preactivated with forskolin or $Ca^{2+}$/calmodulin (Taussig *et al.*, 1994). Pilot experiments with our Sf9 membranes confirmed both of these findings (data not shown).

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*Calculation and Expression of Results* — Experiments presented under "*Results*" are the average of three or more similar experiments. Data expressed as concentration-response curves were fit to sigmoid curves using the fitting routines in the GraphPad Prism™ software. The $EC_{50}$ and $V_{max}$ values shown in Tables II and II were taken from these fits. Statistical differences between the fitted curves were determined using all the individual data points from multiple experiments to calculate the F statistic (Motulsky and Ransnas, 1987).

*Materials* — All reagents used in the culture of Sf9 cells and for the expression and purification of G protein $\beta\gamma$ subunits have been described in detail (Graber *et al.*, 1996). The baculovirus transfer vector, pVL1393, was purchased from Invitrogen; BaculoGold® viral DNA from PharMingen; 10% Genapol C-100 and phosphatidylinositol 4,5-bisphosphate from Calbiochem®; phosphatidylethanolamine (bovine heart) from Avanti Polar Lipids, Inc.; [*inositol*-2-$^3$H]phosphatidylinositol 4,5-bisphosphate from NEN Life Science Products; CHAPS from Roche Molecular Biochemicals; BSA (fatty acid-free) from Sigma; the pCNTR shuttle vector and the Morph™ Site-Specific Plasmid DNA Mutagenesis Kit from 5 Prime $\rightarrow$ 3 Prime, Inc. (Boulder, CO). All other reagents were of the highest purity available.

## Results

The $\beta_1\gamma_1$ and $\beta_1\gamma_{11}$ dimers are measurably less active than the $\beta_1\gamma_2$ dimer in its ability to activate $K^+$ channels (Cabrera-Vera *et al.*, 2003), PLC-$\beta$ (Myung *et al.*, 1999), type II adenylyl cyclase (Myung *et al.*, 1999) and PtdIns 3-kinase (Kerchner *et al.*, 2004), or to inhibit type I adenylyl cyclase (McIntire *et al.*, 2001). As the dimers tested in these experiments all contained the $\beta_1$ subunit, differences in the primary amino acid sequence of the $\gamma$ subunits, in their prenyl modification or both must explain the differences in activity. While it was initially attractive to ascribe the unique properties of the $\gamma_1$ subunit to its specialized role in visual transduction (Cabrera-Vera *et al.*, 2003), the fact that $\gamma_{11}$ and $\gamma_8$, have similar amino acid sequences and prenyl modifications (see Figure 1A) and

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are widely expressed (Downes and Gautam, 1999), makes it important to understand the reasons for the differential activity of this subfamily of $\gamma$ subunits. Figure 1A presents the differences in the N and C termini of the $\gamma_1$, $\gamma_{11}$ and $\gamma_2$ subunits. Note that the N-terminal 20 amino acids of the very similar $\gamma_1$ and $\gamma_{11}$ subunits have six negatively charged amino acids whereas this region of the $\gamma_2$ subunit has only one negative charge and that the C-terminal amino acids of the $\gamma$ subunits also show marked differences in charge distribution. Thus, differences in the amino acid sequences and charges in the N- and C-terminal regions of these proteins may be important for the signaling-specificity of the $\beta\gamma$ dimer. To address this issue, we constructed a set of 4 chimeric $\gamma$ subunits in which the N- and C-terminal amino acids of $\gamma_1$ and $\gamma_2$ were exchanged. To complete the set, an additional 4 mutants with altered prenyl modifications was prepared. Figure 1B presents the 8 chimeras which were made and the nomenclature used to describe them. The 12 $\gamma$ subunits shown in Figure 1B were expressed with $\beta_1$ subunit and the dimers purified from baculovirus-infected Sf9 insect cells by $\alpha_{i1}$-agarose affinity chromatography. The activity of the complete set of $\beta\gamma$ dimers was examined in a panel of *in vitro* assays measuring the affinity of the dimer for the $G_{i1}$ $\alpha$ subunit, the ability to support coupling of the $\alpha$ subunit to the bovine A1 adenosine receptor, to activate PLC-$\beta$ and to regulate type I or type II adenylyl cyclases.

*Receptor Coupling* — The C-terminal domain of the mammalian $\gamma$ subunit and its prenyl group are known to be important for the interaction of the $\beta\gamma$ dimer with $\alpha$ subunits and receptors (Yasuda *et al.*, 1996;Azpiazu *et al.*, 1999). Interestingly, the affinity of the $\alpha$ subunit for the $\beta\gamma$ dimer is an important component of the interaction of the heterotrimer with the receptor (Sarvazyan *et al.*, 1998) but this parameter is not commonly measured. Moreover, the differences noted with the different forms of the $\gamma$ subunit can be minimized when high concentrations of the $\alpha$ and $\beta\gamma$ subunits are reconstituted into vesicles or membranes (see Figure 2A). For these reasons, we performed experiments to compare the affinity of dimers containing the chimeric $\gamma$ subunits for the Gi1 $\alpha$ subunit with the $EC_{50}$ values determined for the ability of the dimer to support the high affinity, agonist binding state of the A1 adenosine receptor. Figure 2A shows ability of various concentrations of the $G\alpha$ $i_1$ : $\beta_1\gamma_2$ heterotrimer to reconstitute the high affinity, agonist

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binding conformation of the adenosine A1 receptor over-expressed in Sf9 cell membranes (Yasuda *et al.*, 1996). Four sets of membranes were reconstituted with increasing amounts of heterotrimer and the amount of total agonist ligand bound plotted as a function of ligand concentration. Note that concentrations of heterotrimer in the 40 nM range shift the affinity of the receptor for the agonist (amino-benzyladenosine – ABA) from about 30 nM (open circles - no G protein) to 0.3 nM (inverted triangles - 40 nM $G\alpha_{i1}$ : $\beta_1\gamma_2$). An intermediate shift in the affinity of the receptor is caused by 2-6 nM heterotrimer. When the concentration of the heterotrimer is in the the 2-6 nM range, it is possible to measure differences in the ability of various $\beta\gamma$ combinations to interact with the receptor - $\alpha$ subunit complex and support high affinity binding (McIntire *et al.*, 2002). Therefore, we measured the ability of the 6 C-terminal chimeras to support the establishment of the high affinity, agonist binding conformation of bovine A1 adenosine receptors at 6 nM heterotrimer. The results were expressed as the $EC_{50}$ of the interaction and compared with the results with the affinity of the $\beta\gamma$ dimers for the Gi1 $\alpha$ subunit measured in solution. The data in Figure 2B show that the Kd (x axis) of the $\beta\gamma$ dimer for the $Gi\alpha$ subunit varies about 20 fold from 0.4 nM ($\beta_1\gamma_2$) to 7.7 nM ($\beta_1\gamma_{BAA}$). Note that the $EC_{50}$ for supporting coupling of the Gi1 $\alpha$ subunit to the receptor (y axis) varies 5-6 fold from 0.4 nM ($\beta_1\gamma_2$) to about 2.2 nM ($\beta_1\gamma_{BAA}$). All $\beta\gamma$ dimers containing $\gamma$ subunits modified with the farnesyl group (open squares) had lower affinities for the Gi1 $\alpha$ subunit and lower $EC_{50}$'s for supporting receptor coupling than did those containing the geranylgeranyl moiety. These results confirm and extend the concept that the C-terminal region of the $\gamma$ subunit and its prenyl group are very important for the interaction of the dimer with $\alpha$ subunits and receptors in this assay. Moreover, the data show that there is a rough correlation between the $EC_{50}$ for receptor coupling and the affinity of the $\alpha$ and $\beta\gamma$ subunits themselves.

*Phospholipase C-$\beta$* — Our previous experiments show that the activity of the $\beta_1\gamma_1$ and $\beta_1\gamma_{11}$ dimers on PLC-$\beta$ is significantly less than that of the $\beta_1\gamma_2$ dimer and the activity is affected by the type of prenyl group on the $\gamma$ subunit (Myung *et al.*, 1999). To determine how the N and C terminal regions of the $\gamma$ subunit affected the interaction of the $\beta\gamma$ dimer with PLC-$\beta$, dimers containing the 12 $\gamma$ subunits shown in Figure 1B were tested for their

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ability to stimulate avian PLC-β *in vitro.* The data in Figure 3A show that β1γ2 markedly activates PLC-β with an $EC_{50}$ of about 5 nM (closed squares) and that β1γ1 (open circles) is about 5 fold less potent ($EC_{50}$ of 25 nM – see Table II). The activity of the β1γ1 dimer can be made equal to the β1γ2 dimer by replacing the central and C-terminal amino acids of the γ1 subunit with those from the γ2 subunit (open triangles - $\beta1\gamma_{ABB}$ ). A chimeric γ subunit containing the first 50 amino acids of the γ1 subunit and the C terminus of the γ2 subunit ($\beta1\gamma_{AAB}$) is also equally effective as $\beta1\gamma_{ABB}$ (see Table II). These results suggest that the C terminal 23 amino acids of the γ2 subunit are important for potent activation PLC-β in vitro, a result confirmed by the data in Figure 3C which shows replacing the last 23 amino acids and the geranylgeranyl group of γ2 with the amino acids and farnesyl group of γ1 ($\beta1\gamma_{BBA}$) yields a dimer with activity very similar to β1γ1. The amino acids in the central region do not seem to affect the interaction with PLC-β, as $\beta1\gamma_{BAA}$ has an activity equal to $\beta1\gamma_{BBA}$ and β1γ1 (see Table II).

Chimeric γ subunits with switched prenyl groups indicate that the differences the activity of the dimers shown in Figures 3A and C are due both to the differences in the C terminal 23 residues and the composition of the prenyl group. Note that switching the prenyl group on the $\beta_1\gamma_2$ dimer from farnesyl to geranylgeranyl ($\beta1\gamma_{BAA\text{-}S74L}$) provides a dimer with intermediate activity on PLC-β (closed diamonds – Figure 3B). Exchanging the prenyl group on β1γ1 ($\beta1\gamma1\text{-}S74L$) produces a similar result (closed circles). In fact, all dimers containing γ subunits with exchanged prenyl groups were intermediate in either their $EC_{50}$ or their maximal activity on PLC-β (see Table II). These results indicate that the βγ dimers containing the C-terminal sequence of the $\gamma_2$ subunit modified with a geranylgeranyl group are more potent and effective in activating PLC-β *in vitro* than those with the C-terminal region of the $\gamma_1$ subunit and/or modified with farnesyl group.

*Type I Adenylyl Cyclase* — The data in Figure 4 present the ability of five representative βγ dimers to inhibit type I adenylyl cyclase. Figure 4A shows that the $\beta_1\gamma_2$ dimer inhibited type I adenylyl cyclase with an $IC_{50}$ value of 15 nM (closed squares) and was about 2-fold more potent and effective than the $\beta_1\gamma_1$ dimer ($IC_{50}$ of 36 nM - see Table II). However, a

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chimeric $\gamma$ subunit with the C terminal 23 amino acids of the $\gamma_1$ subunit and its prenyl group exchanged with those of the $\gamma_2$ subunit ($\gamma_{AAB}$) produces a $\gamma$ subunit with activity equal to that of the $\gamma_2$ subunit (solid triangles – Figure 4A). In contrast, replacing the C terminus of $\gamma_2$ and the geranylgeranyl group of $\gamma_2$ with the C terminus of $\gamma_1$ and the farnesyl group ($\gamma_{BBA}$) produces a dimer with activity simliar to that of $\beta_1\gamma_1$ (open triangles - Figure 4C). The data in Table II indicate that other dimers in which the C terminus of the $\gamma_1$ subunit is intact and modified with farnesyl were roughly equal to the native $\beta_1\gamma_1$ dimer in their ability to inhibit type I adenylyl cyclase (***e.g.*** $\beta1\gamma_{BAA}$ or $\beta1\gamma_{BBA}$). These observations indicate that the C-terminal region of $\gamma_2$ is most critical for the inhibition of type I adenylyl cyclase *in vitro*. As was the case with PLC-$\beta$, both the C terminal amino acids and the prenyl group appear to be important for the interaction of the $\beta\gamma$ dimer with Type I cyclase. Note that if the prenyl group on the $\beta_1\gamma_{AAB}$ dimer is changed to farnesyl ($\beta_1\gamma_{AAB\text{-}L71S}$), the activity was intermediate between that of $\beta_1\gamma_2$ and $\beta_1\gamma_1$ (closed triangles – Figure 4B). Similar data was obtained with the 6 other dimers containing $\gamma$ subunits with modified prenyl groups (see Table II). Overall, the data in Figures 3-4 demonstrate that the C-terminal amino acid sequence of the $\gamma_2$ subunit and its native geranylgeranyl group are important determinants for the interaction of the $\beta\gamma$ subunit with effectors such as PLC-$\beta$ and type I adenylyl cyclase.

*Type II Adenylyl Cyclase* — The $\beta_1\gamma_2$ subunit activates type II adenylyl cyclase more than ten-fold with a nanomolar $EC_{50}$ and surprisingly, neither the $\beta_1\gamma_1$ nor the $\beta_1\gamma_{11}$ dimer activate type II adenylyl cyclase well (Myung ***et al.***, 1999). In addition, phosphorylation of the $\gamma_{12}$ subunit in the $\beta_1\gamma_{12}$ dimer significantly inhibits its ability to stimulate type II adenylyl cyclase (Yasuda ***et al.***, 1998). The phosphorylation site has been determined to be at $Ser^1$ in the N-terminus of the molecule (Asano ***et al.***, 1998). There are more negative charges in the N-terminal region of $\gamma_1$ or $\gamma_{11}$ as compared to the $\gamma_2$ subunit (Figure 1A), potentially explaining the inability of dimers containing the $\gamma_1$ or $\gamma_{11}$ subunits to activate type II adenylyl cyclase. Taken together, these results suggest that introduction of negative charges in the N-terminal regions of the $\gamma$ subunit inhibit the interaction of the dimer with the type II adenylyl cyclase. Thus, we examined the ability

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of $\beta\gamma$ dimers containing N-terminal chimeric $\gamma$ subunits to activate type II adenylyl cyclase.

The data in Figure 5 present the ability of 6 representative $\beta\gamma$ dimers to activate type II adenylyl cyclase performed using concentrations of dimers ranging from 0.1 nM to 100 nM. As expected (Yasuda *et al.*, 1998), the $\beta_1\gamma_2$ dimer (closed squares) activated type II adenylyl cyclase with an estimated $EC_{50}$ value of 13 nM and was far more potent and effective in activating this effector enzyme than the $\beta_1\gamma_1$ dimer (Figure 5A). Adding the N-terminal region of the $\gamma_2$ subunit to the $\gamma_1$ subunit and changing its prenyl group to geranylgeranyl ($\beta_1\gamma_{BAA\text{-}S74L}$) generated a molecule with activity equal to $\beta_1\gamma_2$ (closed diamonds – Figure 5A). A similar result was observed with $\beta_1\gamma_{BBA\text{-}S74L}$ (see Table III). In contrast, a dimer with the poor activity of $\beta_1\gamma_1$ could be created using a chimeric $\gamma$ subunit containing the N terminal 23 amino acids of $\gamma_1$ and a farnesyl group ($\beta_1\gamma_{AAB\text{-}L71S}$ - closed circles – Figure 5C). These results suggest that both the N-terminal region and the geranylgeranyl group are very important for the activation of type II adenylyl cyclase *in vitro*. Indeed, Figure 5B shows that the $\beta_1\gamma_{BAA}$ dimer which contained the N-terminal amino acids of $\gamma_2$ and the farnesyl modification was intermediate in the activation of type II adenylyl cyclase. Indeed, the data in Table III show that every chimeric $\gamma$ subunit which is intermediate in activity on type II cyclase is missing either the N terminal amino acids of $\gamma_2$ and or the geranylgeranyl group. These results indicate that the $\beta\gamma$ dimers containing the N-terminal sequence of the $\gamma_2$ subunit which has one negatively charged amino acid are more potent and effective in activating type II adenylyl cyclase than those with the N-terminal region of the $\gamma_1$ subunit which has six negatively charged amino acids. A C terminus modified with the geranylgeranyl group is also important. Similar results are observed with the effector PtdIns 3-kinase in that dimers containing $\gamma_1$ or $\gamma_{11}$ weakly activate the enzyme (Kerchner *et al.*, 2004).

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## Discussion

There are three major findings of this *in vitro* study. First, βγ dimers with a γ subunit containing the C-terminal 23 amino acid sequence of the $\gamma_2$ subunit and modified with a geranylgeranyl group have the highest affinity for the Gi1 α subunit and are the most effective in coupling of the $G_{i1}$ α subunit to bovine A1 adenosine receptors (Figure 2). These data provides an important demonstration of the correlation between the affinity of the α subunit for the βγ dimer and the ability of the complex to support high affinity receptor – G protein coupling. Second, dimers with these attributes are most effective at activating PLC-β and inhibiting type I adenylyl cyclase *in vitro* (Figures 3-4). Finally, βγ dimers containing the N-terminal 19 residues of the $\gamma_2$ subunit are far more effective at activating type II adenylyl cyclase than those containing the N-terminal 22 residues of the $\gamma_1$ subunit. The full activity on type II adenylyl cyclase also required modification of the γ subunit with a geranylgeranyl group, but experiments with dimers such as $\beta_1\gamma_{1\text{-S74L}}$ and $\beta_1\gamma_{11\text{-S73L}}$ (Table II) which are modified with the geranylgeranyl lipid, indicate that the C-terminal prenyl group is of secondary importance to the N terminal amino acid residues in the activation of this effector (Myung *et al.*, 1999).

The finding that the C-terminal region of the γ subunit and its prenyl group is much more important than the N terminus for the interaction with α subunits and receptors confirms and extends a large amount of data obtained with rhodopsin in the visual system (Jian *et al.*, 2001), the A1 adenosine receptor (Yasuda *et al.*, 1996), the 5-$HT_{1A}$ receptor (Butkerait *et al.*, 1995), the $\alpha_{2A}$ receptor (Richardson and Robishaw, 1999;Lim *et al.*, 2001) and bombesin receptors (Jian *et al.*, 1999). Experiments performed with a similar set of chimeric γ subunits made between $\gamma_1$ and $\gamma_2$ were tested for their ability to support coupling between rhodopsin and the Gt α subunit (Jian *et al.*, 2001). These experiments also indicated that the C terminus of the $\gamma_2$ subunit and its geranygeranyl group contribute to better coupling between receptor and the heterotrimer (Jian *et al.*, 2001).

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Moreover, experiments using synthetic peptides to probe the rhodopsin : $\gamma$ subunit interaction found that the C-terminal 12 amino acids of the $\gamma$ subunit (DKNPFKELKGGC in $\gamma_1$ or SENPFREKKFFC in $\gamma_2$) are clearly involved in interacting with $\alpha$ subunits and the receptor. When the $\gamma_1$ peptide was altered to change $Phe^{64}$ to Thr and $Leu^{67}$ to Ser, it markedly decreased the interaction with rhodopsin (Azpiazu *et al.*, 1999). Taken together, the results indicate that the C terminus of the mammalian $\gamma$ subunit is very important for the interaction between G protein-coupled receptors and the heterotrimer. Interestingly, the receptor also appears to contact multiple domains in the $\alpha$ subunit and a region near the C terminus of the $\beta$ subunit (Cabrera-Vera *et al.*, 2003). Thus, each subunit in the heterotrimer is used for efficient activation of the $\alpha$ subunit.

While data obtained with mammalian cells point to a role for the C terminus of the $\gamma$ subunit in coupling $\beta\gamma$ dimers to receptors, studies with the *S. cerevisiae* pheromone mating pathway provide evidence that the C-terminal region of the yeast $\gamma$ subunit is not important in receptor coupling. Using yeast strains in which the Ste18 locus (the only yeast $\gamma$ subunit) was disrupted and pheromone signaling inhibited, a variety of mutated $\gamma$ subunits subunits were tested for their ability to rescue signaling. Yeast $\gamma$ subunits with a variety of C-terminal sequences, mutated by alanine scanning or by deletion of up to 8 amino acids immediately preceding the CAAX box were all able to rescue pheromone signaling (Chinault and Blumer, 2003). These data suggests that this region of the yeast molecule was not important for signaling through yeast G protein coupled receptors *in vivo* (Chinault and Blumer, 2003). More studies with mammalian cells are needed to reconcile these differences, although the diversity of the G$\beta$ and G$\gamma$ subunits expressed in the mammalian genome will complicate these studies.

While the C termini of $\gamma$ subunits contain sequences capable of interacting with multiple receptors, subtle differences in the residues and charges must be important in determining specificity. For example, the $\beta_1\gamma_1$ dimer couples the $G_{i1}$ $\alpha$ subunit to the $\alpha_2$-adrenergic receptor poorly (Richardson and Robishaw, 1999), whereas the $\beta_1\gamma_{11}$ dimer couples well (Lim *et al.*, 2001), yet there are only two differences in the C-terminal 12

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amino acids of $\gamma_1$ and $\gamma_{11}$ (see Figure 1A). Experiments performed with receptors reconstituted with G protein heterotrimers using various protocols show relatively modest differences in the interaction of the various $\gamma$ subunits with receptors (Hou *et al.*, 2001;Akgoz *et al.*, 2002;Lim *et al.*, 2001;Yasuda *et al.*, 1996). However, antisense experiments performed with intact cells suggest an exquisite selectivity between receptors, $\alpha$ subunits and certain $\beta\gamma$ combinations (Kleuss *et al.*, 1993). Thus, in spite of considerable effort, the factors dictating the specificity that must exist in intact cells remain elusive. These data, combined with the studies performed in yeast, suggest that additional work with mammalian cells is needed to reconcile these differences. The diversity of the G$\beta$ and G$\gamma$ subunits in the mammalian genome will make these studies complex, but the observation that mice with the $\gamma_7$ subunit knocked out have an altered phenotype (Schwindinger *et al.*, 2003) offers hope that these studies will provide interesting results.

The data in Figure 3 indicate that, as with receptors, both the prenyl group and the C-terminal amino acids of the $\gamma$ subunit contribute significantly to the interaction of the dimer with PLC-$\beta$ in vesicles. This finding is consistent with experiments showing that native dimers containing the $\gamma_1$ or $\gamma_{11}$ subunits have reduced activity on this effector (Myung *et al.*, 1999). In addition, experiments performed with dimers containing the $\gamma_5$ subunit modified to contain a shortened or extended C terminus are less effective at activating PLC-$\beta$ (Akgoz *et al.*, 2002). A possible explanation for the role of the prenyl group in the activation of effectors may be provided by the hypothesis that the prenyl group on the $\gamma$ subunit interacts with the C-terminal region of the $\beta$ subunit and participates in a conformational change that, in part, determines the activity of the dimer at effectors (Myung and Garrison, 2000). Alternatively, the prenyl group may interact directly with PLC-$\beta$ as suggested by experiments with prenylated peptides (Akgoz *et al.*, 2002). It is also possible that both mechanisms come into play. A more complete understanding of the role of this region of the $\beta$ and $\gamma$ subunits in these interactions awaits structural information or biophysical experiments determining the exact sites of contact between these regions of the dimer and receptors or effectors.

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A surprising finding is that the C-terminal region of the γ subunit appears to predominate in the inhibition of type I adenylyl cyclase whereas the N-terminal region of the molecule is the most important for the activation of type II adenylyl cyclase. However, the regulation of adenyl cyclase is complex, with multiple binding sites identified for the known regulatory molecules (Taussig *et al.*, 1994). The differences identified for regulation by the γ subunit in the dimer are in keeping with these findings. The observations that the N-terminal domain of γ is important for the activation of type II cyclase is consistent with earlier experiments showing that phosphorylation of the $\gamma_{12}$ subunit in the $\beta_1\gamma_{12}$ dimer with protein kinase C decreases its ability to stimulate type II adenylyl cyclase (Yasuda *et al.*, 1998). The phosphorylation site in $\gamma_{12}$ is $Ser^1$ at the N terminus (Asano *et al.*, 1998), suggesting that negative charges in this region decrease the interaction of the dimer with type II cyclase. Interestingly, the $\gamma_1$, $\gamma_8$ and $\gamma_{11}$ subunits contain six negatively charged amino acids in their N terminus (Figure 1A) and thus dimers containing these γ's might be predicted to interact poorly with type II cyclase. Indeed, dimers containing $\gamma_1$, $\gamma_{11}$ or chimeras such as $\gamma_{ABB\text{-}L71S}$ or $\gamma_{ABB}$ are not able to activate the enzyme well (see Table III and (Myung *et al.*, 1999)). Together, these results strongly suggest that negatively charged amino acids in the N terminus of the γ subunit lead to an inability to activate type II adenylyl cyclase *in vitro*. The observation that inhibition of the type I isoform of adenyl cyclase is very dependent on the sequences in the opposite end of the γ subunit indicates that the different isoforms of adenyl cyclase must bind to different regions of the β and γ subunits in the βγ dimer. In keeping with this finding, mutations on the top surface of the β subunit that dramatically inhibit activation of type II cyclase do not alter the inhibition of type I cyclase (Li *et al.*, 1998). Thus, the precise determinants of the interaction of the type I and type II cyclases with βγ dimers appear to be different.

The isoform of the β subunit is also important in the interaction of βγ dimers with effectors and certain regions on the top surface of the β subunit have been identified as critical for interaction of the βγ dimer with effectors (Ford *et al.*, 1998). A major concept arising from these studies is that while different effectors may interact with distinct or overlapping regions of the β subunit, certain regions in the β subunit interacting with

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effectors are in the domain covered by the $\alpha$ subunit in the heterotrimer (Ford *et al.*, 1998). Thus, formation of the basal state of the $\alpha$:$\beta\gamma$-receptor complex is an efficient mechanism to terminate $\beta\gamma$ signaling. The data in Figure 2B shows that the C terminus of the $\gamma$ subunit and its prenyl group are important determinants of the affinity of the $\alpha$ subunit for the $\beta\gamma$ dimer. Thus, this domain of the $\gamma$ subunit may be important for the rapid formation of a high affinity, $\alpha : \beta\gamma$ complex and which speeds reversal of the effects of the $\beta\gamma$ dimer on targets such as PLC-$\beta$ or adenylyl cyclase. The present study also demonstrates that the nature of the N and C terminal regions of the $\gamma$ subunit imparts significant specificity to $\beta\gamma$ signaling. Thus, $\beta\gamma$ dimers containing $\gamma$ subunits from subfamily I such as $\gamma_1$, or $\gamma_{11}$ (and probably $\gamma_8$, although it has not been studied extensively) appear to have unique signaling properties in that they are unable to activate type II adenylyl cyclase or other important effectors such as phosphatidylinositol (4,5) 3-kinase (Kerchner *et al.*, 2004). The distribution of $\gamma_1$ is restricted to retinal rods (Fung, 1983) and the original report of $\gamma_8$ (termed $\gamma_c$) suggested that it was restricted to cone cells (Ong *et al.*, 1995). However, the $\gamma_{11}$ subunit is widely expressed (Morishita *et al.*, 1998) and it now appears that $\gamma_8$ is also widely expressed (Downes and Gautam, 1999). Thus, it is important to define the signaling properties of dimers containing these farnesylated $\gamma$ subunits in multiple cellular systems. The appealing concept that certain $\beta\gamma$ dimers may associate selectively with distinct $\alpha$ subunits and/or receptors has been employed to account for the diversity of these subunits in the G protein family (McIntire *et al.*, 2001;Richardson and Robishaw, 1999;Cabrera-Vera *et al.*, 2003;Hildebrandt, 1997). This hypothesis also predicts that unique $\beta\gamma$ dimers will be released upon receptor activation in intact cells, allowing a given receptor to release a $\beta\gamma$ dimer with selective signaling properties. If the $\beta\gamma$ dimers released were from subfamily I, they might have very selective signaling properties at effectors. The data in this report support this idea, however future experiments will need to concentrate on the functions of this diverse family of proteins in intact cellular systems.

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## Footnotes

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## Figure Legends

**Fig. 1.** Strategy for constructing the chimeric $\gamma$ subunits. (***A)***, Comparison of the primary sequences and charged amino acids in the N- and C-terminal regions of the $\gamma_1$ and $\gamma_{11}$ subunits with those of $\gamma_2$. The QLK and DPL sites and the C-terminal *CAAX* motif are highlighted. The center section of the molecules is omitted. ***(B)***, Schematic view of the chimeric $\gamma$ subunits. The sequence of the $\gamma_1$ subunit is indicated in *white*, and the $\gamma_2$ subunit in *grey*. Both the $\gamma_1$ and $\gamma_2$ subunits are divided into three regions at the QLK and DPL sites. The molecules were designated as AAA for the $\gamma_1$ subunit and BBB for the $\gamma_2$ subunit. The farnesyl group (C-15) on the $\gamma_1$ subunit is indicated in *light grey*, and the geranylgeranyl group (C-20) on the $\gamma_2$ subunit is indicated in *dark grey*. The nomenclature used in the text to identify each chimera is indicated above the schematic representation.

**Fig. 2.** Comparison of the ability of native $\beta\gamma$ dimers and those containing chimeric $\gamma$ subunits to support the high affinity, agonist binding state of the A1 adenosine receptor. **(*A)***, Sf9 cell membranes expressing recombinant, bovine A1 adenosine receptors were reconstituted with the Gi1 $\alpha$ subunit and the $\beta\gamma$ dimer at concentrations of 0 nM, 2 nM, 6 nM or 40 nM. The ratio of receptor : $\alpha$:$\beta\gamma$ was approximately 1:25. The amount of agoinst binding to the receptor was measured with graded concentrations of $^{125}$I-ABA as described in *Experimental Procedures*. The figure indicates the recovery of high-affinity, agonist binding conformation of the receptor as a function of the total amount of G protein heterotrimer reconstituted into the membrane. Each data point is an average of three similar experiments performed in triplicate. **(*B)*,** A plot of the affinity of the Gi1 $\alpha$ subunit for eight different $\beta\gamma$ dimers measured in the flow cytometer against the $EC_{50}$'s for formation of the high affinity, agonist binding state of the receptor measured at 6 nM Gi1 $\alpha$ and concentrations of $\beta\gamma$ ranging from 0-100 nM. Each data point is an average of three similar experiments performed in triplicate. Details of both assays are found in *Experimental Procedures.* The open squares represent dimers with farnesyl moieties and the closed squares represent dimers with geranylgeranyl moieties.

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**Fig. 3.** Comparison of the ability of native βγ dimers and those containing chimeric γ subunits to stimulate PLC-β. **(*A*),** The indicated concentrations of βγ dimers were reconstituted with recombinant, turkey PLC-β in phospholipid vesicles containing [$^3$H]-$PIP_2$ and PLC-β activity was measured as described in *Experimental Procedures*. The activity of β1γABB (*open triangles*) was compared with β1γ2 (*closed squares, thick solid line*) and β1γ1 (*open circles, thick dotted line*). The effect of the β1γABB dimer was statistically different from β1γ1 ($p < 0.0001$; see Table II), but not from β1γ2. **(*B*),** An analogous experiment performed with β1γ1-S74L and β1γBAA-S74L and compared with the effects of β1γ1 and β1γ2. See Table II for the activity of dimers containing other chimeric γ subunits. **(C*)*,** An analogous experiment performed with β1γBBA and compared with the effect of β1γ1 and β1γ2. The effect of the β1γBAA dimer was statistically different from β1γ2 , but not from β1γ1 ($p < 0.0001$; see Table II). Each data point is an average of three independent experiments, each performed in duplicate.

**Fig. 4.** Comparison of the ability of native βγ dimers and those containing chimeric γ subunits to inhibit type I adenylyl cyclase. ***(A),*** Sf9 cells were infected with a recombinant baculovirus encoding the type I adenylyl cyclase, membranes prepared, and the cyclase reaction performed with the indicated concentrations of βγ dimers. Cyclic AMP was measured as described in *Experimental Procedures*. The ability of β1γAAB (*closed triangles*) to inhibit type I cyclase was compared with β1γ2 (*closed squares, thick solid line*) and β1γ1 (*open circles, thick dotted line*). The effect of β1γAAB was statistically significant different from β1γ1 but not from β1γ2 (see Table II). Each data point is an average of three independent experiments, each performed in duplicate. **(*B*)** An analogous experiment performed with β1γAAB-L71S (*closed triangles*) and compared with the effect of β1γ1 and β1γ2. Each data point is an average of three independent experiments, each performed in duplicate. **(C*)*,** An analogous experiment performed with β1γBBA (*open triangles*) and compared with the effect of β1γ1 and β1γ2. The effect of the β1γBBA dimer was statistically different from β1γ2 , but not from β1γ1 ($p < 0.0001$; see Table II). Each data point is an average of three independent experiments, each performed in duplicate.

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**Fig. 5.** Comparison of the ability of native βγ dimers and those containing chimeric γ subunits to stimulate type II adenylyl cyclase. ***(A),*** Sf9 cells were infected with a recombinant baculovirus encoding the type II adenylyl cyclase, membranes prepared, and the cyclase reaction performed with the indicated concentrations of βγ dimers as described in *Experimental Procedures*. The ability of β1γBAA-S74L (*closed diamonds*) to activate type II adenylyl cyclase was compared with β1γ2 (*closed squares, thick solid line*) and β1γ1 (*open circles, thick dotted line*). The effect of β1γBAA-S74L was statistically significant different from β1γ1 (see Table III). ***(B)*** An analogous experiment performed with β1γBAA and β1γBBA and compared with the effects of β1γ1 and β1γ2. Each data point is an average of three independent experiments, each performed in duplicate. ***(C)*** An analogous experiment performed with β1γABB-L71S (*closed circles*) and compared with the effects of β1γ1 and β1γ2. The difference between the effect of β1γABB-L71S and β1γ2 was statistically significant (see Table III). Each data point is an average of three independent experiments, each performed in duplicate.

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Table I

*Molecular mass of recombinant, chimeric $\gamma$ subunits.*

The molecular weight of the $\gamma$ subunits in the dimers used in this study was measured by mass spectrometry as described "*Experimental Procedures*". Molecular masses were determined on two independent preparations of each $\beta\gamma$ dimer and were in agreement. The data presented are from one determination.

| | $\gamma$ Subunits | Calculated Mass (*Da*) | Observed Mass (*Da*) |
|---|---|---|---|
| **Native** | $\gamma_1$ | 8,332 | 8,330 |
| | $\gamma_2$ | 7,751 | 7,751 |
| **Exchanged Prenyl Group** | $\gamma_{1\text{-S74L}}$ | 8,400 | 8,400 |
| | $\gamma_{2\text{-L71S}}$ | 7,683 | 7,681 |
| **C-Terminal Chimeras** | $\gamma_{AAB}$ | 8,551 | 8,552 |
| | $\gamma_{AAB\text{-L71S}}$ | 8,483 | 8,482 |
| | $\gamma_{BBA}$ | 7,532 | 7,531 |
| | $\gamma_{BBA\text{-S74L}}$ | 7,600 | 7,602 |
| **N-Terminal Chimeras** | $\gamma_{BAA}$ | 7,818 | 7,818 |
| | $\gamma_{BAA\text{-S74L}}$ | 7,886 | 7,888 |
| | $\gamma_{ABB}$ | 8,265 | 8,265 |
| | $\gamma_{ABB\text{-L71S}}$ | 8,197 | 8,199 |

Table II

Comparison of the ability of βγ dimers containing native or chimeric γ subunits to activate PLC-β or to inhibit Type I adenylyl cyclase. The $EC_{50}$, $IC_{50}$, or $V_{max}$ values for PLC obtained with the indicated βγ dimers were determined by fitting each data set to sigmoid curves as described in "*Experimental Procedures.*" Vinhib is the level of cyclic AMP production (as nmole cyclic AMP/mg membrane protein/min) observed with $10^{-7}$m of the indicated βγ dimer. Control levels of ACI activity were 0.9 nmoles cyclic AMP/mg protein/min. Values are the average of three similar experiments expressed as means ± S.E.M.

| Type | βγ Dimers | Phospholipase C-β | | Type I Adenylyl Cyclase | |
|---|---|---|---|---|---|
| | | $EC_{50}$ (nM) | $V_{max}$ (μmol/mg PLC/min) | $IC_{50}$ (nM) | $V_{inbib}$ (nmol/mg protein/min) |
| **Chimeras with activity similar to $\beta_1\gamma_2$** | | | | | |
| | $\beta_1\gamma_2$ | 5.2 ± 1.1 | 3.40 ± 0.07 | 15.4 ± 1.4 | 0.26 ± 0.06 |
| | $\beta_1\gamma_{ABB}$ | 6.2 ± 1.2† | 3.24 ± 0.13† | 18.1 ± 2.2† | 0.34 ± 0.24† |
| | $\beta_1\gamma_{AAB}$ | 7.8 ± 1.1† | 3.16 ± 0.09† | 14.2 ± 1.3† | 0.39 ± 0.03† |
| **Chimeras with activity similar to $\beta_1\gamma_1$** | | | | | |
| | $\beta_1\gamma_1$ | 24.6 ± 1.2 | 2.14 ± 0.16 | 36.4 ± 2.6 | 0.58 ± 0.09 |
| | $\beta_1\gamma_{BBA}$ | 51.2 ± 1.3* | 2.57 ± 0.29* | 96.0 ± 15.1* | 0.48 ± 0.28* |
| | $\beta_1\gamma_{BAA}$ | 25.8 ± 1.3* | 1.95 ± 0.15* | 60.7 ± 6.6* | 0.49 ± 0.25* |
| | $\beta_1\gamma_{AAB\text{-}L71S}$ | 19.1 ± 1.3§ | 2.32 ± 0.13§ | 24.6 ± 1.5§ | 0.54 ± 0.03§ |
| ***Chimeras with Intermediate activity*** | | | | | |
| | $\beta_1\gamma_{1\text{-}S74L}$ | 15.9 ± 1.2§ | 3.21 ± 0.20§ | 18.5 ± 1.7§ | 0.53 ± 0.04§ |
| | $\beta_1\gamma_{2\text{-}L71S}$ | 15.0 ± 1.2§ | 2.83 ± 0.14§ | 26.2 ± 2.6§ | 0.43 ± 0.15§ |
| | $\beta_1\gamma_{BBA\text{-}S74L}$ | 12.2 ± 1.5§ | 2.94 ± 0.22§§ | 16.2 ± 2.2§ | 0.51 ± 0.08 |
| | $\beta_1\gamma_{BAA\text{-}S74L}$ | 12.2 ± 1.2§ | 2.34 ± 0.22§§ | 22.9 ± 1.9§ | 0.37 ± 0.14 |
| | $\beta_1\gamma_{ABB\text{-}L71S}$ | 15.6 ± 1.3§ | 2.66 ± 0.17§ | 26.1 ± 2.2§ | 0.62 ± 0.11§ |

* Significant differences in responses to βγ dimers in comparison with native $\beta_1\gamma_2$; * $p < 0.0001$.

† Significant differences in responses to βγ dimers in comparison with native $\beta_1\gamma_1$; † $p < 0.0001$.

§ Significant differences in responses to βγ dimers containing γ subunits altered *CAAX* sequence to direct modification with different prenyl groups with those containing γ subunits with native prenyl groups; § $p < 0.0001$, §§ $p < 0.001$.

Table III

Comparison of the ability of βγ dimers containing native or chimeric γ subunits to activate Type II adenylyl cyclase. The $EC_{50}$ and $V_{max}$ values obtained with each βγ dimer was determined by fitting each data set to sigmoid curves as described in "*Experimental Procedures.*" Values are the average of three similar experiments expressed as means ± S.E.M.

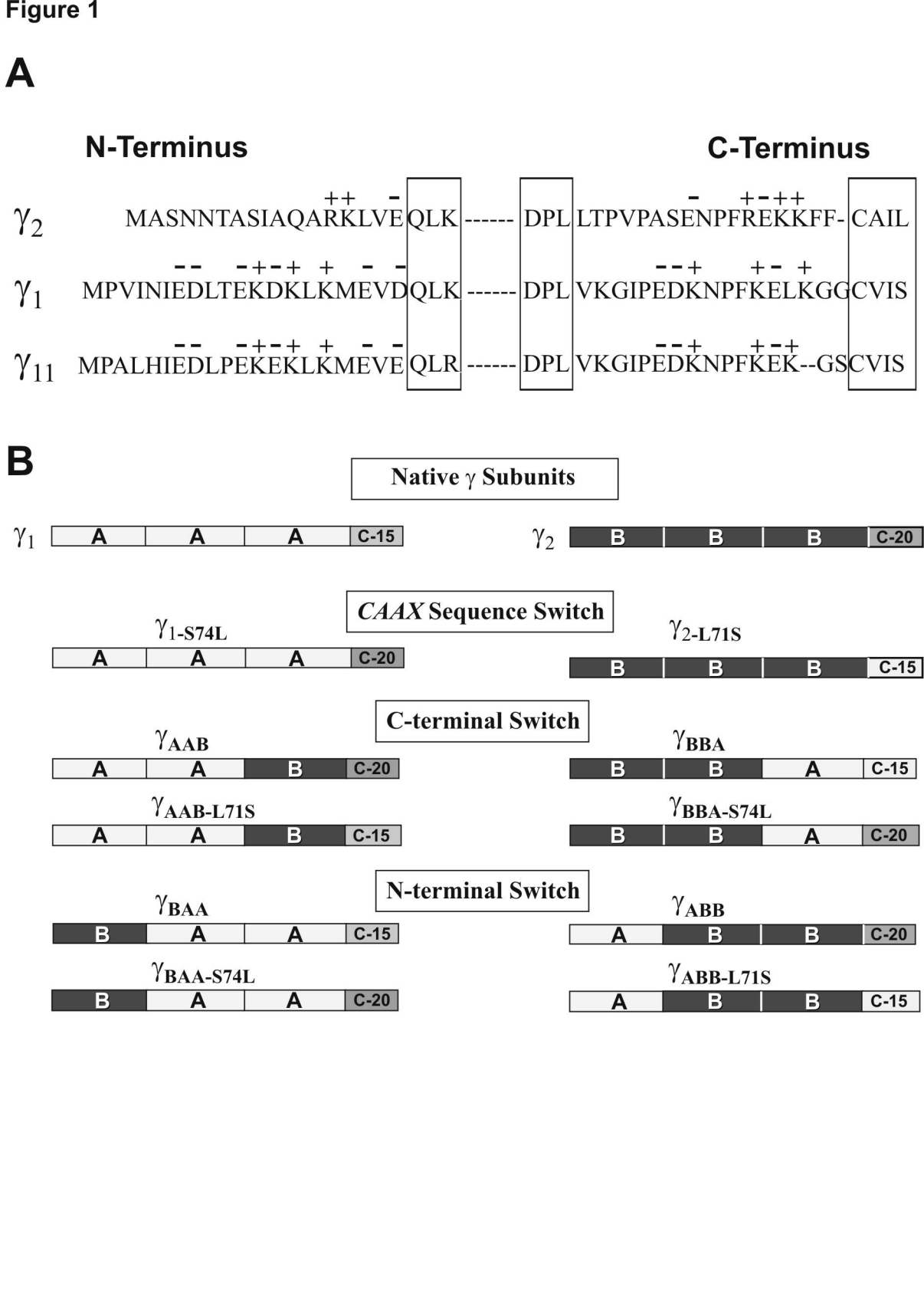
| *Type* | **βγ Dimers** | Type II Adenylyl Cyclase | |
|---|---|---|---|
| | | *$EC_{50}$* *(nM)* | *$V_{max}$* *(μmol/mg protein/min)* |
| **Chimeras with activity similar to $\beta_1\gamma_2$** | | | |
| | $\beta_1\gamma_2$ | 13.3 ± 1.2 | 29.05 ± 1.72 |
| | $\beta_1\gamma_{BAA\text{-}S74L}$ | 15.6 ± 1.2[§] | 27.8 ± 1.74[§] |
| | $\beta_1\gamma_{BBA\text{-}S74L}$ | 18.2 ± 1.4[§] | 24.07 ± 1.85[§] |
| **Chimeras with activity similar to $\beta_1\gamma_1$** | | | |
| | $\beta_1\gamma_1$ | 46.7 ± 1.9 | 6.31 ± 0.65 |
| | $\beta_1\gamma_{ABB\text{-}L71S}$ | 48.7 ± 2.1[§] | 6.89 ± 1.09[§] |
| | $\beta_1\gamma_{AAB}$ | 61.8 ± 2.0[*] | 9.13 ± 2.31[*] |
| | $\beta_1\gamma_{AAB\text{-}L71S}$ | 93.3 ± 11.9[§] | 25.91 ± 9.45[§] |
| | $\beta_1\gamma_{1\text{-}S74L}$ | 33.1 ± 1.6[§§] | 8.39 ± 1.68 |
| ***Chimeras with Intermediate activity*** | | | |
| | $\beta_1\gamma_{BAA}$ | 24.6 ± 1.3[†] | 13.52 ± 1.11[†] |
| | $\beta_1\gamma_{BBA}$ | 35.7 ± 1.2[††] | 14.05 ± 1.07[†] |
| | $\beta_1\gamma_{2\text{-}L71S}$ | 41.7 ± 1.3[§] | 16.19 ± 1.3[§] |
| | $\beta_1\gamma_{ABB}$ | 25.2 ± 1.3[*] | 23.17 ± 1.66[††] |

* Significant differences in responses to βγ dimers in comparison with native $\beta_1\gamma_2$; * $p < 0.0001$, ** $p < 0.001$.

† Significant differences in responses to βγ dimers in comparison with native $\beta_1\gamma_1$; † $p < 0.0001$, †† $p < 0.001$.

§ Significant differences in responses to βγ dimers containing γ subunits altered *CAAX* sequence to direct modification with different prenyl groups with those containing γ subunits with native prenyl groups; § $p < 0.0001$, §§ $p < 0.001$.

Figure 1


A
N-Terminus
C-Terminus
γ2 MASNNTASIAQARKLVEQLK------DPLLTPVPASENPFREKKFF-CAIL
γ1 MPVINIEDLTEKDKLKMEVDQLK------DPLVKGIPEDKNPFKELKGGCVIS
γ11 MPALHIEDLPEKEKLKMEVEQLR------DPLVKGIPEDKNPFKEK--GSCVIS
B
Native γ Subunits
γ1
A
A
A
C-15
γ2
B
B
B
C-20
CAAX Sequence Switch
γ1-S74L
A
A
A
C-20
γ2-L71S
B
B
B
C-15
C-terminal Switch
γAAB
A
A
B
C-20
γBBA
B
B
A
C-15
γAAB-L71S
A
A
B
C-15
γBBA-S74L
B
B
A
C-20
N-terminal Switch
γBAA
B
A
A
C-15
γABB
A
B
B
C-20
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A
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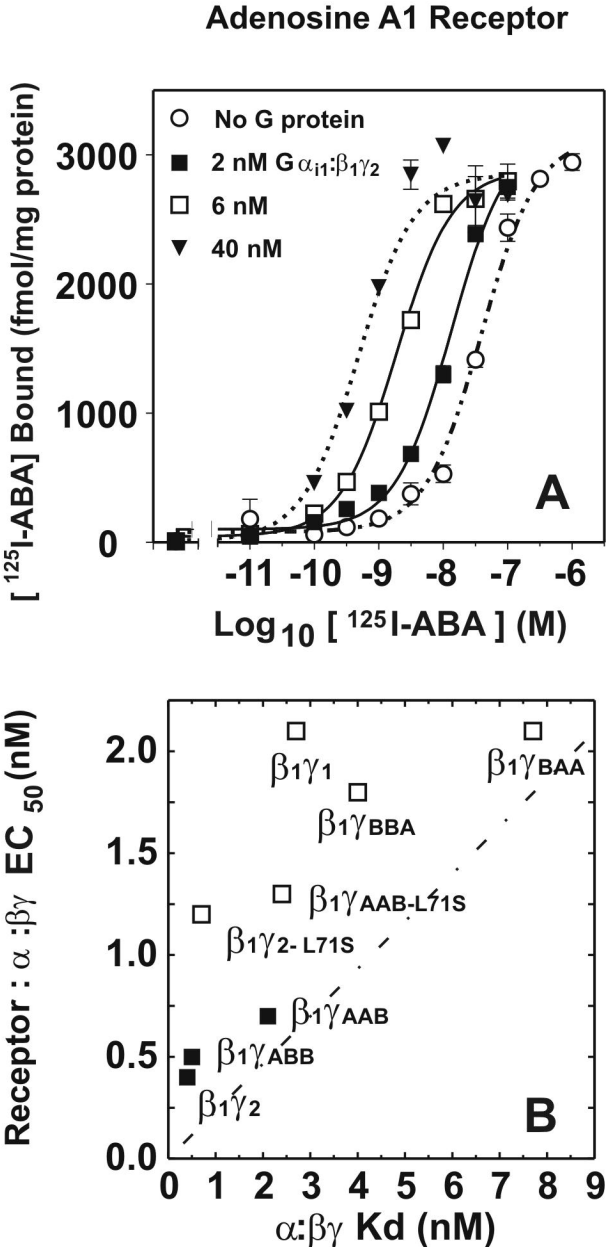
C-20
γABB-L71S
A
B
B
C-15

# Figure 2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2 nM Gαi1:β1γ2
6 nM
40 nM
[125I-ABA] Bound (fmol/mg protein)
3000
2000
1000
0
A
-11 -10 -9 -8 -7 -6
Log10 [125I-ABA] (M)
Receptor : α :βγ EC 50 (nM)
2.0
1.5
1.0
0.5
0.0
β1γ1
β1γBAA
β1γBBA
β1γAAB-L71S
β1γ2- L71S
β1γAAB
β1γABB
β1γ2
B
0 1 2 3 4 5 6 7 8 9
α:βγ Kd (nM)

# Figure 3

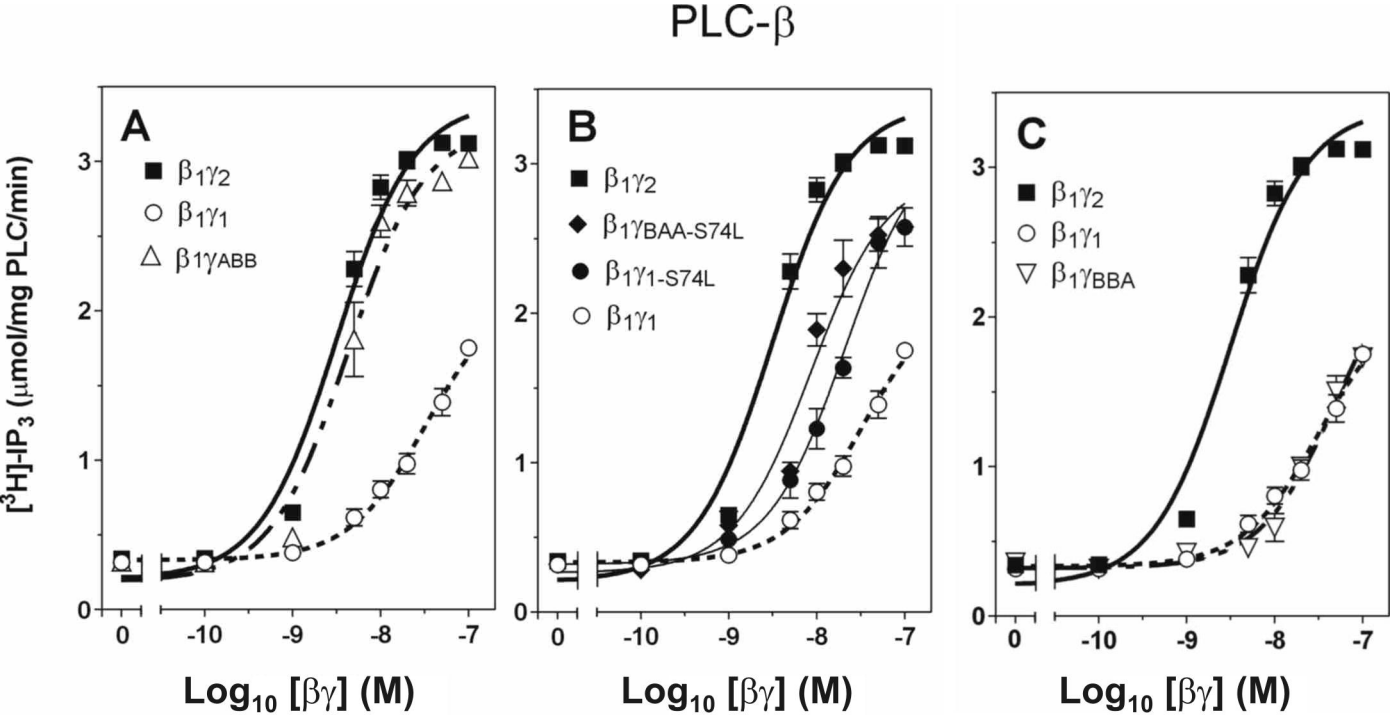
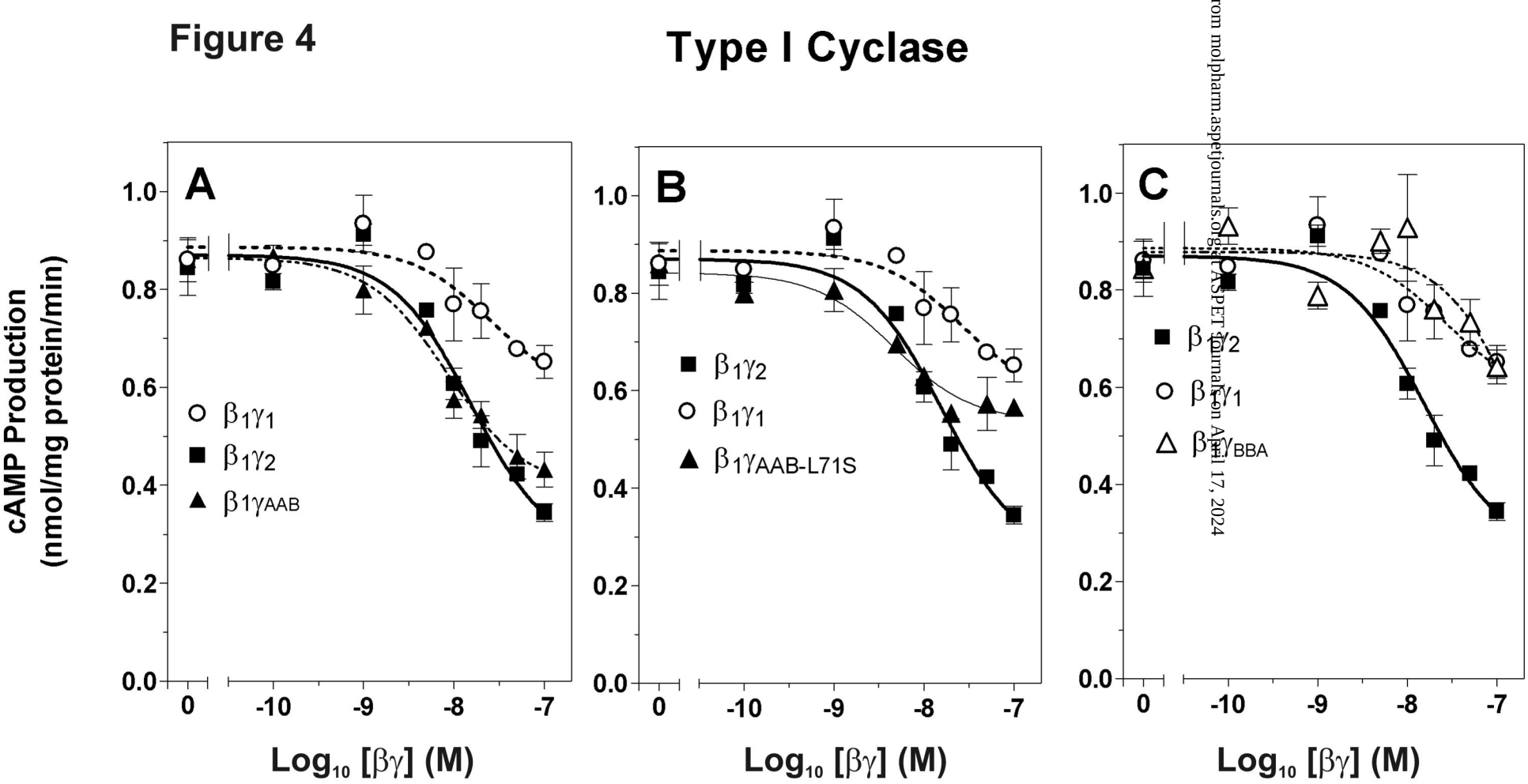
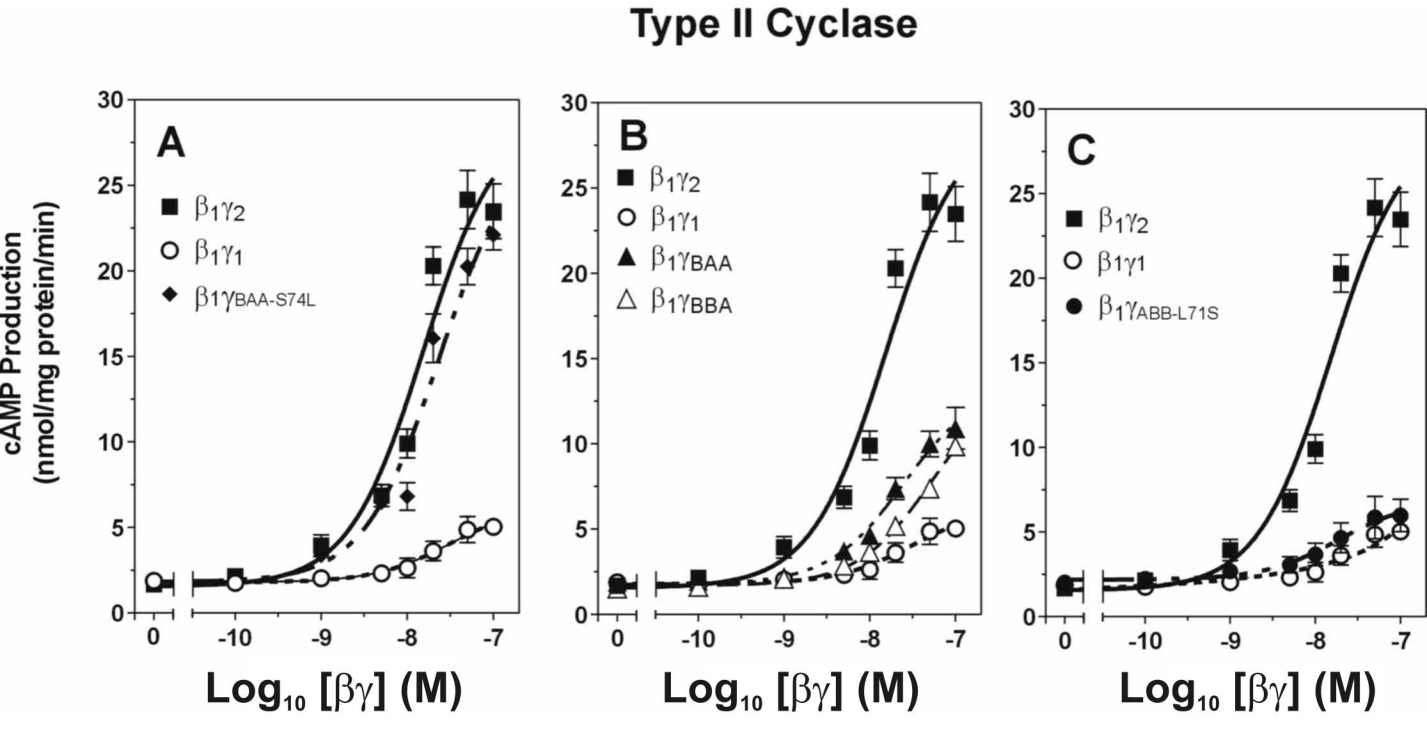

PLC-β
A
B
C
β1γ2
β1γ1
β1γABB
β1γBAA-S74L
β1γ1-S74L
β1γBBA
[3H]-IP3 (μmol/mg PLC/min)
Log10 [βγ] (M)
0
1
2
3
-10
-9
-8
-7

Figure 4



# Figure 5


Type II Cyclase
A
B
C
β1γ2
β1γ1
β1γBAA-S74L
β1γBAA
β1γBBA
β1γABB-L71S
cAMP Production (nmol/mg protein/min)
Log10 [βγ] (M)
0
5
10
15
20
25
30
-10
-9
-8
-7