BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2011,** *50,* 207–220 207
DOI: 10.1021/bi101227y

Binding of $\beta_4\gamma_5$ by Adenosine A_1 and A_{2A} Receptors Determined by Stable Isotope Labeling with Amino Acids in Cell Culture and Mass Spectrometry[†]

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Received August 2, 2010; Revised Manuscript Received December 1, 2010

ABSTRACT: Characterization of G protein $\beta\gamma$ dimer isoform expression in different cellular contexts has been impeded by low levels of protein expression, broad isoform heterogeneity, and antibodies of limited specificity, sensitivity, or availability. As a new approach, we used quantitative mass spectrometry to characterize native $\beta\gamma$ dimers associated with adenosine A_1:α_{i1} and adenosine A_{2A}:α_S receptor fusion proteins expressed in HEK-293 cells. Cells expressing A_1:α_{i1} were cultured in media containing [$^{13}C_6$]Arg and [$^{13}C_6$]Lys and $\beta\gamma$ labeled with heavy isotopes purified. Heavy $\beta\gamma$ was combined with either recombinant $\beta\gamma$ purified from Sf9 cells, $\beta\gamma$ purified from the A_{2A}:α_S expressed in HEK-293 cells cultured in standard media, or an enriched $\beta\gamma$ fraction from HEK-293 cells. Samples were separated by SDS−PAGE, protein bands containing β and γ were excised, digested with trypsin, and separated by HPLC, and isotope ratios were analyzed by mass spectrometry. Three β isoforms, β_1, β_2, and β_4, and seven γ isoforms, γ_2, γ_4, γ_5, γ_7, γ_{10}, γ_{11}, and γ_{12}, were identified in the analysis. β_1 and γ_5 were most abundant in the enriched $\beta\gamma$ fraction, and this $\beta\gamma$ profile was generally mirrored in the fusion proteins. However, both A_{2A}:α_S and A_1:α_{i1} bound more β_4 and γ_5 compared to the enriched $\beta\gamma$ fraction; also, more β_4 was associated with A_{2A}:α_S than A_1:α_{i1}. Both fusion proteins also contained less γ_2, γ_{10}, and γ_{12} than the enriched $\beta\gamma$ fraction. These results suggest that preferences for particular $\beta\gamma$ isoforms may be driven in part by structural motifs common to adenosine receptor family members.

The G protein[1] $\beta\gamma$ dimer participates in the initiation of signaling cascades by coupling Gα subunits to G protein coupled receptors (*1*), and once activated, $\beta\gamma$ dimers can interact with and regulate a multitude of signaling proteins (*2*). Function of the Gα isoforms has been well established with respect to specific receptor coupling and downstream signaling pathways. However, the 5 β and 12 γ isoforms form a diverse constellation of $\beta\gamma$ dimers (*3*−*5*), the functional significance of which is only beginning to be appreciated. A number of powerful genetic approaches, including homologous recombination (*6*, *7*) and RNA interference (*8*, *9*), have emerged to allow deletion or attenuation of β or γ genes of interest. Results of these studies revealed that regulation of specific β and γ isoforms is tightly integrated to many elements of G protein coupled receptor signaling pathways. Furthermore, the advent of real time PCR has enabled the analysis of transcriptional regulation with great precision (*9*). In contrast, characterization of β and γ isoforms at the protein level has relied predominantly on antibodies; limitations in this approach, such as cross-reactivity and poor sensitivity, make quantitative characterization of this family of highly related proteins fraught with difficulty.

One advance in proteomics has been the development of SILAC (*s*table *i*sotope *l*abeling with *a*mino acids in *c*ell culture) for the quantitation of proteins by mass spectrometry (*10*). An advantage of SILAC is that protein standards can be combined with samples and treated identically during the sample preparation steps necessary for mass spectrometry, thus allowing protein quantitation with great precision. This study describes a general procedure for purifying endogenous $\beta\gamma$ dimers from cells by expressing either an epitope-tagged adenosine A_1 receptor:α_{i1} (A_1:α_{i1}) fusion protein or adenosine A_{2A} receptor:α_S (A_{2A}:α_S) fusion protein. After immobilization as an R:G complex on affinity beads, the receptor fusion protein can release native $\beta\gamma$ when activated with AlF_4^-. When used in conjunction with SILAC and LC MS/MS mass spectrometry, this technique demonstrates femtomole sensitivity, the capability to identify and quantify individual β and γ isoforms in a mixed $\beta\gamma$ population, and the precision to discern differences in $\beta\gamma$ composition among different adenosine receptor G protein complexes and the overall $\beta\gamma$ profile in a cell. These attributes combine to provide a powerful approach that can be used to characterize G protein β and γ isoforms under a variety of experimental conditions.

[†]The work was supported by American Heart Association National Scientist Development Grant 0535350N, a Pilot/Feasibility Award from the UVa Silvio O. Conte Digestive Disease Research Center, and National Institutes of Health Grant R01-DK-19952.

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[1]Abbreviations: G protein, guanine nucleotide-binding regulatory protein; SILAC, stable isotope labeling with amino acids in cell culture; Sf9 cells, *Spondoptera frugiperda* cells; HEK cells, human embryonic kidney cells; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; DDM, *n*-dodecyl β-D-maltoside; CHS, cholesteryl hemisuccinate; SDS, sodium dodecyl sulfate; MALDI, matrix-assisted laser desorption/ionization.

Published on Web 12/03/2010

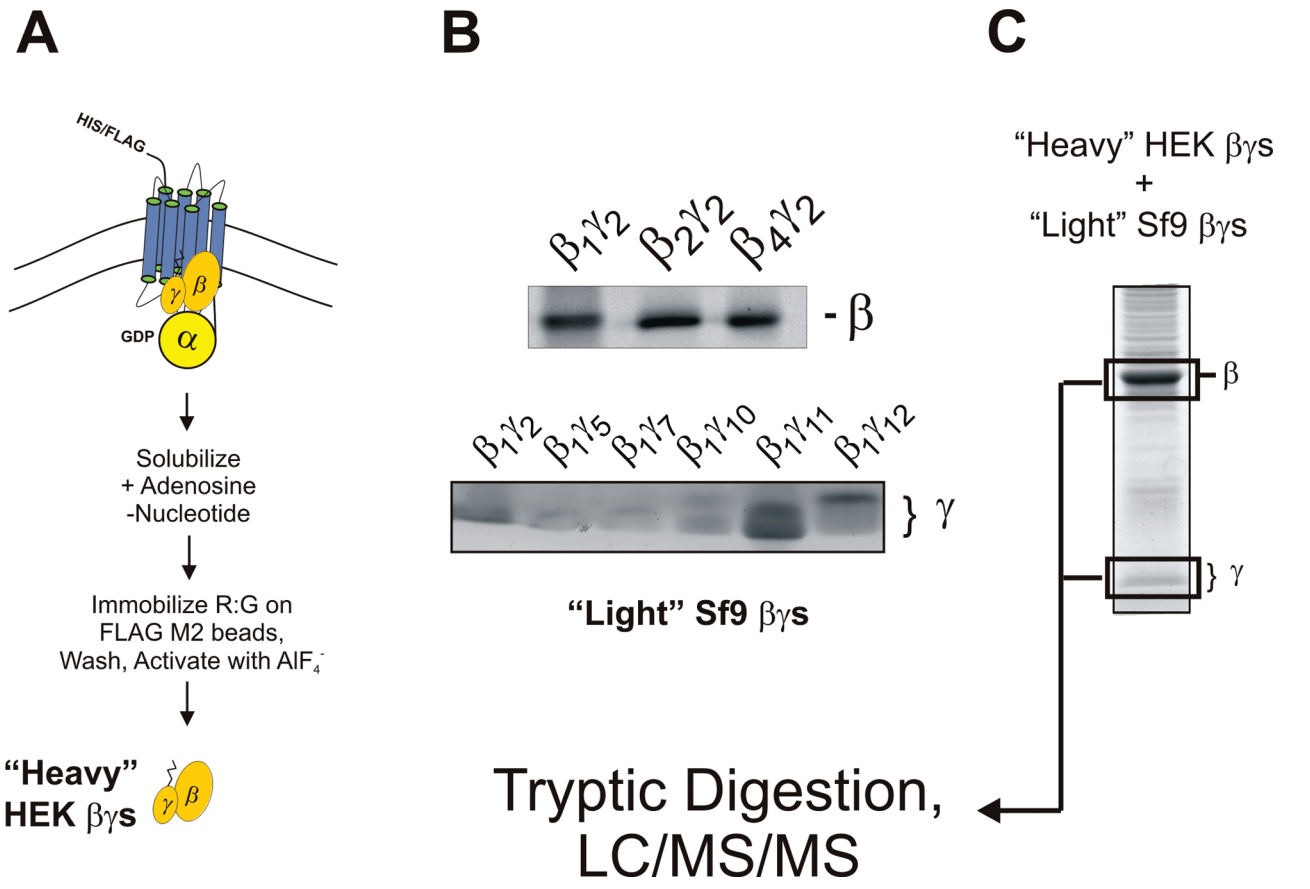

FIGURE 1: (A) Flow chart illustrating expression of epitope-tagged receptor fusion protein in HEK-293 cells cultured in heavy SILAC media, purification of R:G complex, and recovery of native heavy $\beta\gamma$ dimers associated with the receptor fusion protein. (B) Example of purified recombinant $\beta\gamma$ dimers from Sf9 cells cultured in light media used for the quantitation of β and γ isoform levels. 250 ng each of $\beta_1\gamma_2$, $\beta_2\gamma_2$, and $\beta_4\gamma_2$ was stained with Coomassie blue (above), and 50 ng each of $\beta_1\gamma_2$, $\beta_1\gamma_5$, $\beta_1\gamma_7$, $\beta_1\gamma_{10}$, $\beta_1\gamma_{11}$, and $\beta_1\gamma_{12}$ was stained with silver (below). (C) Native $\beta\gamma$ in (A) combined with Sf9 standard $\beta\gamma$ in (B) and separated by SDS−PAGE, in preparation for mass spectrometric analysis (see arrow).

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses. The baculovirus expressing the human γ_5 subunit was engineered by digestion of the pcDNA3.1+ plasmid containing γ_5 (Missouri S&T cDNA Resource Center) with *Pme*I in order to generate a blunt end γ_5 insert. The baculovirus expression vector pFastBac1 (Invitrogen) was digested with *Stu*I to generate linear DNA with blunt ends; the γ_5 insert was ligated into the vector, and cDNAs from positive clones were screened for correct orientation of insert and verified for correct sequence. DH10bac cells (Invitrogen) were transformed with the pFastBac1 vector containing γ_5, and screening of bacmid DNA from positive clones for correct transposition was achieved by PCR. The origin of the baculoviruses encoding $_{6HIS}$-G_{i1} α, β_1, β_2, β_4, γ_2, γ_7, γ_{10}, γ_{11}, and γ_{12} has been published elsewhere (*11*, *12*).

Expression and Purification of Recombinant βγ Dimers in Sf9 Cells. Recombinant baculoviruses encoding the desired combination of the $_{6HIS}$-G_{i1} α subunit and $\beta\gamma$ dimer were used to infect Sf9 cells at an MOI of 3, which were then harvested and used to purify recombinant $\beta\gamma$ dimers as described (*11*). This purification scheme yields a highly pure preparation of recombinant $\beta\gamma$ dimer of defined composition (Figure 1).

Design and Validation of β- and γ-Specific PCR Primers. PCR primers for individual β and γ isoforms were designed using Beacon Designer software and tested using end point PCR to verify a single amplicon product. Amplicons were sequenced to verify the fidelity of the primer target interaction; validated primer sets are listed in Table 1.

Quantitation of mRNA by Real Time rtPCR. Total cell RNA was extracted using the RNeasy minikit (Qiagen); cDNAs were created with 1 μg of RNA using the iScript cDNA synthesis kit (Bio-Rad), and quantitative real time PCR was performed using the iQ SYBER green supermix (Bio-Rad) in an iCycler PCR machine (Bio-Rad). The ribosomal protein 13A was used as an internal control reference gene. Normalization of the target gene was accomplished by using the formula $2^{E_t - R_t}$, where E_t and R_t are the threshold cycles for the experimental and reference genes, respectively (*13*).

Construction of Plasmids. A plasmid encoding a fusion protein of the human adenosine A_1 receptor and rat $G_{i1}\alpha$ was kindly provided by Dr. Graeme Milligan (University of Glasgow, Scotland, U.K.). Restriction enzymes *Bam*HI and *Eco*RV were used to generate an insert consisting of the 3′ end of the A_1 receptor and the entire G_{i1} α subunit. The same restriction enzymes were used to digest the vector pDoubleTrouble containing the adenosine A_1 receptor (*14*), and the fusion protein insert was ligated into the purified linearized pDoubleTrouble vector containing the HIS and FLAG epitope tags and the 5′ end of the A_1 receptor.

Vector pcDNA3.1+ containing the gene for the human G_S α short (Missouri S&T cDNA Resource Center) was modified by PCR mutagenesis in order to facilitate the fusion of human

Table 1: Human PCR Primers Used for Quantitation of β and γ mRNA Transcripts in HEK-293 Cells

Target Gene	Sense Primer	Antisense Primer
β_1	GACTGCTGTTGGATTCTG	CACTACTGCTGCTATGAAG
β_2	CGCCTGTGATGCCTCTATC	GCCGTTGGGGAAGAAAGC
β_3	ACCTGTCCATCCTTCTCTG	CCTCAAACTGTGCTCCTC
β_4	AATGTGAGAGTAAGCCGAGAGTTG	TCCAGAATGCCCAGTGAATGTG
β_5	GCTCTCCGCTTCCCTCTC	CTTGGCTCGCTCCTCCTC
γ_1	CCAAATGTTGTGAAGAAG	GCTTAGTAGTAATAGTATGC
γ_2	TTTCTTTCTTCCTTCTCCTCTACCC	ACCAGTCCAGCCTTATCTCCAC
γ_3	CATCCCATCCCTAACCCTTG	CCATCCCTCTCCATTGTCTG
γ_4	GGGCAGTAGAATGAAAGAGG	CACACGGAGTTAGAGAATGG
γ_5	ATCCAGTGATATTCAAGAGAGC	GACGAAAGTAGAAGTTTGTATATTATG
γ_7	GCAGGAATGGCAGGAAGG	AGATGGCTCGTTGGAAAGG
γ_8	CGCAAGACGGTGGAACAG	CTCGCAGAAAGCCAGGAG
γ_9 ($\gamma_{8\ cone}$)	AGGTGGCTGTCTGATAAG	CTGTGATGAAGAGAAGGTG
γ_{10}	ACACTCAAGGTCTCTCAG	AAGGCAGTCATTCATCAC
γ_{11}	TCTCAAACTTAACCCTCATC	GTCCCGAAACAACTGAAG
γ_{12}	TCCTCGCCTCTTCCCAACAAC	AAACAGTAACCCAACATAAAGCCATAG
γ_{13}	GACCTGATGAAGAACAAC	TACAAGATGGAGTGAGTG

adenosine A_{2A} receptor to G_S α. Two endogenous *Sma*I sites were changed in order to eliminate the restriction site: a C to G mutation in the noncoding backbone region of the plasmid DNA; the other site was internal to the G_S α cDNA in which nucleotide 963(G) was mutagenized to a (C), resulting in a silent mutation at residue R321. During the same multimutagenesis reaction (Stratagene, La Jolla, CA) a *Sma*I restriction site was incorporated at the 5′ end of the G_S α cDNA. Nucleotides 1A, 2T, 3G, and 6C of the G_S α cDNA were changed to CCC and G, respectively, resulting in a *Sma*I site. Construction of the A_{2A}:G_S α fusion was completed utilizing standard PCR techniques to amplify the wild-type A_{2A} gene using modified primers encoding exogenous restriction sites *Kpn*I at the 5′, TTA AAC TTA AGC TTG GTA CCA TGC CCA TCA TGG GCT CCT, and *Nco*I at the 3′, CCC GAG GCA GCC CAT GGA CAC TCC TGC TCC ATC CT, termini. The PCR product was digested with *Nco*I and filled in using Klenow to generate a blunt end. Following subsequent digestion with *Kpn*I, the product was subcloned by ligation into the modified pcDNA3.1+ G_S α vector that had been digested with *Kpn*I and *Sma*I. The A_{2A}:G_S α fusion protein construct was subcloned into the vector pDoubleTrouble by digestion of the pcDNA3.1+ vector containing A_{2A}:G_S α with *Bst*EII and *Pme*I to produce the fusion protein insert with a blunt 3′ end. A pDoubleTrouble vector containing the A_{2A} receptor (*14*) was digested with *Bst*EII and *Eco*RV to produce a linearized empty vector with a blunt end at the EcoRV site; the fusion protein insert was then ligated into the vector, resulting in a pDoubleTrouble vector containing an A_{2A}:G_S α fusion protein with a HIS/FLAG tag at the N-terminus.

Cell Culture. Human HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. Stable cell lines expressing the adenosine A_1 or A_{2A} receptor fusion proteins were generated by supplementing the media with G418 (500 μg/mL, final concentration). For SILAC conditions used to generate heavy $\beta\gamma$ dimers associated with the A_1:α_{i1} fusion protein, DMEM Flex media (Invitrogen) was supplemented with 200 g/L glucose, 200 mM L-glutamine, 10 g/L phenol red, 10% dialyzed FBS (Invitrogen) (10000 MW cutoff), 179.6 mg/L L-lysine:2HCl [$^{13}C_6$], and 86.2 mg/L L-arginine:HCl [$^{13}C_6$] (Cambridge Isotope Laboratories). In order to fully incorporate the heavy amino acids, cells were cultured in SILAC media for five doubling times. Cells cultured in either light or heavy media were harvested by trituration with PBS containing 5 mM EDTA, washed with PBS, collected by centrifugation, and resuspended in buffer containing 20 mM HEPES, pH 7.4, 1 mM EGTA, 100 μg/mL Pefabloc SC Plus, 2 μg/mL pepstatin, leupeptin, and aprotinin, and 20 μg/mL benzamidine before flash freezing and storage at −80 °C.

Preparation of Enriched βγ Fraction from HEK-293 Cells. Membranes from HEK-293 cells were collected after lysis with a 21 g needle and centrifugation at 53000 rpm in a 90 Ti rotor for 45 min at 4 °C. Buffer containing 20 mM Tris, pH 8.0, 1 mM EDTA, 1% cholate, 1 mM DTT, 5 μM GDP, 100 μg/mL Pefabloc SC Plus, 2 μg/mL pepstatin, leupeptin, and aprotinin, and 20 μg/mL benzamidine was used to extract $\beta\gamma$ dimers, either alone or in heterotrimeric form. The cholate extract was subjected to DEAE chromatography as described in Graber et al. (*15*). Fractions containing β-common immunoreactivity determined by SDS−PAGE and Western blotting were pooled, concentrated, and resolved by Superose 6 size exclusion chromatography as described in McIntire et al. (*11*). Fractions from the Superose 6 separation containing β-common immunoreactivity were pooled, concentrated, and frozen at −80 °C.

Purification of Native βγ Dimers Using Receptor Fusion Proteins. Cell pellets from approximately 30 15-cm plates of HEK-293 cells expressing the adenosine A_1:α_{i1} or A_{2A}:α_S fusion protein were lysed by nitrogen cavitation, and membranes were collected by centrifugation at 35000 rpm in a 45 Ti rotor for 45 min at 4 °C. Membranes were washed with HNG buffer (20 mM HEPES, pH 7.4, 20 mM NaCl, and 10% glycerol) containing 100 μg/mL Pefabloc SC Plus, 2 μg/mL pepstatin, leupeptin, and aprotinin, 20 μg/mL benzamidine, and 5 μM GDP and resuspended to a volume of 10 mg/mL protein with HNG buffer containing 1 mM EDTA, 1% *n*-dodecyl β-D-maltoside (DDM), 0.02% cholesteryl hemisuccinate, 100 μM adenosine, 100 μg/mL Pefabloc SC Plus, 2 μg/mL pepstatin, leupeptin, and aprotinin, and 20 μg/mL benzamidine. All buffers used in the purification procedure were 0.22 μm filtered, and all steps were performed at 4 °C unless otherwise noted (see Figure 1 for flow chart of purification). After being stirred for 2 h, the DDM extract containing the receptor fusion protein was clarified by centrifugation as described above and diluted to approximately 0.5% DDM with HNG buffer containing 1 mM EDTA, 100 μM adenosine, 100 μg/mL Pefabloc SC Plus, 2 μg/mL pepstatin, leupeptin, and aprotinin, and 20 μg/mL benzamidine. The diluted extract was allowed to incubate with 200 μL of FLAG M2

affinity resin for 1 h, rocking end over end. FLAG beads were collected with a 5 mL centrifuge column and washed with five 1 mL volumes of HGN buffer containing 1 mM EDTA (HNGE buffer), 0.1% DDM, and 100 μM adenosine. The column was then washed with two 1 mL volumes of HNGE buffer containing 1% cholate and 100 μM adenosine. HNGE buffer containing 100 μM adenosine and 1% cholate was supplemented with AlF_4^- (activation buffer) for elution of $\beta\gamma$ dimers associated with the receptor fusion protein; 200 μL of activation buffer warmed to room temperature was added to the column and collected. The column was then capped, and a second 200 μL was added and allowed to incubate for 15 min at room temperature in order to facilitate dissociation of $\beta\gamma$ from receptor fusion protein. After the incubation, the second volume was collected, along with four more 200 μL volumes, and fractions containing $\beta\gamma$ dimer were pooled, concentrated in an Amicon concentrator, and exchanged twice with buffer containing 20 mM HEPES, pH 7.4, 20 mM NaCl, 1 mM EDTA, 0.1% CHAPS, and 1 mM DTT. The column-bound receptor fusion protein and FLAG elution buffer (HGNE buffer containing 0.1% DDM, 100 μM adenosine, and 0.5 mg/mL FLAG peptide) were warmed to room temperature, and 200 μL of the elution buffer was applied to the column and collected. The column was then capped, and a second 200 μL elution volume was applied and allowed to incubate for 15 min. The column was then uncapped, and a total of five more 200 μL elution volumes were collected to recover the receptor fusion protein.

MALDI Mass Spectrometric Analysis of Intact γ Subunits. Samples were prepared based on the thin-layer method described by Cadene et al. (*16*). Briefly, a thin-layer matrix solution was prepared by diluting a saturated solution of cyanohydroxycinnamic acid in a 1:2 mixture of water:acetonitrile 4-fold with 2-propanol. A sample matrix solution was prepared by sonicating a cyanohydroxycinnamic acid saturated solution in a 3:1:2 mixture of formic acid:water:acetonitrile for 10 min, followed by centrifugation. The thin-layer matrix was prepared by applying 10−20 μL of thin-layer matrix solution on a plate and allowing it to spread. When only traces of solvent were remaining, the plate was gently wiped to leave only a thin film of matrix. The sample matrix solution was used to dilute the purified $\beta\gamma$ samples 20-fold; within 10 min, 0.5 μL was loaded on the plate containing the thin layer, allowed to dry, and washed with 2 μL of 0.1% TFA. Samples were then analyzed on a Bruker MicroFlex MALDI mass spectrometer in linear mode using the manufacturer's standard settings and collecting 200 shots.

SDS−PAGE and Western Blotting. Prior to gel electrophoresis, samples were incubated with 6× sample buffer at room temperature for 1 h without boiling. Proteins were separated using 12% polyacrylamide gels and visualized by staining with silver or Coomassie blue; alternatively, gels were transferred to nitrocellulose for Western blotting with a β-common (sc-378; Santa Cruz) or α-common (NEI-800; DuPont NEN) antibody. Polyacrylamide gels used for generation of samples for mass spectrometric analysis were prepared by 0.22 μm filtration of the separating and stacking solutions, as well as the running buffer; this step is important for removal of common protein contaminants, such as keratin, that can obscure the detection of sample proteins. Gels were stained in a 0.1% Coomassie Brilliant blue solution of 45:45:10 methanol:water:acetic acid, followed by destaining in a 45:45:10 methanol:water:acetic acid solution. Once protein bands were adequately visualized, gels were stored in a 10% acetic acid solution. The β protein, which separates from γ during SDS−PAGE, has an electrophoretic mobility of approximately 36 kDa, while the γ protein is present at the dye front. These portions of the gel were excised in order to recover protein for mass spectrometric analysis.

Tryptic Digestion of Gel Bands and LC/MS/MS Analysis. Gel pieces were transferred to siliconized tubes and washed and destained in 200 μL of 50% methanol overnight. The gel pieces were dehydrated in acetonitrile, rehydrated in 30 μL of 10 mM dithiolthreitol in 0.1 M ammonium bicarbonate, and reduced at room temperature for 0.5 h. The DTT solution was removed and the sample alkylated in 30 μL of 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 h. The reagent was removed, and the gel pieces were dehydrated in 100 μL of acetonitrile. The acetonitrile was removed, and the gel pieces were rehydrated in 100 μL of 0.1 M ammonium bicarbonate. The pieces were dehydrated in 100 μL of acetonitrile, the acetonitrile was removed, and the pieces were completely dried by vacuum centrifugation. The gel pieces were rehydrated in 20 ng/μL trypsin in 50 mM ammonium bicarbonate on ice for 10 min. Any excess enzyme solution was removed and 20 μL of 50 mM ammonium bicarbonate added. The sample was digested overnight at 37 °C, and the peptides formed were extracted from the polyacrylamide in two 30 μL aliquots of 50% acetonitrile/5% formic acid. These extracts were combined and evaporated to 15 μL for MS analysis.

The LC-MS system consisted of a Thermo Electron LTQ Orbitrap XL mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm × 75 um i.d. Phenomenex Jupiter 10 μm C18 reversed-phase capillary column. Volumes (7.5 μL) of the extract were injected and the peptides eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.4 μL/min over 1 h. The nanospray ion source was operated at 2.5 kV. The digest was analyzed by acquiring a full scan mass spectrum using Fourier transform ion cyclotron resonance at 100K resolving power to determine peptide molecular weights followed by 10 product ion spectra in the ion trap to determine amino acid sequence in sequential scans. This mode of analysis produces approximately 10000 MS/MS spectra of ions ranging in abundance over several orders of magnitude. The data were analyzed by database searching using the Sequest algorithm against Human International Protein Index (v3.66).

Determination of Ratios of Heavy and Light Peptides. Peptide spectra putatively identified by Sequest as belonging to G protein γ or β isoforms were manually verified. H:L peptide ratios were calculated using areas obtained from QualBrowser (Xcalibur 2.1) for the monoisotopic m/z (±0.01 Da) for the heavy and light forms. The H:L ratio for each protein was obtained by taking the average peptide ratio for all peptides observed for a particular isoform.

Calculation of Protein Concentration. For A_1:α_{i1} fusion protein $\beta\gamma$ dimers purified from HEK-293 cells cultured in media containing [$^{13}C_6$]Arg and [$^{13}C_6$] Lys, protein concentration was determined by Western blotting with the sc-378 β-common antibody (Santa Cruz) against a standard curve of purified recombinant $\beta\gamma$ dimer from Sf9 cells. For β isoform quantitation by mass spectrometry, purified recombinant $\beta_1\gamma_2$, $\beta_2\gamma_2$, and $\beta_4\gamma_2$ dimers from Sf9 cells were each added to the A_1:α_{i1} fusion protein $\beta\gamma$ dimers at a 1:10 molar ratio. For γ isoform quantitation, purified recombinant $\beta_1\gamma_2$, $\beta_1\gamma_5$, $\beta_1\gamma_7$, $\beta_1\gamma_{10}$, $\beta_1\gamma_{11}$, and $\beta_1\gamma_{12}$ dimers from Sf9 cells were also each added at a 1:10 ratio. The combined samples were separated by SDS−PAGE (Figure 1)

and processed according to the procedure outlined above in SDS−PAGE and Western Blotting and Tryptic Digestion of Gel Bands and LC/MS/MS Analysis.

Typically, several peptides from each β or γ isoform (see Table 3) produced ion pairs that were used to determine an average peak area ratio between heavy and light ion traces (H:L). Expression of β and γ isoforms as micrograms per microliter of sample was determined by multiplying the average (H:L) ratio for each isoform by the micrograms of each standard isoform added. The amount of Sf9 γ isoform added in a standard was determined indirectly as a function of β concentration, assuming a 1:1 β:γ ratio. In experiments comparing $\beta\gamma$ dimers between fusion proteins, or between the A_1:α_{i1} fusion protein and the enriched HEK-293 $\beta\gamma$ fraction, the known heavy β and γ concentrations from the A_1:α_{i1} fusion protein were used along with the H:L ratios to determine the relative amounts of light β and γ isoforms associated with the A_{2A}:α_S fusion protein or present in the enriched $\beta\gamma$ fraction from HEK-293 cells. In order to normalize the relative levels of β and γ isoforms present, each β and γ isoform was expressed as a percent of the total β and γ protein quantified, respectively, for each sample.

Statistical Analysis. H:L ratios were first analyzed for variability, and ratios that were greater than two standard deviations from the mean of data sets containing at least five values were excluded from further analysis. *n* values higher than the number of peptides observed (Table 3, A_1:α_{i1} vs enriched HEK-293 $\beta\gamma$ fraction) occur when several charge states of the same peptide generate unique H:L ratios. Prior to pooling data from separate experiments, H:L ratios were converted into moles of β or γ and then expressed as percent of total moles of β or γ detected. In order to determine statistical significance, data sets were compared using the unpaired *t* test in GraphPad Prism 5 to calculate two-tailed *p* values.

Materials. Reagents for Sf9 cell culture and purification of $\beta\gamma$ dimers have been described previously (*17*−*20*): GDP, CHS, adenosine, HEPES, and anti-FLAG M2 agarose from Sigma; DDM from MP Biochemicals; CHAPS from Roche Molecular Biochemicals; 10% Genapol C-100 from CalBiochem; Ni^{2+}-NTA Superflow resin from Qiagen; Centricon 30 concentrators from Millipore; Superose 6 HR 10/30 column from Pharmacia; all other materials were of the highest available purity. FLAG peptide was synthesized at the University of Virginia Biomolecular Research Facility. Mass spectrometric analysis of peptides was performed at the W. M. Keck Biomedical Mass Spectrometry Laboratory.

RESULTS

Methodology. A graphical overview of the process for the isolation and biochemical characterization of native $\beta\gamma$ dimers from cells is provided in Figure 1. The scheme for $\beta\gamma$ purification from a receptor:α:$\beta\gamma$ complex after expression of epitope-tagged adenosine receptor:α fusion protein in cells cultured in "heavy" SILAC media to introduce ^{13}C-labeled Lys and Arg into cellular proteins is presented in Figure 1A. This general scheme could be used for any combination of receptor and α engineered in a fusion protein construct. Figure 1B illustrates recombinant $\beta\gamma$ dimers purified from Sf9 cells cultured in normal or "light" media. The $\beta\gamma$ dimers containing different β isoforms (stained with Coomassie blue, Figure 1B, above) and different γ isoforms (stained with silver, Figure 1B, below) were used to quantify β and γ isoforms isolated from HEK-293 cells in Figure 1A.

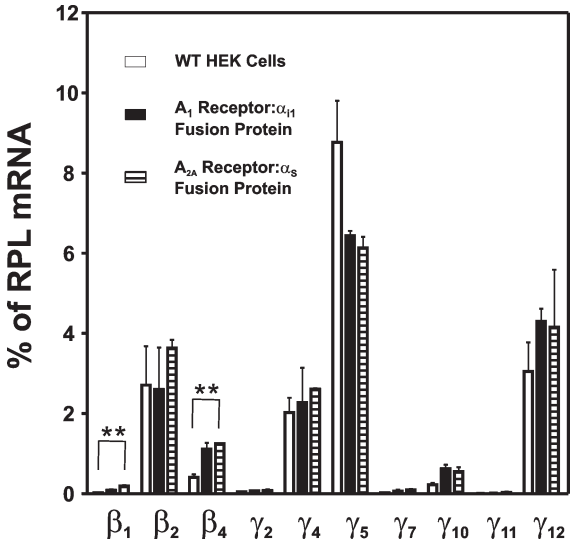

FIGURE 2: Real time QPCR analysis of β and γ mRNA transcript levels in HEK-293 cells. Isoform-specific human primers (see Table 1) were used to measure the mRNA levels of various β and γ isoforms, expressed as a percent of the housekeeping gene ribosomal protein 13A (RPL) in wild-type HEK-293 cells (white bars), HEK cells expressing the $_{HIS/FLAG}$adenosine A_1:α_{i1} receptor fusion protein (black bars), and HEK cells expressing the $_{HIS/FLAG}$adenosine A_{2A}:α_S receptor fusion protein (hatched bars). β_1 mRNA was ~8-fold higher in A_{2A}:α_S cells over wild-type HEK cells (0.20% vs 0.03% of RPL); β_4 mRNA was ~3-fold higher in A_{2A}:α_S cells over wild-type HEK cells (1.25% vs 0.4% of RPL). ** = $p < 0.01$.

A known amount of light $\beta\gamma$ standard is combined with the native mixture of heavy $\beta\gamma$ derived from the receptor fusion protein for quantitative analysis by mass spectrometry. Figure 1C illustrates the purity of the combined $\beta\gamma$ dimers from both sources after separation by SDS−PAGE and staining with Coomassie blue. The β and γ proteins from the gel in Figure 1C (see boxes) are excised, digested with trypsin, and analyzed by LC/MS/MS as described in Materials and Methods.

Analysis of β and γ mRNA Transcript Levels in HEK-293 Cells. Although the main focus of this study was to characterize the β and γ isoforms at the protein level, it was also important to correlate β and γ protein with mRNA levels. Validated human PCR primers designed to target specific β and γ isoforms are listed in Table 1; all primers were validated by sequence verification of the amplicon, with the exception of the γ_2 primers, which specifically recognized a plasmid containing the target sequence. Since this study examined the $\beta\gamma$ dimers from HEK-293 cells, the β_3 and β_5 primers, as well as the γ_1, γ_3, γ_8, γ_9, and γ_{13} primers, were excluded from this analysis, as these isoforms were not observed experimentally at the protein level (*21*, *22*) and thus do not appear to have a prominent role in HEK-293 cell G protein signaling. Of the β isoforms, β_2 was the most abundant, with transcript levels over 10-fold higher than β_1 or β_4 in wild-type cells (Figure 2, white bars). The γ_4, γ_5, and γ_{12} isoforms were all transcribed at high levels in wild-type cells; however, transcripts for γ_2, γ_7, γ_{10}, and γ_{11} were also detected (Figure 2, white bars). β and γ mRNA levels were also compared to HEK-293 cell lines expressing the adenosine A_1:α_{i1} fusion protein (Figure 2, black bars) or the adenosine A_{2A}:α_S fusion protein (Figure 2, hatched bars) to control for possible changes in transcription caused by fusion protein expression. There were no significant differences in β or γ transcript levels between the adenosine A_1 and A_{2A} receptor fusion protein stable cell lines; in addition, there were no differences in β or γ mRNA between the

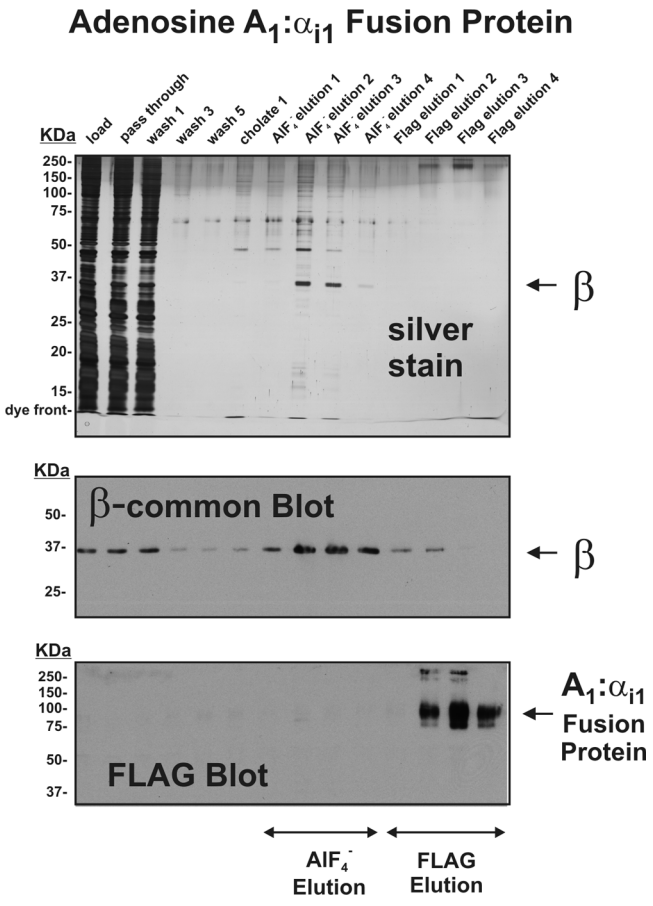

FIGURE 3: Purification of native $\beta\gamma$ dimers using the $_{HIS/FLAG}$adenosine A_1:α_{i1} receptor fusion protein expressed in HEK-293 cells. Native $\beta\gamma$ dimers associated with the receptor fusion protein were released after incubation with AlF_4^-; subsequently, FLAG peptide was used to elute the remaining adenosine A_1:α_{i1} receptor fusion protein. Fractions from the purification were separated using SDS−PAGE and either stained with silver (above) or transferred to nitrocellulose and blotted with β-common (middle) and FLAG (below) antibodies. The silver-stained gel illustrates the purity of the $\beta\gamma$ released from the fusion protein, and the β-common Western blot confirms the identity of the protein band at 36 kDa. The FLAG Western blot illustrates elution of adenosine A_1:α_{i1} fusion protein, with a predicted molecular mass of approximately 80 kDa.

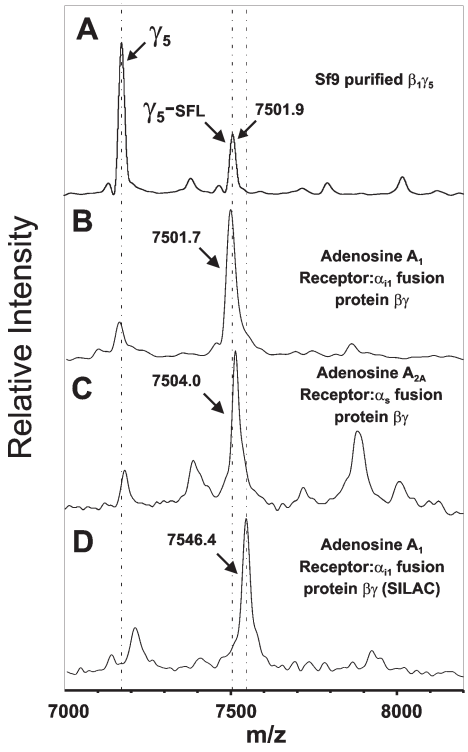

FIGURE 4: MALDI mass spectrometric analysis of intact γ Isoforms. (A) Light $\beta_1\gamma_5$ purified from Sf9 cells grown in standard media. (B) Purified light $\beta\gamma$ released from A_1:α_{i1} fusion protein expressed in HEK-293 cells cultured in standard media. (C) Purified light $\beta\gamma$ released from A_{2A}:α_S fusion protein expressed in HEK-293 cells cultured in standard media. (D) Purified heavy $\beta\gamma$ released from A_1:α_{i1} fusion protein expressed in HEK-293 cells cultured in media containing [$^{13}C_6$]Arg and [$^{13}C_6$]Lys. Dashed lines align with peaks coinciding with expected average masses for conventionally processed γ_5 (geranylgeranylation, methylation at C65, and loss of C-terminal SFL sequence mass) and γ_5-SFL (geranylgeranylation at C65 and retention of C-terminal SFL sequence) in spectra A, B, and C. Dashed line bisecting peak in spectrum D indicates expected mass of γ_5-SFL containing [$^{13}C_6$]Arg and [$^{13}C_6$]Lys.

wild-type HEK-293 cells and the cell line expressing the A_1:α_{i1} fusion protein. However, the HEK-293 cell line expressing the A_{2A}:α_S fusion protein displayed an approximately 8-fold increase in β_1 mRNA, increasing from 0.03% in wild-type cells to 0.20% in the A_{2A}:α_S stable cell line (Figure 2). Further, there was a 3-fold increase in β_4 mRNA, from 0.4% in wild-type cells to 1.25% in the A_{2A}:α_S stable cell line (Figure 2).

Purification of Native βγ Dimers Associated with the Adenosine A_1:α_{i1} Fusion Protein Expressed in HEK-293 Cells. The high-affinity agonist binding state of a receptor occurs when it is bound to heterotrimeric G protein, which makes agonist binding a useful parameter for measuring the interaction between receptor and G protein. The fusion of α_{i1} onto the C-terminus of the adenosine A_1 receptor did not interfere with the ability of the receptor to interact with the complete heterotrimeric G protein, as measured by high-affinity agonist binding (*23*). Thus the fact that the A_1:α_{i1} fusion protein was functionally similar to the analogous nonfused proteins (*23*) made it a natural initial choice for the examination of interactions between the adenosine A_1 receptor, α_{i1}, and β and γ isoforms.

Figure 3 illustrates the purification of native HEK-293 $\beta\gamma$ dimers from the 6HIS/FLAG-tagged A_1:α_{i1} fusion protein; the β subunit at 36 kDa can clearly be seen in both the silver-stained gel and the β-common Western blot (AlF_4^- elutions 2 and 3, Figure 3). Figure 1C demonstrates the purity of native HEK-293 $\beta\gamma$ dimers combined with Sf9-purified $\beta\gamma$ dimers after staining with Coomassie blue. The A_1:α_{i1} fusion protein can also be recovered by elution with FLAG peptide; the expected electrophoretic mobility of the receptor fusion protein is approximately 80 kDa. In agreement with this, FLAG immunoreactivity was observed in the FLAG elution fractions of the Western blot between the 75 and 100 kDa molecular mass standards (Figure 3, FLAG blot, elutions 2−4). The faint visualization of the receptor fusion protein in the silver-stained gel of Figure 3 may be due to glycosylation-induced band broadening of the fusion protein or differential protein staining with silver. Similar results were observed for the purification of $\beta\gamma$ from the A_{2A}:α_S fusion protein (data not shown).

MALDI Mass Spectrometric Analysis of Intact γ Subunits. Figure 4A displays a mass spectrum of purified $\beta_1\gamma_5$ from Sf9 cells using MALDI mass spectrometry, a technique which is able to ionize intact γ subunits in the $[M + H]^+$ charge state. The largest peak at approximately m/z 7160 (Figure 4A, left dashed line) corresponds to the predicted mass of the γ_5 subunit which

Table 2: Determination of N-Terminal Structure of γ Isoforms by ESI-MS/MS Mass Spectrometry[a]

Isoform	N-Terminal Sequence	Structure of Gγ N-Terminal Residue	
		βγ from A_1 R:G	βγ from A_{2A} R:G
γ_2	*MASNN	Ac-A	Ac-A
γ_4	MKEGM	#M	#M
γ_5	*MSGSS	Ac-S	Ac-S
γ_7	*MSATN	Ac-S	Ac-S
γ_{10}	*MSSGA	Ac-S	Ac-S
γ_{11}	MPALH	n.d.	n.d.
γ_{12}	MSSKT	n.d.	n.d.

[a]* = cleavage of N-terminal methionine observed. # = no modifications observed. Ac = acetylation; n.d., not determined.

undergoes the conventional posttranslational processing of prenylation, cleavage of the C-terminal three amino acids, and methylation of the prenylated cysteine (*5*). At the higher *m*/*z* of 7501.9 (Figure 4A, middle dashed line), a smaller peak corresponds to the predicted mass (7501.3) of the γ_5 lacking proteolytic cleavage of the three C-terminal SFL residues (*22*). In contrast, the largest peak at *m*/*z* of 7501.7 in the mass spectrum for $\beta\gamma$ purified from the A_1:α_{i1} fusion protein expressed in HEK-293 cells (Figure 4B, middle dashed line) corresponds to a geranylgeranylated γ_5 lacking proteolytic cleavage of the three C-terminal SFL residues with a predicted mass of 7501.3. Similar results were observed for the $\beta\gamma$ purified from the A_{2A}:α_S fusion protein (Figure 4C, middle dashed line), with the experimental *m*/*z* of 7504.0 in good agreement with the predicted mass of 7501.3. The mass spectrum for the "heavy" $\beta\gamma$ purified from the A_1:α_{i1} fusion protein expressed in HEK-293 cells under SILAC conditions was observed to have a peak (Figure 4D, right dashed line) that was approximately 45 Da higher than the major peak in Figure 4B. This peak with an *m*/*z* of 7546.4 was in agreement with the predicted mass of 7549.3 for a geranylgeranylated γ_5 subunit lacking proteolytic cleavage of the three C-terminal SFL residues, in which all of the arginines and lysines have been replaced with [$^{13}C_6$]Arg and [$^{13}C_6$]Lys, respectively. The experimental implications of differential posttranslational modification (*24*) are illustrated in the silver-stained gel of purified γ subunits in Figure 1, where significant heterogeneity in electrophoretic mobility of different γ isoforms was observed under the separating conditions (12% SDS−PAGE).

ESI-MS/MS Analysis of Modifications to γ Isoforms. Differential N-terminal processing was also observed in the MS/MS analysis of peptides from γ isoforms. Table 2 lists all of the γ isoforms characterized in this study, with the N-terminal sequence translated from the open reading frame of each γ gene and the N-terminal structure for each γ isoform purified from the A_1:α_{i1} and A_{2A}:α_S fusion proteins as determined by mass spectrometry. Although levels of the γ_4 isoform were not quantified, data from the mass spectrometric analysis still allowed qualitative characterization of posttranslational processing of this isoform. With the exception of γ_4, all the γ isoforms for which N-terminal peptides were observed (γ_2, γ_5, γ_7, and γ_{10}) had undergone cleavage of the N-terminal methionine, followed by acetylation (Table 2). The presence of the N-terminal methionine in γ_4 can be accounted for by the lysine at position 2 (Table 2), which has been reported to provide a poor binding environment for methionine aminopeptidase (*25*). N-Terminal acetylation of proteins containing a Met-Lys at positions 1 and 2 has been reported to be variable in humans (*26*). A study of bovine brain derived γ_3, which also contains an N-terminal Met-Lys motif, and is thus expected to retain the N-terminal methionine, found more than half of the protein to be acetylated at the N-terminal methionine (*24*). Although the characterization of N-terminal processing of γ isoforms in this study is not quantitative, only unacetylated N-terminal peptides were detected for γ_4 (Table 2). The absence of N-terminal peptides for γ_{11} was probably due to the low abundance of this isoform, and the Lys at position 4 of γ_{12} (Table 2) likely resulted in a tryptic N-terminal peptide that was too small for successful analysis. Other studies, however, have reported that the γ_{12} isoform undergoes cleavage of the N-terminal methionine, followed by acetylation of the resulting N-terminal serine (*24*). This differential processing of the N-termini of γ isoforms implies a point of functional regulation in the γ subunit, which is discussed below.

Quantitative Mass Spectrometric Analysis of βγ Purified from the Adenosine A_1:α_{i1} Fusion Protein. The use of SILAC allows the simultaneous biochemical processing of chemically identical heavy and light proteins and peptides, which can be differentiated and measured in a mass spectrometer as heavy and light ion pairs. The ratio of the signal intensity of the ion pairs can thus be translated into quantitative information about the proteins in the sample. Ion pairs from the LC/MS/MS analysis of heavy $\beta\gamma$ tryptic peptides from SILAC treated adenosine A_1:α_{i1} fusion protein and light $\beta\gamma$ tryptic peptides from Sf9-purified $\beta\gamma$ were identified and quantified by mass spectrometry.

The use of SILAC technology enables the characterization of heavy and light ion pairs by mass spectrometry over a wide dynamic range. Figure 5A illustrates the ion pair for the peptide KVVQQLR from the abundant γ_5 isoform. Since the peptide has both a lysine and an arginine, the net mass difference between the heavy and light peptides is 12 Da; however, since the ions have a charge of 2+, the *m*/*z* difference is only 6. The inset (Figure 5A) illustrates the relationship between the retention time by HPLC and the ion traces for the monoisotopic heavy and light ions indicated by arrows (Figure 5). Since peptides with the heavy isotopes are chemically identical to their light counterparts, all ion pairs will have identical retention times and thus will be affected equally by any ionization-influencing artifacts introduced by the sample. The ratio of the signal intensities of the ion current peaks (Figure 5A, inset) for the heavy and light peptides in the ion pair is used to quantify protein levels. An example of an ion pair from the less abundant γ_{11} comes from the peptide SGEDPLVK (Figure 5B); in the large spectrum, only the light ion is visible. However, when the part of the *x*-axis containing the heavy ion is magnified 100× (Figure 5B, gray box in inset), the heavy ion becomes visible. The SGEDPLVK ion is also $[M + H]^{2+}$; however, since there is only one amino acid that can be exchanged for a heavy isotope, the net mass change is only 6 Da and the *m*/*z* difference is 3. Table 3 contains a complete list of all the peptides that produced ion pairs used to quantify protein levels of γ isoforms associated with the A_1:α_{i1} fusion protein.

Ion pairs for β isoforms were also examined, and Figure 6A illustrates an example from the ELAGHTGYLSCCR peptide from the most abundant β_1 isoform. A peptide from the least abundant β_4, TFVSGACDASSK, yields an ion pair that is illustrated in Figure 6B with a 2+ charge state. All of the peptides

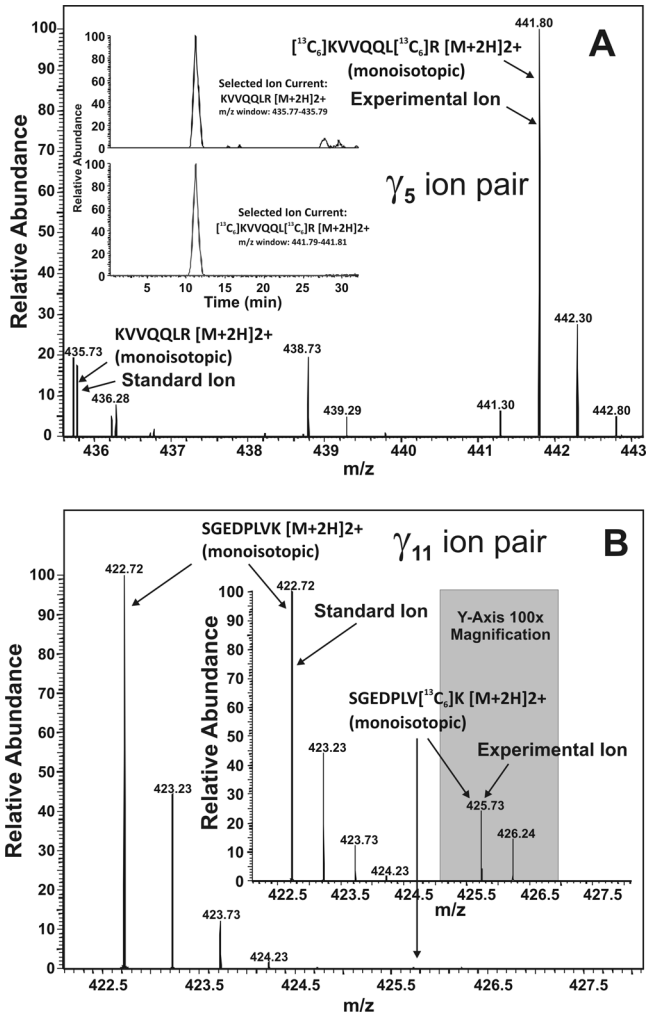

FIGURE 5: Example comparison of heavy and light ion pairs used to determine H:L ratio for γ_5 and γ_{11}. (A) Light (from Sf9 $\beta_1\gamma_5$) and heavy (from A_1:α_{i1} fusion protein $\beta\gamma$) $[M + 2H]^{2+}$ ions from the KVVQQLR peptide derived from the γ_5 isoform. Inset illustrates the selected ion currents from the light and heavy ions plotted against retention time by HPLC; H:L ratios were determined from these peaks as described in Materials and Methods under Determination of Ratios of Heavy and Light Peptides. (B) Light and heavy $[M + 2H]^{2+}$ ions from the SGEDPLVK peptide derived from the γ_{11} isoform. Note that the heavy ion is not visible in the main spectrum; however, upon 100× enlargement of the *y*-axis (inset spectrum), the heavy ion becomes evident.

from which ion pairs were observed for the quantification of β isoforms associated with the A_1:α_{i1} fusion protein are listed in Table 3.

The protein concentration from the known Sf9 $\beta\gamma$ standards and the ratios of the heavy and light ion pairs were used to calculate the moles of each β and γ isoform purified from the A_1:α_{i1} fusion protein. After expressing each β and γ isoform as a percent of the total β and γ protein observed, respectively, levels of each β isoform (Figure 7A) and γ isoform (Figure 7B) purified from the A_1:α_{i1} fusion protein were compared to every other β and γ isoform member (respectively) for differences in the percentage levels. All of the β isoforms were different from each other at the $p < 0.001$ level (Figure 7A), with β_1 over 12-fold more abundant than β_4 (see Figure 7C for bar graph expression of data in Figure 7A). Significant differences were observed among many of the γ isoforms; notably, γ_5 was estimated to be 78% of total γ isoforms, while γ_{11} was only 0.03% of total γ isoforms (Figure 7B). Although γ_5 was the most abundant γ isoform, γ_2, γ_7, γ_{10}, and γ_{12} all presented between 2% and 12% of total γ isoforms (Figure 7C). Data in Figure 7B are expressed in bar graph format in Figure 7D.

Quantitation of β and γ Isoforms in HEK-293 Cells and Associated with the A_{2A}:α_S Receptor Fusion Protein. One important question arising from quantitation of the β and γ isoform composition of the A_1:α_{i1} fusion protein is the relationship of the A_1:α_{i1} $\beta\gamma$ profile to that of another receptor or to the $\beta\gamma$ profile in the whole cell. Enrichment of the $\beta\gamma$ fraction in HEK-293 cells was necessary to reduce background protein signal and increase the strength of β and γ peptide signals in the mass spectrometric analysis. Protein concentration of the enriched $\beta\gamma$ fraction from HEK-293 cells based on quantitative Western blotting was 7.9 ng of β/μg of protein (sc-378; Santa Cruz) and 3.1 ng of α/μg of protein (NEI-800; DuPont NEN); thus, the enriched $\beta\gamma$ fraction likely contained both free $\beta\gamma$ and heterotrimeric G protein. The A_{2A}:α_S fusion protein was chosen for comparison purposes as a member of the same receptor family with distinctly different Gα coupling preferences. For the experimental comparisons, heavy $\beta\gamma$ purified from the A_1:α_{i1} fusion protein was added to a similar amount of light $\beta\gamma$ purified from the A_{2A}:α_S fusion protein and to the enriched $\beta\gamma$ fraction from HEK-293 cells. The samples were then separated by SDS−PAGE and stained with Coomassie blue, and the gel bands containing the heavy and light β isoforms, and the heavy and light γ isoforms, were excised and analyzed as described in Materials and Methods.

Using the heavy $\beta\gamma$ dimers purified from A_1:α_{i1} as standards, β and γ isoform levels in both the whole cell and specifically associated with A_{2A}:α_S were calculated (see Table 3 for a list of peptides used to determine H:L ratios). Figure 8 illustrates that the A_{2A}:α_S fusion protein bound ~30% more β_4 (6.8% of total β) compared to the A_1:α_{i1} fusion protein (5.3% of total β). Further, the A_1:α_{i1} fusion protein contained ~40% higher levels of β_4 than the enriched $\beta\gamma$ fraction from HEK-293 cells (Figure 8: 5.3% vs 3.7% of total β), and the A_{2A}:α_S fusion protein contained ~80% higher levels of β_4 than the enriched $\beta\gamma$ fraction (Figure 8: 6.8% vs 3.7% of total β). Differences in β_1 and β_2 levels were not significantly different among A_1:α_{i1}, A_{2A}:α_S, or total HEK-293 $\beta\gamma$; however, levels of β_2 were trending lower in both A_1:α_{i1} and A_{2A}:α_S compared to total HEK-293 $\beta\gamma$ (Figure 8), offsetting the higher levels of β_4 in both fusion proteins. No differences were observed in types of γ isoforms between A_1:α_{i1} and A_{2A}:α_S; however, the A_1:α_{i1} and A_{2A}:α_S fusion proteins contained 25% and 21% higher levels of γ_5, respectively, than total HEK-293 cell $\beta\gamma$. These higher levels of γ_5 were offset by lower levels of γ_2 (A_1:α_{i1}, 41% Δ; A_{2A}:α_S, 40% Δ), γ_{10} (A_1:α_{i1}, 61% Δ; A_{2A}:α_S, 54% Δ), and γ_{12} (A_1:α_{i1}, 36% Δ; A_{2A}:α_S, 29% Δ) compared to total HEK-293 cell $\beta\gamma$ (Figure 9). Though not significantly different, the γ_7 isoform appeared to also trend lower in A_1:α_{i1} and A_{2A}:α_S compared to total HEK-293 cell $\beta\gamma$ (Figure 9).

Numerical values (±SEM) for β and γ protein levels associated with the A_{2A}:α_S the fusion protein and the enriched $\beta\gamma$ fraction from HEK-293 cells are reported in Table 4. Interestingly, levels of mRNA detected for β isoforms (Figure 2) did not correlate with β protein levels in the HEK-293 enriched $\beta\gamma$ fraction (Figure 8). Whereas β_2 mRNA was 10-fold higher than β_1 mRNA, β_1 protein was actually 2-fold higher than β_2 protein, suggesting that the β_1 protein is relatively long-lived in the cell. In contrast to the β isoform, protein levels for γ isoforms (Figure 9) correlated roughly with the mRNA detected by QPCR

Table 3: Peptides from β and γ Isoforms Used for H:L Ion Pair Analysis[a]

Isoform	M.W.	A_1:α_{i1} vs Sf9 $\beta\gamma$ (Two experiments)	A_1:α_{i1} vs A_{2A}:α_S (Two experiments)	A_1:α_{i1} vs Enriched HEK-293 $\beta\gamma$ Fraction (One experiment)
β_1	37377	ACADATLSQITNNIDPVGR LFVSGACDASAK ELAGHTGYLSCCR **n = 6**	ACADATLSQITNNIDPVGR LFVSGACDASAK **n = 4**	ACADATLSQITNNIDPVGR LFVSGACDASAK LLLAGYDDFNCNVWDALK KACADATLSQITNNIDPVGR ADQELMTYSHDNIICGITSVSFSK ELAGHTGYLSCCR **n = 13**
β_2	37344	ACGDSTLTQITAGLDPVGR LIIWDSYTTNK TFVSGACDASIK IYAMHWGTDSR LLVSASQDGK **n = 10**	ACGDSTLTQITAGLDPVGR LIIWDSYTTNK TFVSGACDASIK **n = 6**	ACGDSTLTQITAGLDPVGR LLLAGYDDFNCNIWDAMK TFVSGACDASIK ADQELLMYSHDNIICGITSVAFSR FLDDNQIITSSGDTTCALWDIETGQQ TVGFAGHSGDVMSLSLAPDGR KACGDSTLTQITAGLDPVGR QTFIGHESDINAVAFFPNGYAFTTGS DDATCR **n = 19**
β_4	37354	KACNDATLVQITSNMDSVGR TFVSGACDASSK IYAMHWGYDSR **n = 6**	TFVSGACDASSK MHAIPLR **n = 4**	TFVSGACDASSK KACNDATLVQITSNMDSVGR LLLAGYDDFNCNVWDTLK ACNDATLVQITSNMDSVGR ADQELLLYSHDNIICGITSVAFSK VSCLGVTDDGMAVATGSWDSFLR **n = 12**
γ_2	7750	MEANIDR KLVEQLK EDPLLTPVPASENPFR **n = 6**	MEANIDR KLVEQLK EDPLLTPVPASENPFR **n = 6**	AAADLMAYCEAHAK KLVEQLK EDPLLTPVPASENPFR **n = 6**
γ_4		EDPLIIPVPASENPFR -MKEGMSNNSTTSISQAR **N.D.**	EDPLIIPVPASENPFR -MKEGMSNNSTTSISQAR **N.D.**	EDPLIIPVPASENPFR -MKEGMSNNSTTSISQAR EGMSNNSTTSISQAR VSQAAADLLAYCEAHVR **N.D.**
γ_5	7501	KVVQQLR VKVSQAAADLK **n = 4**	KVVQQLR VKVSQAAADLK **n = 4**	VSQAAADLK KVVQQLR LEAGLNR **n = 5**
γ_7	7379	NDPLLVGVPASENPFK **n = 2**	NDPLLVGVPASENPFK **n = 2**	NDPLLVGVPASENPFK KLVEQLR **n = 4**
γ_{10}	7105	LEAGVER DALLVGVPAGSNPFR DALLVGVPAGSNPFREPR **n = 6**	LEAGVER DALLVGVPAGSNPFR DALLVGVPAGSNPFREPR **n = 4**	VSQAAAELQQYCMQNACK DALLVGVPAGSNPFR **n = 3**
γ_{11}	8268	SGEDPLVK NYIEER LKMEVEQLR CSEEIKNYIEER **n = 7**	SGEDPLVK **n = 2**	SGEDPLVK **n = 1**
γ_{12}	7864	LEASIER RTVQQLR TASTNNIAQAR ASADLMSYCEEHAR SDPLLIGIPTSENPFK SDPLLIGIPTSENPFKDK **n = 12**	LEASIER RTVQQLR TASTNNIAQAR ASADLMSYCEEHAR SDPLLIGIPTSENPFK SDPLLIGIPTSENPFKDK **n = 12**	LEASIER TASTNNIAQAR ASADLMSYCEEHAR SDPLLIGIPTSENPFK SDPLLIGIPTSENPFKDK **n = 12**

[a] *n* values indicate the number of H:L ratios used from each comparison in the analysis of β and γ isoform levels. N.D. = not determined; a protein standard for γ_4 was not used in this study.

(Figure 2), suggesting that the γ_4 isoform (which was not quantified) is moderately expressed in HEK-293 cells. These discrepancies suggest that QPCR data should be interpreted with caution and, if at all possible, verified with quantitative data at the protein level.

β:γ Ratios Associated with A_1:α_{i1} and A_{2A}:α_S Receptor Fusion Proteins and Enriched $\beta\gamma$ Fraction from HEK-293 Cells. Quantitation of β and γ isoforms allowed examination of the ratio between β and γ subunits in dimers purified from the A_1:α_{i1} and A_{2A}:α_S receptor fusion proteins, as well as in the enriched

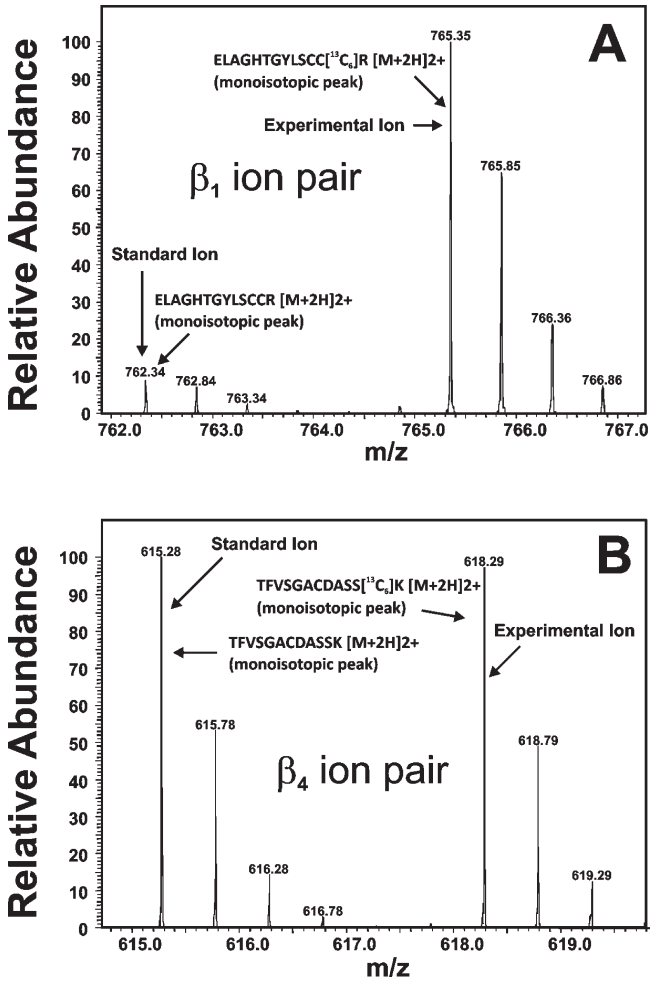

FIGURE 6: Comparison of heavy and light ion pairs used to determine H:L ratio for β_1 and β_4. (A) Light and heavy $[M + 2H]^{2+}$ ions from the ELAGHTGYLSCCR peptide derived from the β_1 isoform. (B) Light and heavy $[M + 2H]^{2+}$ ions from the TFVSGACDASSK peptide derived from the β_4 isoform.

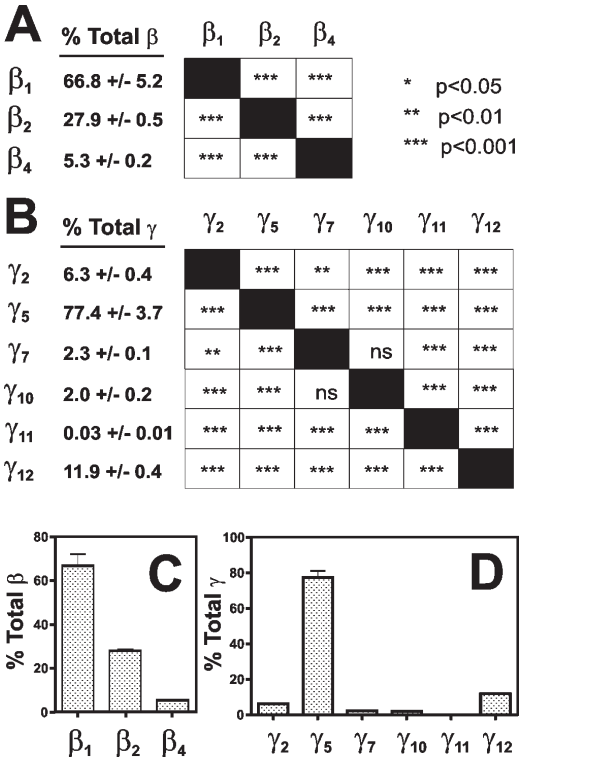

FIGURE 7: Quantitation of β and γ isoforms associated with the A_1:α_{i1} fusion protein. (A) Matrix of β isoforms. Each isoform is expressed as a percentage of the sum of all β protein in each sample. Asterisks illustrate significant differences in comparison of β isoform levels. (B) Matrix of γ isoforms. Each isoform is expressed as a percentage of the sum of γ protein in each sample. Asterisks illustrate significant differences in comparisons between γ isoform levels; ns, not significant. (C) Bar graph representation of the data expressed in (A). (D) Bar graph representation of the data expressed in (B). (See Table 3 for a list of β and γ peptides used to determine H:L ratios and *n* numbers.)

$\beta\gamma$ fraction from HEK-293 cells. This was done by dividing the total moles of γ isoforms in a sample by the total moles of β isoforms in a sample. In theory there is a 1:1 ratio of β:γ subunits in a given sample of purified $\beta\gamma$ dimer. Using quantitative values from this study, there were 0.69 and 0.72 mol of γ for every mole of β in the $\beta\gamma$ dimers purified from the A_1:α_{i1} and A_{2A}:α_S receptor fusion proteins, respectively; similarly, the ratio of γ to β in the enriched HEK-293 cell fraction was 0.71. This was somewhat expected, as γ_4 was not included in the total estimates of γ protein for each receptor fusion protein. Furthermore, reports of instability of $\beta\gamma$ dimers containing γ_{11} (*11*, *27*) suggest that γ_{11} levels in the analysis may be underestimates of the actual level of γ_{11} present prior to the steps used to either purify the receptor−G protein complex or enrich the fraction of $\beta\gamma$ from HEK-293 cells. The theoretical $\beta\gamma$ ratio of 1:1 is an important issue in the choice of Sf9 $\beta\gamma$ standards, as the γ concentration was calculated indirectly from β. Most cases of unstable $\beta\gamma$ dimer combinations involve β_2, β_3, β_4, or β_5 (see review in ref *5*); for this reason, β_1 was expressed with different γ isoforms for the generation of recombinant $\beta\gamma$ standards used to calculate γ protein levels.

The preference of adenosine A_1 and A_{2A} receptors for β_4 and γ_5 isoforms is in agreement with previous reconstitution studies that demonstrated that $\beta_2\gamma_2$ and $\beta_4\gamma_2$ were more efficient than $\beta_1\gamma_2$ at coupling G_S α to the adenosine A_{2A} receptor (*28*). This study refines that work by identifying the β_4 isoform as the preferred binding partner of the two fusion proteins. The previous work is also expanded through the demonstration that the adenosine A_1 and A_{2A} receptors share a preference for the γ_5 isoform. Together, these studies suggest that, in the case of adenosine receptors, binding specific $\beta\gamma$ dimer combinations is more strongly determined by the receptor family than the identity of Gα subunit that binds a particular receptor isoform.

DISCUSSION

G protein $\beta\gamma$ dimers exist at the beginning of a signaling event as part of a receptor:Gα$\beta\gamma$ ternary complex (*29*), with $\beta\gamma$ binding to both receptor and Gα subunit (*11*). The crystal structure of a heterotrimeric G protein provided evidence that binding sites for $\beta\gamma$ on Gα family members are highly conserved (*30*), and few accounts of specificity, such as between G_qα and β_5 (*17*), have been reported. Broad diversity in both potential $\beta\gamma$ dimer combinations (*11*) and G protein coupled receptor isoforms (*31*) suggests that receptor, or possibly both receptor and Gα, influences the composition of β and γ isoforms in a receptor−G protein complex. Understanding preferences of specific receptors for particular combinations of α, β, and γ will help to elucidate the structural determinants that favor these combinations.

Two limitations had to be overcome to successfully quantify β and γ dimers associated with specific receptors. The first limitation concerned the isolation of $\beta\gamma$ in sufficient quantity and

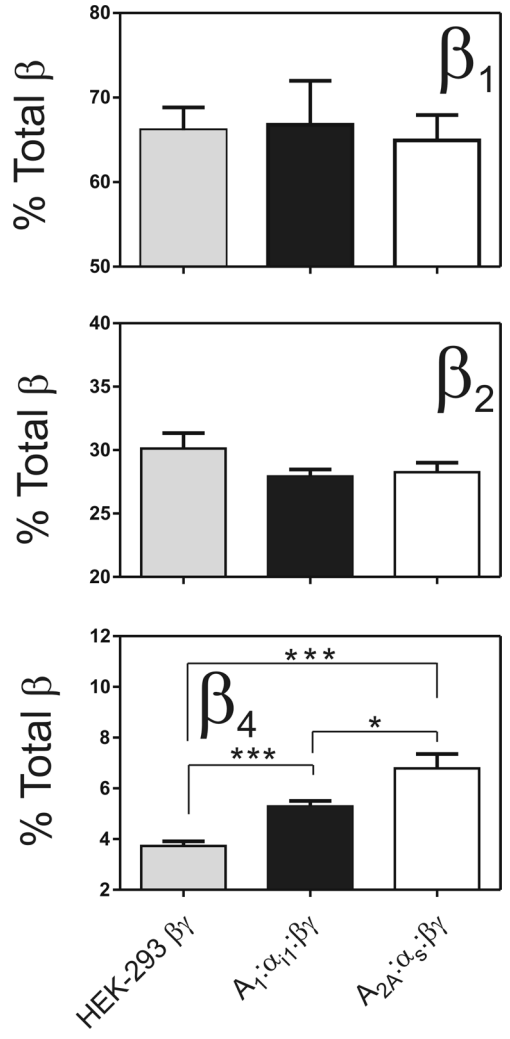

FIGURE 8: Comparison of β isoforms associated with A_1:α_{i1}, A_{2A}:α_S, and enriched HEK-293 $\beta\gamma$. H:L ratios derived from β tryptic peptides were used to calculate the concentration of the β_1, β_2, and β_4 isoforms purified from the two fusion proteins or present in the enriched HEK-293 $\beta\gamma$ fraction. (See Table 3 for a list of β peptides used to determine H:L ratios and *n* numbers.) The concentration of each β isoform was converted to moles, and the moles of each β isoform was divided by the sum total moles of all the β isoforms in the sample. Each β isoform is expressed as a percent of the total moles quantified ± SEM (* = $p < 0.05$; *** = $p < 0.001$). Numerical values for the data are presented in Figure 7A and Table 4.

purity for biochemical analysis. Receptors have been precipitated with associated G proteins; however, affinity and stoichiometry between G protein and receptor can be variable (*32*) and often not adequate for biochemical analysis (unpublished observation). Thus, a receptor fusion protein strategy was employed in order to preserve the interactions among $\beta\gamma$, receptor, and Gα during purification of the complex (Figure 1). This approach allows a more consistent purification and would likely be applicable to any receptor−α combination.

The second limitation involves the availability of immunological reagents for characterizing β and γ isoforms. Many of the antibodies available do not have sufficient sensitivity or specificity to distinguish between isoforms and thus are not quantitative. Mass spectrometry in conjunction with SILAC was chosen as an innovative and powerful approach to quantify β and γ isoforms for several reasons: (1) obviation of the need for specific antibodies; (2) femtomole sensitivity; (3) linearity of heavy:light ion ratios at all signal strengths; (4) absolute specificity with respect to protein isoform and species; (5) ability to characterize covalent modifications of protein isoforms.

Posttranslational modification of proteins is critical to understand because it can have the effect of increasing the functional heterogeneity of a protein. Covalent modifications of γ isoforms were probed by both MALDI and ESI mass spectrometry. MALDI mass spectrometry was able to initially characterize the modification state of the γ_5 subunits from $\beta\gamma$ populations purified from both adenosine receptor fusion proteins in HEK-293 cells. In contrast to the γ_5 protein observed from the $\beta_1\gamma_5$ dimer purified from Sf9 cells (Figure 4A) and other mammalian γ isoforms which appear to exist predominantly in the prenylated and C-terminally processed state (*24*), the γ_5 protein associated with both A_1:α_{i1} and A_{2A}:α_S receptor fusion proteins was mostly prenylated without C-terminal proteolytic processing. This pattern of processing for γ_5 is in agreement with the results reported by Kilpatrick et al. (*22*), which the authors suggest may be related to protein−protein interactions.

ESI-MS/MS analysis was also able to reveal the N-terminal modification state of many of the γ isoform derived peptides identified in this study. The significance of differential N-terminal acetylation of γ isoforms was emphasized by a recent study in *Saccharomyces cerevisiae* that suggested N-terminal acetylation is a degradation signal in the N-end rule pathway (*33*). In the study, the Doa10 ubiquitin ligase preferentially recognized N-acetylated proteins, which targets the protein for ubiquitylation, resulting in shorter half-lives from increased degradation. This likely has relevance for G protein stability, as the γ_2 isoform has been shown to be a substrate for ubiquitylation (*34*). Proteins with a lysine at position 2, such as γ_4 (Table 2), regardless of N-terminal acetylation status, were found to bind poorly to the Doa10 ubiquitin ligase (*33*); interestingly, the only other γ isoform with a lysine at position 2 is γ_3. Although the N-terminus of γ_{11} was not characterized in this study, the proline at position 2 (Table 2) suggests that it is a poor substrate for N-terminal acetyltransferase (*26*) and thus a poor target for Doa10 ligase; only one other γ isoform, γ_1, contains a proline at position 2. Taken together, the lack of or limited acetylation in γ_1, γ_3, γ_4, and γ_{11}, in addition to the lysine at position 2 in γ_3 and γ_4, suggests that these isoforms have the capacity for metabolic stability and may mark a functional divide in the γ isoform family. Extrapolating the effects of acetylation on a physiological system, the degree of acetylation of $\beta\gamma$ dimers contained in a receptor:G protein complex may affect the duration of $\beta\gamma$ signaling through regulation of its half-life.

A previously published report demonstrated that purified $\beta_2\gamma_2$ and $\beta_4\gamma_2$ were more efficient than $\beta_1\gamma_2$ at coupling G_S α to the adenosine A_{2A} receptor in a reconstitution assay (*28*). A distinction should be made that the present study measures interactions of receptors with the endogenous pool of $\beta\gamma$ dimers in a cell, allowing for differences in both stoichiometry and subcellular localization to influence formation of an R:G complex. One interpretation of the two studies is that $\beta\gamma$ dimers containing either β_2 or β_4 are able to couple G_S α to the A_{2A} receptor with high efficiency; however, in the context of the HEK-293 cell, both adenosine A_1 and A_{2A} receptors have a preference for $\beta\gamma$ dimers containing the β_4 isoform, likely $\beta_4\gamma_5$. It should also be noted that the adenosine A_1 receptor has been documented to have a preference for the G_{i3} α subunit over G_{i1} α, G_{i2} α, or G_O α (*35*). Although little specificity between Gα and $\beta\gamma$ has been reported (*21*), it is possible that Gα isoforms modulate the specificity of receptor $\beta\gamma$ interactions, and this may be reflected

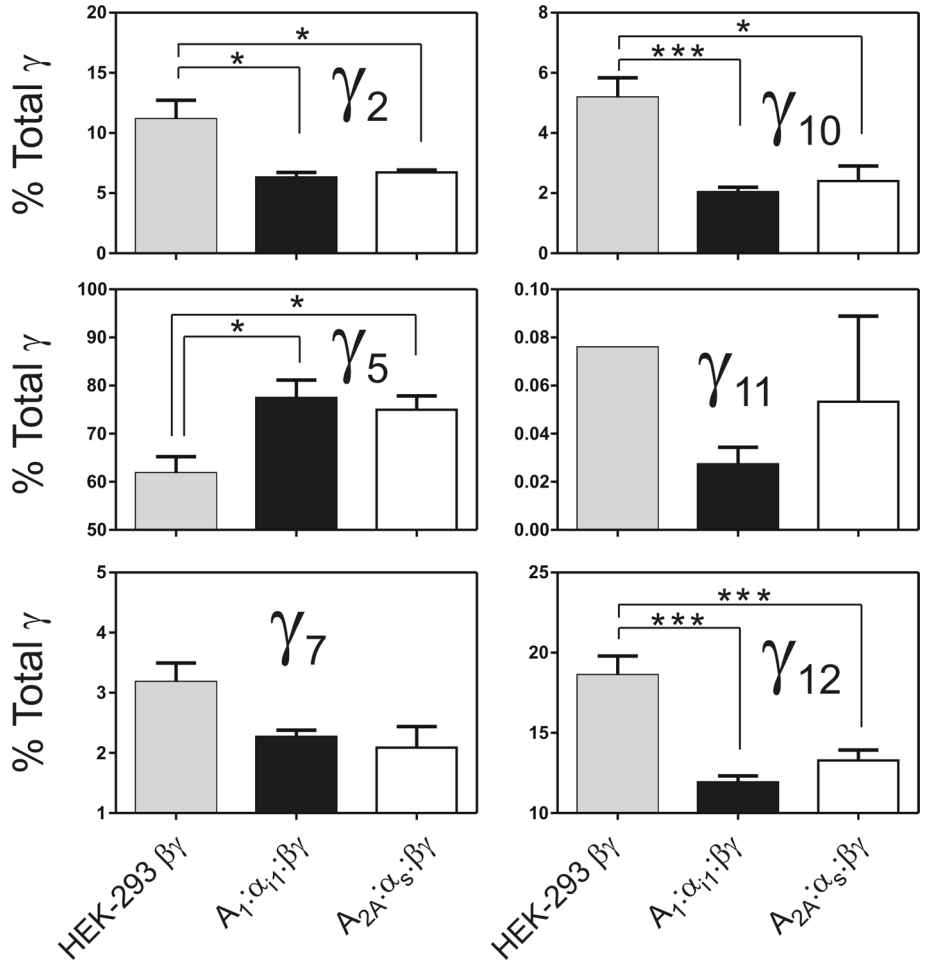

FIGURE 9: Comparison of γ isoforms associated with A_1:α_{i1}, A_{2A}:α_S, and enriched HEK-293 $\beta\gamma$. H:L ratios derived from γ tryptic peptides were used to calculate the concentration of the γ_2, γ_5, γ_7, γ_{10}, γ_{11}, and γ_{12} isoforms purified from the two fusion proteins or present in the enriched HEK-293 $\beta\gamma$ fraction. (See Table 3 for a list of γ peptides used to determine H:L ratios and *n* numbers.) The concentration of each γ isoform was converted to moles, and the moles of each γ isoform was divided by the sum total moles of all the γ isoforms in the sample. Each γ isoform is expressed as a percent of the total moles quantified ± SEM (* = $p < 0.05$; *** = $p < 0.001$). Numerical values for the data are presented in Figure 7B and Table 4.

in the differences in affinity observed between A_1:α_{i1} and A_{2A}:α_S fusion proteins for dimers containing β_4. The variability in Gα isoform may also account for the preference of the adenosine A_{2A} receptor for β_2 and γ_7 in striatum, where A_{2A} receptor mediated elevation of cAMP occurs primarily via G_{olf} α instead of G_S α (*36*). Cell type and differences in transcription may also contribute to the identity of a heterotrimeric G protein that can interact with a receptor. Thus, the increase in β_4 mRNA levels resulting from A_{2A}:α_S fusion protein expression in this study (Figure 2) may be related to the higher levels of β_4 protein observed with A_{2A}:α_S (Figure 9). Interestingly, this correlates with another study that reported a decrease in β_4 mRNA levels after ablation of G_S α expression using RNAi (*9*). This suggests that signaling components within a transduction cascade can be regulated in concert beginning at the level of transcription, and thus there are likely many points of control that determine the final makeup of a receptor:G protein complex.

In this analysis, β and γ isoforms are expressed as a percent of the total quantified, which is essentially a zero sum situation where increases in one isoform must be offset by decreases in others. This can be explained by Figure 9, which illustrates an ~15 percentage point increase in γ_5 associated with A_1:α_{i1} over HEK-293 $\beta\gamma$. The combined percentage point decrease in γ_2, γ_7, γ_{10}, and γ_{12} associated with A_1:α_{i1} relative to HEK-293 $\beta\gamma$ agrees very closely with this value (Figures 7 and 9 and Table 4). In contrast, the increase in β_4 levels associated with A_1:α_{i1} compared to HEK-293 $\beta\gamma$, although significant, is less than 2 percentage points (Figure 8). This increase is likely offset by the slight trend lower of β_2 associated with A_1:α_{i1} compared to HEK-293 $\beta\gamma$ (Figure 8). One interpretation of these differences is that increases in γ_5 levels in A_1:α_{i1} over HEK-293 $\beta\gamma$ cannot be explained by increases in preference for the $\beta_4\gamma_5$ dimer alone. Thus, A_1:α_{i1} (and A_{2A}:α_S to a lesser extent) likely also has preferences for $\beta_1\gamma_5$ and/or $\beta_2\gamma_5$ dimers.

This specificity for γ_5 may be related to the physiological properties of adenosine receptors. For instance, activation of the adenosine A_{2A} receptor has been shown to attenuate the inflammatory effects of *Helicobacter pylori* induced gastritis (*37*). It has also been shown that *H. pylori* infection upregulates γ_5 mRNA levels in a human gastric cancer cell line (*38*); increased transcription of γ_5 may be related to a mechanism by which adenosine receptors interact with specific G protein combinations to signal and counter the effects of inflammation. Indeed, there is mounting evidence that β and γ levels are dynamic and respond to extracellular cues. For instance, the γ_3 transcript was upregulated in rat hippocampus following oxidative stress (*39*) and in activated CD4+ T-cells (*40*). LPS stimulation of the microglial cell line BV-2 resulted in a transient increase in γ_{12} levels (*41*).

Table 4: Mean Total Percent of β and γ Isoforms Quantified in Enriched HEK-293 Fraction (A and B) and in $\beta\gamma$ Dimers Purified from A_{2A}:α_s Fusion Protein (C and D) ± SEM[a]

Enriched HEK-293 βγ Fraction

A

% Total β	β1	β2	β4
β1 66.1 +/- 2.7		***	***
β2 30.1 +/- 1.2			***
β4 3.7 +/- 0.2			

* p<0.05
** p<0.01
*** p<0.001

B

% Total γ	γ2	γ5	γ7	γ10	γ11	γ12
γ2 11.2 +/- 1.6		***	**	*		**
γ5 61.8 +/- 3.4			***	***		***
γ7 3.2 +/- 0.3				*		***
γ10 5.2 +/- 0.7						***
γ11 0.08						***
γ12 18.6 +/- 1.2						

βγ Purified from A_{2A}:α_s

C

% Total β	β1	β2	β4
β1 65.0 +/- 3.0		***	***
β2 28.3 +/- 0.7			***
β4 6.8 +/- 0.6			

* p<0.05
** p<0.01
*** p<0.001

D

% Total γ	γ2	γ5	γ7	γ10	γ11	γ12
γ2 6.7 +/- 0.2		***	***	***	***	***
γ5 75.0 +/- 2.9			***	***	***	***
γ7 2.1 +/- 0.4				ns	*	***
γ10 2.4 +/- 0.5					*	***
γ11 0.05 +/- 0.03						***
γ12 13.3 +/- 0.7						

[a]Asterisks indicate significant differences in comparisons between levels of β isoforms (A and B) and γ isoforms (B and D) within a sample. ns = not significant. Only one observed γ_{11} peptide in one experiment (B) precluded statistical comparison with other γ isoforms.

Levels of β_4 mRNA and protein increased in human microvascular endothelial cells in response to IL-1 and TNF-α (*42*), and IFN-β was shown to increase β_4, γ_2, and γ_{11} transcripts in Ubp43$^{-/-}$ bone marrow derived macrophages (*43*). These examples suggest that extracellular stimuli may prime a cell to express a particular profile of β and γ isoforms; it is of critical importance to determine if specific receptors within the cellular context have inherent preferences for the resulting $\beta\gamma$ dimers.

As an example of how dynamic regulation of β and γ transcription may influence signaling, the IFN-β-mediated increase in β_4 mentioned above may be examined in the context of adenosine receptor signaling. Increased β_4 expression could facilitate the population of adenosine A_{2A} receptor complexes containing $\beta_4\gamma$ dimers which, compared to A_{2A} receptor complexes containing $\beta_1\gamma$ dimers, have the ability to shift the equilibrium of the A_{2A} receptor population toward more high-affinity agonist binding sites (*44*). This would have the effect of lowering the concentration of adenosine required for activation of the A_{2A} receptor. Interestingly, a similar mechanism for increased adenosine receptor signaling was proposed after the discovery that IFN-β induced the expression of CD73, an ecto-5′-nucleotidase that increases adenosine production (*45*); the authors proposed that increased adenosine receptor signaling may be one way that IFN-β ameliorates the progression of multiple sclerosis.

The question of how β and γ subunit diversity translates into signaling specificity has been enigmatic. It is possible that multiple mechanisms exist for heterogeneity of β and γ isoforms to influence cellular signaling. Regardless, this innovative approach will allow the question to be fully addressed through the quantitative measurement of changes in both β and γ isoforms with high precision under a variety of experimental conditions.

ACKNOWLEDGMENT

We thank Dr. James Garrison for support during the course of these studies and for helpful discussions during the preparation of the manuscript. Thanks go to Dr. Joel Linden for the generous gift of the pDoubleTrouble vectors containing adenosine A_1 and A_{2A} receptors. We also thank Dr. Thurl Harris, Jessica Ng, and Jiping Zhou for expert technical assistance. We acknowledge the University of Virginia Pratt Committee for its generous support of the Biomedical Research Facility.

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