G Protein $\beta\gamma$ Dimer Formation: Gβ and Gγ Differentially Determine Efficiency of in Vitro Dimer Formation†

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ABSTRACT: The Gβ and Gγ subunit of the heterotrimeric G proteins form a functional dimer that is stable once assembled in vivo or in vitro. The requirements, mechanism, and specificity of dimer formation are still incompletely understood, but represent important biochemical processes involved in the specificity of cellular signaling through G proteins. Here, seven Gβ and 12 FLAG-epitope-tagged Gγ subunits were separately synthesized in vitro using a rabbit reticulocyte lysate expression system. The translation products were combined and dimers isolated by immunoprecipitation. Gβ1 and Gβ4 formed dimers with all Gγ subunit isoforms, generally with Gβ/Gγ stoichiometries between 0.2:1 and 0.5:1. Gβ5, Gβ5L, and Gβ3s did not form significant amounts of dimer with any of the γ subunit isoforms. Gβ2 and Gβ3 formed dimers with selected Gγ isoforms to levels intermediate between that of Gβ1/Gβ4 and Gβ3s/Gβ5/Gβ5L. We also expressed selected G$\beta\gamma$ in HEK293 cells and measured PLCβ2 activity. G$\beta\gamma$ dimer-dependent increases in IP3 production were seen with most Gβ1, Gβ2, and Gβ5 combinations, indicating functional dimer expression in intact cells. These results define the complete set of G protein $\beta\gamma$ dimers that are formed using a single biochemical assay method and suggest that there are Gβ isoform-specific factors in rabbit reticulocyte lysates that determine the efficacy of G$\beta\gamma$ dimer formation.

The heterotrimeric G proteins are an integral part of cellular signaling machinery, sorting incoming signals, and generating responses unique to each receptor. They are composed of one each of 16 α, 5 β, and 12 γ subunits (*1*−*10*). Higher eukaryotic cells generally express multiple G protein-coupled receptors (GPCRs),[1] and signaling specificity resides, in part, in these receptors interacting with specific populations of G protein heterotrimers. In fact, one series of studies investigating subunit isoform requirements by an antisense inhibition strategy concluded that receptors target heterotrimers made up of specific isoforms of each of its three subunits (*11*−*13*). However, the exact nature and the mechanisms by which cells construct unique combinations of subunits into heterotrimers, or even which ones are formed, are still not fully understood (*10*).

G proteins mediate GPCR signaling through two functional components, an α subunit with bound guanine nucleotide and a $\beta\gamma$ dimer (*14*−*17*). Following synthesis and assembly, G$\beta\gamma$ dimers undergo several modifications (*18*) in transit to heterotrimer formation and localization in the plasma membrane (*19*). The G$\beta\gamma$ dimer plays a role in targeting α subunits (*20*), in maintaining the G protein in an inactive conformation responsive to activated receptor (*21*), in specificity of receptor recognition of the heterotrimer (*11*, *12*, *22*), in downstream signaling specificity through regulation of effector targets (*3*, *6*), and in receptor desensitization, down regulation, and recycling (*23*−*26*). The dimer formed of Gβ and Gγ is stable unless denatured (*14*), and so it is not generally thought to reversibly associate in cells, but the array of dimers made and the fundamental requirements for functional selectivity of them remain uncertain (*14*−*17*).

Many different approaches have been used to characterize or infer G$\beta\gamma$ dimer formation, including both functional and biochemical assays (*22*, *27*−*32*). Early studies indicated that not all possible combinations of Gβ and Gγ form dimers, for example, Gβ2 and Gγ1 do not (*33*, *34*); but there remains uncertainty about the significance of other G$\beta\gamma$ dimer combinations, such as those containing Gβ5 (*17*, *35*, *36*). In fact, the actual requirements, mechanism, and full specificity of G$\beta\gamma$ dimer formation remain uncertain. Although dimerization is a cellular process, understanding its components, requirements, and possibly even regulation will ultimately require definition and full replication of the assembly process in a cell-free system. Here, we evaluate G protein dimer formation with subunits expressed in rabbit reticulocyte lysates. This approach was one of the first used to study formation of dimers (*34*, *37*) and may provide the only truly direct method for characterization of the complex biochemical events involved in dimer formation. Particularly important in this approach is the ability to quantitatively evaluate the stoichiometry of dimer formation and its relationship to the

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[1] Abbreviations: GPCR, G protein-coupled receptors; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBSBT, buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mg/mL BSA, and 0.1% polyoxyethylene glycol (Thesit) or 0.1% polyoxyethylene 10-lauryl ether [C12E10]; PLCβ2, phospholipase Cβ2; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; DMEM, Dulbecco's modiefied Eagle's medium; HBSS, Hepes-buffered salt solution.

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G protein subunit isoforms involved. Our results define the complete range of dimers formed in this assay and identify isoform-specific components of the assembly process. Most importantly, they indicate that there are Gβ subunit-specific factors involved in assembly that determine the efficiency of dimer formation in vitro, even though these factors do not necessarily appear to be limiting in the context of intact cells. These results have significance for both the mechanism and consequences of G$\beta\gamma$ dimer formation.

MATERIALS AND METHODS

Vectors, cDNAs, and Expression Constructs. All Gγ constructs contained the FLAG epitope at the N-terminus, just after the initial Met. Human Gγ13 was obtained from Guthrie cDNA Resource Center (www.cdna.org) and the FLAG epitope inserted using QuikChange (Stratagene) to generate FLAGγ13. Gγ1 (human) in pBluescript KS was obtained from Henry Fong, transferred to pcDNA3, and the FLAG epitope inserted using QuikChange to give FLAGγ1. FLAGγ2 was created by insertion of the FLAG sequence at the N-terminus of Gγ2 (bovine) in pBlueScript (obtained from Mel Simon) using QuikChange; the construct was transferred into pcDNA3.1(+). Gγ3 (bovine) in pcB6+ was obtained from N. Gautam, transferred to pcDNA3, and the FLAG epitope inserted with QuikChange to give FLAGγ3. Human Gγ4 and bovine Gγ7, both in pcDNA3, were obtained from William Simonds, and the FLAG epitope was inserted with QuikChange to give FLAGγ4 and FLAGγ7. Gγ5 was obtained from rat liver DNA using PCR, cloned into pCRII, transferred into pcDNA3.1(+), and the FLAG epitope inserted with QuikChange to give FLAGγ5. Human Gγ8cone (Gγ8c) in pT7T3D-Pac was obtained as a dbEST from ATCC (clone no. 388278), cloned into pcDNA3.1(−), and the FLAG epitope inserted with QuikChange to give FLAGγ8c. Rat Gγ8olf (Gγ8o) in pBlueScriptKS was obtained from Nicholas Ryba, transferred into pcDNA3.1-(+), and the FLAG epitope inserted to give FLAGγ8o. Gγ10 was obtained from human brain RNA (Clontech, no. 64020-1) using RT-PCR, cloned into pCRII, transferred into pcDNA3.1(+), and the FLAG epitope inserted with Quik-Change to give FLAGγ10. Human Gγ11 in pT7T3D-Pac was obtained as a dbEST from ATCC (clone no. 388278), transferred into pcDNA3.1(+), and the FLAG epitope inserted with QuikChange to give FLAGγ11. Human Gγ12 pT7T3D-Pac was obtained as a dbEST clone from ATCC (clone no. 3528027), subcloned into pcDNA3, and the FLAG epitope inserted with QuikChange to give FLAGγ12. Bovine Gβ1, human Gβ2, and mouse Gβ5 constructs were obtained from Mel Simon, and were transferred into pcDNA3. Human Gβ3 in pGEM3z was obtained from Michael Levine and transferred into pcDNA3. Human Gβ4 and Gβ5L in pcD-NA3.1(+) were obtained from the Guthrie cDNA Resource Center (www.cdna.org).

In Vitro Translation and Transcription. In vitro translation of subunits was carried out using the TnT Quick Coupled Transcription/Translation Systems (Promega). Each Gβ and each Gγ cDNA were expressed separately in vitro using the TNT Quick-Coupled translation kit (Promega) and [^{35}S]-Met without carrier. Sample volumes varied from 40 to 100 μL and contained either [^{35}S]-Met (5−10 μCi at 1000 Ci/mmol) or 20 μM unlabeled Met, and 0.5−2.0 μg of DNA. The transcription/translation mixture was incubated at 30 °C, usually for 60−90 min. The reaction was stopped either by addition of sample buffer for gel electrophoresis or by freezing at −80 °C. To quantify the protein synthesized, a sample equivalent to 1−5% of the total expressed protein was quantitated in a filter binding assay, as described below, and the remaining expressed protein was stored at −80 °C. Alternatively, the samples were separated on an SDS−PAGE gel and autoradiographed to determine expression.

Quantification of ^{35}S-labeled Gγ and Gβ expression was done in one of two ways. For Gβ, the percent incorporation of label was determined with a filter binding assay (as described by Promega in the technical manual for the translation kit), and the incorporation was defined as the percent of total label added that bound to glass fiber filters (Whatman GF/C). The total amount of protein translated (pmol) was determined from the specific activity of the ^{35}S-Met, the percent incorporation, and the number of Met residues present in each isoform (Table 1). For Gγ, a fraction of the translation mix was run on an 8−16% gel (Bio-Rad Criterion Tris), along with Gβ subunits of known incorporation, and after drying, the gel was exposed to a phosphor storage screen for 1−3 days. The radiolabeled proteins were visualized by imaging the screen on a Molecular Dynamics PhosphorImager or a Molecular Dynamics Storm instrument; the radioactivity detected by the screen was quantitated using ImageQuant (Molecular Dynamics). With this software, the labeled bands were quantified, corrected for background, and converted to relative molar ratios by dividing the signal thus determined by the number of Met residues in the sequence of the respective protein (Table 1). Generally, proteins used in dimerization of assays (described below) were synthesized and characterized at the same time with [^{35}S]-Met of the same specific activity. Using [^{35}S]-Met-labeled β subunits synthesized at the same time and characterized on filter binding assays (above), we were able to calculate absolute molar amounts.

G$\beta\gamma$ Dimerization Assay. For the dimerization reaction, Gβ translations were combined with FLAGγ translations with a 2:1 molar ratio of Gβ/Gγ, based upon the quantification of synthesized subunits described above. Reactions were carried out at 37 °C for 2 h and were terminated by dilution with buffer for the immunoprecipitation experiments. Immunoprecipitation was carried out immediately after the dimerization reaction. Agarose beads conjugated with anti-FLAG antibody (Sigma, A-2220) were used for the immunoprecipitation, with 5 μL of beads per sample. The beads were washed twice in 50 vol of TBSBT (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mg/mL BSA, and 0.1% polyoxyethylene glycol (Thesit) or 0.1% polyoxyethylene 10-lauryl ether [C12E10]), then blocked against nonspecific binding by adding 10 vol of TBSBT with 10% reticulocyte lysate and incubating at room temperature for 1 h with gentle mixing. The beads were washed twice and resuspended with TBSBT to 50 vol. The samples were brought to a volume of 250 μL with TBSBT, mixed with 250 μL of the bead suspension, and incubated for 1 h at room temperature with gentle mixing. Samples were then centrifuged for 10 s in a microfuge, and the supernatant was removed. The beads were washed twice with TBSBT, transferring the beads to a new tube after the first wash to control for nonspecific binding to the original sample tube. Electrophoresis was carried out using the procedure of Laemlli et al., using Criterion pre-

Table 1: cDNAs and Constructs Used in These Studies[a]

subunit	source	accession no.	species	percent identity to human	predicted no. Met	N-terminal sequence	N-terminal processing	assumed no. Met
β1	M. Simon	M13236	human	100	8	MSELD	yes	7
β2	M. Simon	M36429	human	100	9	MSELE	yes	8
β3	M. Levine	M31328	human	100	9	MSELE	yes	8
β3s	GRC	M31328	human	100	10	MGEME	yes	9
β4	GRC	AF300648	human	100	11	MATEG	yes	10
β5	M. Simon	L34290	mouse	99	8	MSELE	yes	7
β5L	GRC	AF300650	human	100	11	MATEG	yes	10
FLAGγ1	H. Fong	P63211	human	100	3	MDYKD	no	3
FLAGγ2	M. Simon	M37183	bovine	100	3	MDYKD	no	3
FLAGγ3	N. Gautam	M58349	bovine	100	4	MDYKD	no	4
FLAGγ4	W. Simonds	U31382	human	100	4	MDYKD	no	4
FLAGγ5	PCR from rat liver	M95780	rat	100	2	MDYKD	no	2
FLAGγ7	W. Simonds	P030671	bovine	97	2	MDYKD	no	2
FLAGγ8cone	ATCC dbEST	AF365870	bovine	100	2	MDYKD	no	2
FLAGγ8o	N. Ryba	L35921	rat	98	3	MDYKD	no	3
FLAGγ10	PCR human brain	P50151	human	100	2	MDYKD	no	2
FLAGγ11	ATCC dbEST	U31384	human	100	2	MDYKD	no	2
FLAGγ12	ATCC dbEST	AF365871	human	100	2	MDYKD	no	2
FLAGγ13	GRC	Q9P2W3	human	100	4	MDYKD	no	4

[a] The constructs used for the G$\beta\gamma$ dimer studies are indicated on the left, along with their source and/or reference and an accession number for their sequence; GRC, Guthrie cDNA Resource Center. Also shown in the table are the number of predicted Met in the sequence, the initial five residues of each sequence, the predicted processing of the N-terminal Met (*41*), and resulting number of Met in the expressed proteins assumed in the calculations described here.

cast gels (Bio-Rad). For autoradiography, after electrophoresis, the gel was washed twice for 15 min with 50% methanol and 10% acetic acid, then for 5 min with 7% acetic acid, 7% methanol, and 1% glycerol before drying. Dried gels were then exposed to film overnight at −80 °C or to a phosphor storage screen for 3−5 days at room temperature. The screen was imaged and analyzed using a Molecular Dynamics Phosphorimager and ImageQuant software.

Inositol Phosphate Determination in HEK293 Cells. HEK 293 cells grown in DMEM with 10% FBS at 37°C were transfected in 60 mm dishes with 0.4 μg PLCβ2, 0.5 μg Gβ cDNA, and 0.5 μg Gγ cDNA using Lipofectamine as previously described (*38*). The Gβ and Gγ constructs were in pcDNA3 and are described in Table 1. Human PLCβ2 in pRC/CMV was obtained from Ravi Iyengar. Control cells were transfected with pcDNA3 vector in place of the Gγ cDNA, but otherwise received 0.4 μg PLCβ2 cDNA and 0.5 μg Gβ cDNA. Cells were labeled with 2 μCi/mL myo-[^{3}H]-inositol approximately 24 h prior to assay for IP3 production. Cells were washed once with DMEM followed by incubation in DMEM/20mM NaHepes (or HBSS where indicated) containing 10 mM LiCl for 40 min. The incubation was terminated by the addition of 0.75 mL ice-cold 10 mM formic acid to the cells. [^{3}H]Inositol phosphates were separated from [^{3}H]inositol on Dowex anion-exchange columns (AG1-X8 resin: BioRad No. 140-1444) as described (*39*). Data are presented as the percent of total [^{3}H]inositol recovered as [^{3}H]inositol phosphates, where total [^{3}H]inositol equals [^{3}H]-inositol phosphates plus [^{3}H]inositol.

Statistical Analysis. The extent of G$\beta\gamma$ dimer formation was defined as the molar ratio of Gβ to Gγ, and data are presented as mean ± SEM. Conclusions about whether specific combinations of Gβ and Gγ form a dimer were based upon a one-way ANOVA (*40*). The mean square for error (MSE) from the analysis of variance was used to calculate a baseline for dimer formation based upon 95% and 99% confidence intervals for a mean of 0. Dimer formation is nominally defined as those dimers with a stoichiometry greater than that predicted by the 99% confidence interval value.

RESULTS

The Gβ and Gγ constructs used in these studies are listed in Table 1. All Gβ and Gγ were translated and labeled with [^{35}S]-Met using the Promega reticulocyte lysate translation system. One microliter of each translation reaction was used to determine the amount of protein expressed; this sample was electrophoresed on a gel, dried, exposed to a storage screen, and visualized using the PhosphorImager. From the digital image, the amount of radioactivity in each band (the pixel volume) was determined. Using the values in Table 1, we quantified the protein by dividing the volume for a given band (corresponding to translated protein) by the number of Met residues present in that protein. All of the Gβ proteins are predicted to be N-terminally processed by acetylation after cleavage of the N-terminal Met. Incorporation of the FLAG epitope at the N-terminus of Gγ places an Asp after the initial Met and stabilizes the N-terminal Met against cotranslational cleavage (*41*). Figure 1A shows examples of the translated Gβ subunits, and Figure 1B the Gγ subunits. All of the Gβ constructs were expressed to approximately the same extent. The Gβ subunits have one major band, and sometimes minor bands, which may reflect initiation at an internal Met. In general, the minor bands do not co-immunoprecipitate with FLAGγ (see Figures 2 and 3). There were some differences in the expression of the different FLAGγ constructs, but all expressed adequately for these studies.

The G protein subunits were expressed in different translations (Figure 1), quantified as described in Materials and Methods, and then combined at a 2:1 stoichiometry of Gβ subunits to FLAGγ subunits. This stoichiometry was determined to be optimal, that is, gave maximal dimer stoichiometry, in preliminary experiments (data not shown)

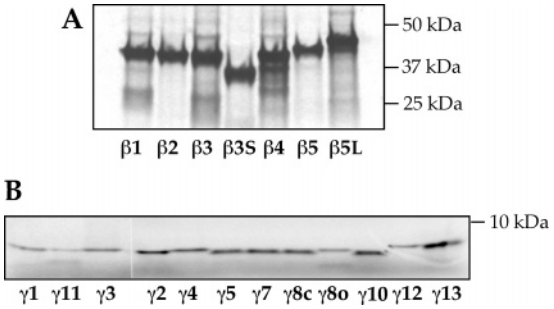

FIGURE 1: Expression of G protein subunits by in vitro translation. The following constructs were translated in vitro: FLAG-tagged Gγ1, Gγ2, Gγ3, Gγ4, Gγ5, Gγ7, Gγ8c, Gγ8o, Gγ10, Gγ11, Gγ12, and Gγ13 and Gβ1, Gβ2, Gβ3, Gβ3s, Gβ4, Gβ5, and Gβ5L. One microliter of each was run on an 8−16% Criterion gel (Bio-Rad); the gel was dried and subjected to autoradiography using a phosphor storage screen. (A) Expression of Gβ constructs. (B) Expression of Gγ constructs. Indicated along the right side of each PhosphorImager profile is the approximate position of molecular weight standards.

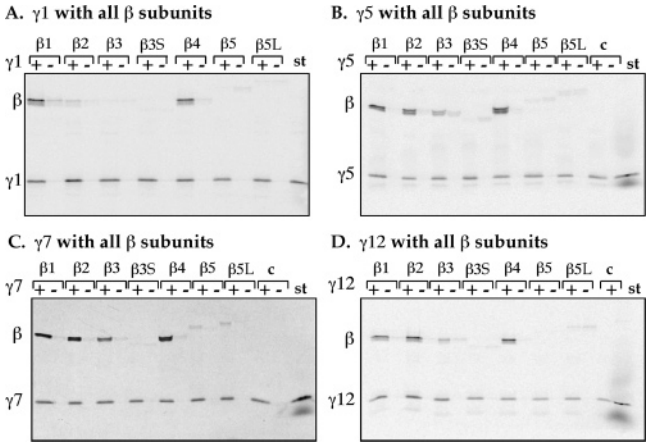

FIGURE 2: Assay of Gβγ dimer formation. The Gβ1, Gβ2, Gβ3, Gβ3s, Gβ4, Gβ5, and Gβ5L isoforms were incubated with and without the indicated FLAGγ isoforms and, after time for dimerization, were immunoprecipitated with anti-FLAG beads. The Gβ isoform is indicated at the top, as is presence or absence of γ. C, Control beads with only Gγ; st, starting amount of Gγ added to each sample. Results are for (A) Gγ1, (B) Gγ5, (C) Gγ7, and (D) Gγ12.

and ensured that Gβ was not rate limiting for dimer formation. After a 2-h incubation at 37 °C, the samples were immunoprecipitated with anti-FLAG antibody immobilized on agarose beads. These beads bind the FLAG-tagged Gγ subunit, precipitating it, and any Gβ subunit bound to it. The complexes were isolated by centrifugation and separated on SDS−PAGE. The dried gel was subjected to autoradiography on a phosphoimager storage screen, and the immunoprecipitated proteins were visualized and quantitated. Background binding was measured by incubating samples of Gβ with anti-FLAG beads in the absence of Gγ. The results of typical experiments are shown in Figure 2, where all the Gβγ subunits were dimerized with FLAGγ1, FLAGγ5, FLAGγ7, or FLAGγ12. These data were analyzed as described (see Materials and Methods) to quantify the stoichiometry of Gβγ dimers formed. One set of results for all Gβγ combinations is shown in Figure 3. Clearly, there is great variation in dimer formation with different pairs of Gβ and Gγ subunits. Gβ1 and Gβ4 form dimers with all Gγ isoforms. Gβ3s, Gβ5, and Gβ5L do not form significant amounts of dimer with any

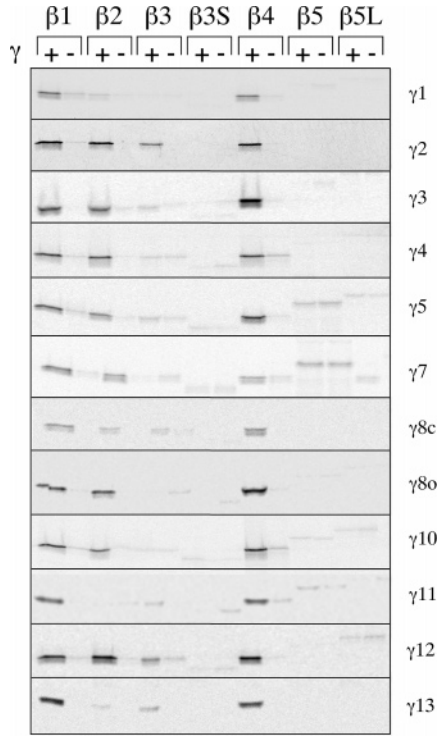

FIGURE 3: Summary of Gβγ dimer formation. In separate experiments similar to that shown in Figure 2, all seven translated Gβ isoforms were incubated with and without a single FLAGγ isoform and, after incubation at 37 °C to allow dimerization to proceed, were immunoprecipitated with anti-FLAG beads. The Gβ isoform is indicated at the top, as is presence or absence of Gγ.

Gγ, while Gβ2 and Gβ3 have Gγ preferences. A graphical summary of the average stoichiometry ratio for each Gβγ pair is shown in Figure 4.

For all dimer combinations the stoichiometry of Gβ/Gγ was less than 1 (Figure 4). This is expected from the nature of the assay, and given the likely complex process of dimer formation, stoichiometries on the order of 0.5:1 Gβ/FLAGγ, compared to the expected value of 1:1, are actually quite reasonable and completely compatible with those previously published results that do exist (*42*, *43*). There was substantial variation, however, among the Gβ isoforms both in their ability and in their efficiency to form productive dimers. Gβ1 and Gβ4 formed dimers with all Gγ subunit isoforms and, generally, with good efficiency. In all cases, the stoichiometry of Gβ1 and Gβ4 dimers formed was significantly greater than 0. Gβ5, Gβ3s, and Gβ5L (data not shown), failed to form significant amounts of dimer with any of the Gγ subunit isoforms, and the average stoichiometry for all of their dimer combinations was less than the 95% or 99% confidence intervals for a mean of 0 (Figure 4). For Gβ2 and Gβ3, results were more varied. For each of them, evidence could be found for significant and variable dimer formation, but there was also a general difference in their efficiency for forming dimers.

Gβ2 formed dimers with most Gγ subunits. It failed to form dimers with two of the three farnesylated γ subunits, Gγ1 and Gγ11, and also did not dimerize with Gγ13 (Figures 3 and 4). We would conclude that it does form a dimer with Gγ8c, but this is with lower efficiency than for the other dimers. Clearly, however, all of the farnesylated Gγ subunits, and Gγ13, form dimers much less efficiently with Gβ2 than do the other Gγ subunit isoforms.

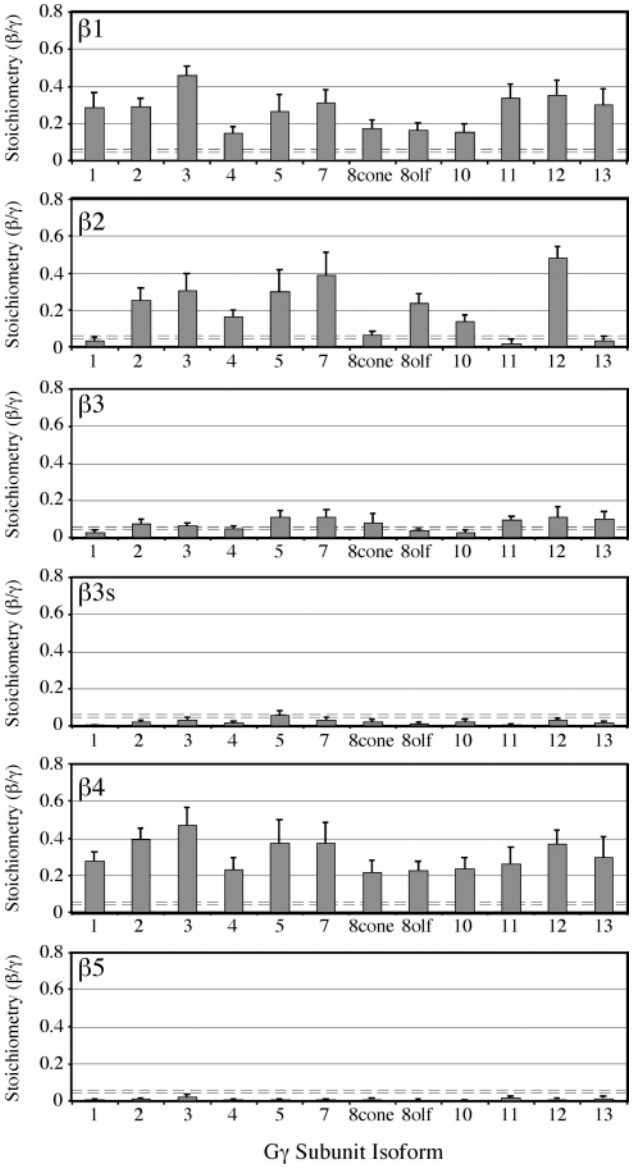

FIGURE 4: Quantitation of G$\beta\gamma$ dimer assay results. Using PhosphorImager analysis and quantitation of the immunoprecipitations as in Figure 3, we determined the stoichiometry of the in vitro binding of each Gβ isoform to each Gγ isoform. At least three separate determinations were done for each combination of Gβ and Gγ. Data are graphed as mean ± SEM from four to nine separate experiments. Data were analyzed by analysis of variance (F = 5.862, df = 71, 356, $p < 0.0001$) (*40*). The dashed lines in the figure represent the upper 99% (upper line) and 95% (lower line) confidence intervals for a mean of 0 based upon the mean square for error from the analysis of variance.

Like Gβ2, the Gβ3 isoform formed productive dimers with some Gγ subunits and not with others, but the overall efficacy for dimer formation in the case of Gβ3 was substantially less than for Gβ2, although still better than for Gβ3s (Figure 4). By the criteria we set, Gβ3 formed dimers with Gγ2, Gγ3, Gγ5, Gγ7, Gγ8c, Gγ11, Gγ12, and Gγ13. Thus, although Gβ2 and Gβ3 share variable dimer formation, they differ in the identity and efficacy with which they select dimer partners.

In the case of Gβ5 and Gβ5L (data not shown), neither formed significant dimers with any Gγ subunits (Figure 4). To further evaluate the Gβ5 construct that we used for these studies, and for a comparison set of dimerization data by an

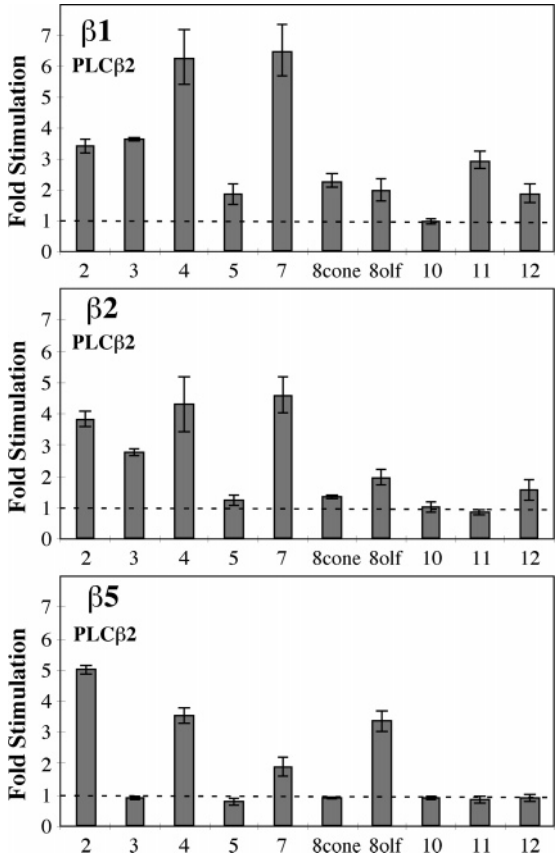

FIGURE 5: Response of IP3 levels in HEK293 cells transfected with a G$\beta\gamma$ stimulated PLCβ2 cDNA and various combinations of different γ subunits with Gβ1, Gβ2, or Gβ5 cDNAs. The dotted line indicates the IP3 levels of cells transfected with the corresponding Gβ cDNA but no Gγ cDNA. Total cDNA was held constant with vector (pcDNA3). Data are mean ± SEM of average values obtained in four independent experiments.

independent approach, we determined the ability of G$\beta\gamma$ combinations to increase the ability of PLCβ2, a G$\beta\gamma$-regulated PLC (*47*, *48*), to generate IP3 in HEK293 cells (Figure 5). Cells were transfected with a PLCβ2 cDNA and with Gβ1, Gβ2, or Gβ5 cDNAs alone or in combination with Gγ2, Gγ3, Gγ4, Gγ5, Gγ7, Gγ8, Gγ8o, Gγ10, Gγ11, and Gγ12 cDNAs (constant total amounts of cDNA). Cellular IP3 levels in response to LiCl treatment were determined by prelabeling the cells with [^{3}H]inositol and isolating [^{3}H]-IP3. In each case, certain Gγ subunit constructs increased the ability of transfected Gβ subunit isoforms to enhance IP3 production by PLCβ2 (Figure 5) by up to 6-fold over control. These data suggest that Gγ2, Gγ4, Gγ7, and Gγ8o are able to interact with Gβ5 sufficiently to stimulate a G$\beta\gamma$-sensitive PLC. In fact, the Gβ5γ2 combination was nearly as effective as the most effective combinations with Gβ1 and Gβ2. These data also indicate that the Gβ5 construct used in the dimerization experiments is functional. These data indicate that the in vitro translation assay provides an important, but not complete, description of the requirements and consequences of G protein $\beta\gamma$ dimer formation and that a stable G$\beta\gamma$ dimer may not be required for all functions.

DISCUSSION

While several studies have addressed the question of G$\beta\gamma$ dimer formation, this is the first to systematically evaluate all possible combinations of these proteins for dimerization.

In our studies, we synthesized the subunits separately and then added them at constant stoichiometry, with an excess of Gβ to Gγ so that we could, theoretically, obtain a 1:1 stoichiometry of Gβ immunoprecipitating with FLAG-Gγ. Despite this, none of the Gβ isoforms achieved this level, although Gβ1 and Gβ4, and for selected dimers with Gβ2, relatively high stoichiomitries were obtained (Gβ/Gγ ~0.5:1). In fact, the most striking thing about the data in Figure 4 is that the general efficacy of dimer formation in this assay varies with the Gβ isoform. Gγ can form dimers with high efficiency with the right partner, but some Gγ isoforms will not make dimers with selective Gβ. Interestingly, G$\beta\gamma$ dimers cannot be assembled in bacteria (*18*), or in an in vitro wheat germ system (*49*), but a Gγ synthesized in the wheat germ system will dimerize with a Gβ synthesized in rabbit reticulocyte lysate, but not the other way around (*49*). This suggests specific requirements for G$\beta\gamma$ dimer formation present in animal systems but absent from bacteria and plants. Our results, because they are under constant conditions and evaluate the range of possible dimers, point to Gβ isoform specific requirements in the rabbit reticulocyte lysate for dimer formation.

We defined a stable dimer, for the purposes of this work, as a Gβ and Gγ that remain associated during the steps of immunoprecipitation, which includes washing with detergent. Although the functionality of all subunit combinations was not directly evaluated, past studies indicate that dimer formation is a stringent, albeit poorly understood, obligatory step for subsequent processing and functional expression (*18*, *42*). One of the important conclusions of this work is that G$\beta\gamma$ dimer formation, at least in vitro, is not an all-or-none process and that there is substantial variability, related to both Gβ and Gγ (but see below), in the efficiency with which dimers are made from different subunit combinations (Figure 4). For a given G$\beta\gamma$ combination, this could not be overcome by increasing the time of dimerization after translation or the ratio of Gβ/Gγ during the dimerization reaction, or by a number of other manipulations of the assay conditions (Wells, Dingus, and Hildebrandt, unpublished observations). This occurs despite the fact that the G$\beta\gamma$ dimer is very stable after synthesis (*14*, *42*), which we also confirmed in additional experiments, where we found that Gβ bound to FLAG-Gγ would not exchange with Gβ in solution (Wells, Dingus, and Hildebrandt, unpublished observations).

There appear to be two different processes going on in the reticulocyte lysate system that determine whether a dimer forms, one related primarily to Gβ and the other related more to Gγ or to the specific interaction between Gβ and Gγ pairs. Thus, the efficiency of dimer formation (Figure 4) relates in an overall way to the Gβ subunit isoform with Gβ4 $\geq$ Gβ1 > Gβ2 > Gβ3 $\gg$ Gβ3s > Gβ5 = Gβ5L, based upon the average stoichiometry of dimers that form. We suggest that this can be explained by the presence of a factor or factors in the lysate that specifically interact(s) with Gβ in an isoform-specific manner, and that this interaction is required for dimer formation. Previous studies have shown that selective point mutants of Gβ1 have a decreased ability to form dimers in vitro, but not in vivo, whereas multiple mutations of this type impair in vivo dimer formation as well (*50*). It was not clear in those studies whether this was a result of requirements for dimerization per se or an effect on Gβ folding or stability. In our case, we are looking at the behavior of different Gβ isoforms, all of which are normally expressed and are not mutants but exhibit a similar kind of behavior, where the efficiency of dimer formation depends both upon the isoform (or mutation profile) and on the context of expression (in vitro or in vivo). Thus, rather than being a property only of Gβ point mutants, variation in efficacy of dimer formation is a normal process associated with different Gβ subunit isoforms.

In contrast to effects on overall dimer efficiency related to Gβ isoform, even when the Gβ makes dimers at high stoichiometry with many Gγ isoforms, it can selectively fail to interact efficiently with other isoforms, as for Gβ2, in particular. This is true even when the specific Gγ, as in the case of Gγ1 and Gγ11, efficiently form dimers with other Gβ isoforms, such as Gβ1 and Gβ4. We suggest that this relates to a specific complementary interaction between Gβ and Gγ partners that is required for dimer formation. In part, sequence determinants of such sites in Gγ have been identified at least for Gγ1 and Gγ2 (*34*, *51*).

One of the things that allowed us to come to a conclusion about the relative ability of Gβ, or Gγ, isoforms to form dimers is the use of a quantitative assay under a defined set of conditions. This, however, is not without its complications. Although we would define dimer formation by immunoprecipitation of Gβ with FLAG-Gγ, there is inherent variability in this assay, as with any other, and we focused on those interactions that efficiently formed dimers. This relates back to our conclusion that dimer formation is not an all-or-none process, but its efficiency depends on the interacting partners. Consequently, it is difficult to come up with a totally satisfactory definition of dimer formation. For example, it may be possible (see below) to demonstrate that specific Gβ/Gγ combinations do form dimers, but that this is at very low efficiency and only observable under conditions where background is stringently minimized. The assay used here was not designed to do this but ustead to focus on those dimers that readily form at significant stoichiometry under a constant set of conditions. Consequently, for the sake of discussion, we have used an admittedly arbitrary statistical definition of dimer formation based upon ANOVA of the pooled results in Figure 4. We would argue that we only have evidence for dimer formation for dimers whose stoichiometry is statistically greater than 0. This is indicated in Figure 4 as the two dashed lines for the 95% and 99% confidence intervals for a difference from 0, based upon the mean square error for all of our data. In an absolute sense, this means that our data will define those combinations that do form dimers but cannot specifically exclude the possibility that dimers can be formed of other combinations, but just at very low efficiency. It remains to be determined what are the functional consequences or meaning of low efficiency; whether this means that there are specific places or times that low-efficiency dimers can be made at high efficiency, and that dimer formation is a regulated process, or if this is a mechanism that limits the amount of a specific dimer pairs in cells. In particular, there are clear differences in the requirements for formation of some dimer combinations, such as Gβ2 with Gγ1 versus Gγ2, that affect the efficacy of dimer assembly under some circumstances, such as in vitro synthesis by rabbit reticulocyte lystate.

Although we have adopted here a statistical evaluation of dimer formation, this not only allows us to compare the

compatibility of dimers within our experiments, but also allows us to compare our results to the conclusions of other studies. For example, Gβ1 and Gβ4 form dimers with all known Gγ subunits. In fact, using this method of assaying dimer formation, Gβ4 had greater efficacy for forming specific dimers than did Gβ1. These two subunits may be widespread components of cellular G$\beta\gamma$ signaling mechanisms. While this work was in progress, other work was published on the ability of Gβ4 to form dimers with Gγ subunits (*30*). Our results are, for the most part, compatible with those, although that work did not include Gγ8o and we observed quantitatively better association of Gβ4 with Gγ1 and Gγ11.

Gβ5 and Gβ5L do not form significant dimers in our assay with any known Gγ subunit. There is disagreement about the physiological role of G$\beta\gamma$ dimers containing Gβ5 (*17*, *36*, *52*), which might be explained by different assay conditions. For example, it has been suggested that detergents modulate the ability to isolate G$\beta\gamma$ dimers containing Gβ5 (17). Although we have not exhaustively investigated these effects, omission of detergents from our dimerization assay did not change the behavior of Gβ5 (Dingus, Wells, and Hildebrandt, unpublished observations). This assay (in vitro dimerization) and others, particularly those based upon cellular expression, may not generate similar conclusions about dimer formation. For example, we demonstrate, as have others (*53*, *54*), that Gβ5, when expressed in HEK293 cells, stimulates a G$\beta\gamma$-responsive phospholipase C to a greater extent when expressed with specific Gγ subunits, suggesting functional dimer formation. Similar data have been found for Gβ3s (*29*). In addition, Gγ subunit partners (2, 3, 4, 5, and 7) have been observed for Gβ5 in a yeast two-hybrid screen (*28*). One explanation for these observations would be that Gβ5 (and perhaps Gβ3s) forms quasi-stable dimers within cells that have some functional behavior but are not stable to isolation. If so, however, the biological role of these dimer sets in G protein signaling should be qualitatively different from that of dimers containing other Gβ isoforms.

Ours is the first study to fully define the Gγ binding specificity of the Gβ2 and Gβ3 isoforms. Gβ2 and Gβ3 each form dimers with a subset of Gγ subunits. Significantly, they have different apparent preferences for Gγ partners. Gβ2 does not form dimers efficiently with the farnesylated γ subunit family, Gγ1, Gγ8c, and Gγ11, or with Gγ13, although it did have a significant interaction with Gγ8c. Gβ3 does form dimers with Gγ8c, Gγ11, and Gγ13, although not significantly with Gγ1. These results are compatible with previously reported co-localization studies of Gβ3 with Gγ8c (*55*), as well as more recent studies of co-immunoprecipitation (*29*). Unlike Gβ2, Gβ3 failed to form dimers with Gγ4, Gγ8o, or Gγ10. These results corroborate the original observation that Gβ2 and Gγ1 do not form dimers (*34*) and that the similar isoform Gγ11 also does not (*56*). In part, the biochemical (sequence) determinants of this specificity for Gβ1 versus Gβ2 has been defined (*33*, *57*), and it is possible that Gβ2 and Gβ3 have more specialized roles in cells, in comparison to Gβ1 and Gβ4, which are thought to be more widely distributed (*9*, *30*).

The Gβ3s isoform is significant in that it originates from a polymorphism of the human Gβ3 gene. This polymorphism results in a gene product lacking 41amino acids, which results in a Gβ3s protein missing one of the seven blades of the characteristic β propeller (*58*). Prevalence of this polymorphism in human populations has been associated with predisposition to hypertension (*58*). The β-propeller that characterizes Gβ structure is a common protein fold, and this structure can accommodate five to eight, or more, blades (*59*). It is remarkable that deletion of part of exon 9 in Gβ3, which includes residues of two adjacent blades, would result in a protein with exactly one blade equivalent missing from the seven-bladed β-propeller of native Gβ subunits. In our assays, we did not find that Gβ3s efficiently formed dimers, although there was a suggestion of dimerization with Gγ5 and Gγ12. Rosskopf et al. (*29*), in assays similar to ours, reported that Gβ3s does form dimers with Gγ5, Gγ8c, and Gγ12, but at a very low level compared to Gβ3, or the other Gβ subunits, and that dimer formation was not seen in trypsin protection assays as an alternative approach. Differences in our results and theirs likely relate in part to differences in sensitivity originating from the design of these respective projects. As discussed above, under conditions where background is minimized and sensitivity is maximized, it is likely that Gβ3s can be shown to form stable dimers with at least selective Gγ subunits. The efficiency, and hence stoichiometry, of this assembly, however, is very low when compared to other Gβ isoforms, such as Gβ1, Gβ2, and Gβ4, in particular, but is notably less than for its related primary splice variant of Gβ3 as well.

Our cellular results evaluating dimerization (Figure 5) and our biochemical results in reticulocyte lysates (Figure 4) do not entirely agree. Thus, we could not demonstrate Gβ5 dimers in vitro, but Gβ5 clearly has effects on PLCβ that are dependent upon its coexpression with specific Gγ isoforms including Gγ2, Gγ4, Gγ7, and Gγ8olf. Others have observed analogous results (*53*, *54*). In other cases, such as dimers of Gβ1 and Gβ2 with Gγ10, dimers are clearly formed in vitro, but we did not observe effects on PLCβ2. A converse example is that of dimers made of Gβ3 with Gγ4. Whereas, Kleuss et al. (*11*−*13*) used antisense injected into GH3 cells to infer that Gβ3γ4 dimers specifically mediate the effects of carbachol on calcium transients via m4 muscarinic receptors, our statistical evaluation did not provide evidence that these form dimers in in vitro translation assays. These discrepancies likely have multiple explanations. For example, a less stringent statistical evaluation would support the existence of Gβ3γ4 dimers, and there are likely to be effector-specific differences in the ability of different dimer combinations to stimulate PLCβ, although this has not been rigorously evaluated here. In addition, our results support the idea that G$\beta\gamma$ dimer assembly is an active process that requires participation of constituents that are rate-limiting in reticulocyte lysates and that especially recognize or select different Gβ isoforms. Similarly, in a cellular context, similar processes or events may facilitate or direct the synthesis of specific G$\beta\gamma$ dimers and, in so doing, modulate the signaling capabilities of the cell. Thus, G$\beta\gamma$ dimer assembly is a likely point of regulation of signaling potential, dependent upon the interaction of specific proteins with selected subunits of the dimer. Such phenomenon may in fact represent part of the effect of phosducin-like protein (PhLP) on G$\beta\gamma$ expression intact cells (*60*).

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