G Protein β_5 Subunit Interactions with α Subunits and Effectors†

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ABSTRACT: When the β_5 (short form) and γ_2 subunits of heterotrimeric G proteins were expressed with hexahistidine-tagged α_i in insect cells, a heterotrimeric complex was formed that bound to a Ni-NTA-agarose affinity matrix. Binding to the Ni-NTA-agarose column was dependent on expression of hexahistidine-tagged α_i and resulted in purification of $\beta_5\gamma_2$ to near homogeneity. Subsequent anion-exchange chromatography of $\beta_5\gamma_2$ resulted in resolution of β_5 from γ_2 and further purification of β_5. The purified β_5 eluted as a monomer from a size-exclusion column and was resistant to trypsin digestion suggesting that it was stably folded in the absence of γ. β_5 monomer could be assembled with partially purified hexahistidine-tagged γ_2 in vitro to form a functional dimer that could selectively activate PLC β2 but not PLC β3. α_o-GDP inhibited activation of PLC β2 by $\beta_5\gamma_2$ supporting the idea that $\beta_5\gamma_2$ can bind to α_o. β_5 monomer and $\beta_5\gamma_2$ only supported a small degree of ADP ribosylation of α_i by pertussis toxin (PTX), but β_5 monomer was able to compete for $\beta_1\gamma_2$ binding to α_i and α_o to inhibit PTX-catalyzed ADP ribosylation. These data indicate that β_5 functionally interacts with PTX-sensitive GDP α subunits and that β_5 subunits can be assembled with γ subunits in vitro to reconstitute activity and also support the idea that there are determinants on β subunits that are selective for even very closely related effectors.

Signal transducing heterotrimeric G proteins consist of three subunits: α, β, and γ. Activation by G protein coupled receptors results in binding of GTP to the α subunits and dissociation of tightly associated β and γ subunits. Both α and $\beta\gamma$ subunits interact with downstream targets to initiate a variety of signal transduction pathways (*1*). There are multiple isoforms of α, β, and γ subunits with 20 different α subunit isoforms and 12 different γ subunit isoforms (*2*, *3*). The β subunits have six different isoforms: β_{1-5} and a long form of β_5. While β subunit isoforms 1−4 have a very high degree of sequence identity (85%), β_5 is clearly different with only 50% homology to the other β subunits (*4*).

It has recently been reported that the β_5 isoform of β subunits of G proteins forms a complex with regulators of G protein signaling (RGS proteins)[1] that contain a G protein γ subunit-like domain (GGL) (*5*, *6*). When cotranslated in vitro the short and long versions of β_5 bound to HA-tagged RGS7 and RGS11 but not to γ_1 or γ_2 (*5*). Purification of β_5 from retina resulted in purification of a complex that contained β_5 and RGS7 (*7*). Purification of RGS9 from retina resulted in the copurification of β_5 (*8*). All of these results indicate that β_5 forms a complex with RGS proteins containing GGL domains and the majority of the β_5 is in a complex with these proteins in the retina. These very interesting results are in direct contrast to the dogma for heterotrimeric G proteins where β subunits only form tight complexes with γ subunits. A major remaining question is what is the function of β_5 protein in the β_5/RGS protein complex.

Other studies of purified $\beta_5\gamma_2$ complexes show that this dimer is capable of selective activation of PLC β2 but not adenylate cyclase (*9*). It has also been reported that $\beta_5\gamma_2$ selectively interacts with α subunits of the Gq family. This is the first example of a β subunit with selectivity toward particular effectors and α subunits and supports the hypothesis that there are different determinants on β subunits for different effectors. In the experiments presented here, we examined the interactions of the β_5 protein, in the presence and absence of γ subunits, with α subunits and effectors. We found that the $\beta_5\gamma_2$ could be assembled in vitro and was capable of functional interactions with G protein α_i and α_o subunits and PLC β2 but not PLC β3. The results have implications with regard to how $\beta\gamma$ subunits function in general as well as defining G protein binding properties of β_5 that may affect the function of β_5/RGS complexes.

EXPERIMENTAL PROCEDURES

Materials. Baculoviruses encoding hexahistidine (6-His)-tagged α_{i1} (6-His-α_i) and wild-type γ_2 and N-terminally 6-His-tagged γ_2 subunits were obtained from Alfred Gilman's (University of Texas Southwestern Medical Center) laboratory. β_5-specific antibody (SGS) and a cDNA encoding the short form of G β_5 was kindly provided by Dr. William Simonds (NIH). Purified myristoylated $G\alpha_o$ was kindly provided by Maurine Linder (Washington University) and was stored in 50 mM NaHepes, pH 8.0, 1 mM EDTA, 1

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[1] Abbreviations: RGS protein, regulator of G protein signaling protein; PIP_2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; PLC, phospholipase C; G protein, GTP binding protein; BSA, bovine serum albumin; GDP, guanosine diphosphate; $C_{12}E_{10}$, polyoxyethylene 10-laurylsulfate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; 6-His, 6-histidine tag; PTX, pertussis toxin.

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mM DTT. Phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids, Ni-NTA-agarose was from Qiagen, and pertussis toxin (PTX) was from List Biologicals.

Expression of G Protein Subunits. A baculovirus vector for expression of β_5 was constructed from the β_5 cDNA using the FASTBac system (Gibco) according to the manufacturer's instructions. For expression of $\beta_5\gamma_2$, 1 L of Sf9 cells at 2.5×10^6 cells/mL was simultaneously infected with the 6-His-α_{i1}, β_5 and γ_2 baculovirus and harvested by centrifugation after 60−70 h. For expression of 6-His-tagged γ_2 subunits alone, a baculovirus expression vector for N-terminally 6-His-tagged γ_2 subunits (*10*) was used to infect 200 mL of Sf9 cells at 2.5×10^6 cells/mL and harvested by centrifugation 48 h later. Harvested cells were frozen in liquid N_2 and stored at −80 °C until they could be processed further.

Purification of β_5 and γ_2 Subunits. β_5 and γ_2 subunits were purified by modification of a published procedure (*10*). Cells were suspended in 15 mL of lysis buffer [50 mM NaHepes, pH 8.0, 0.1 mM EDTA, 100 mM NaCl, 3 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μM GDP, and a cocktail of protease inhibitors including 133 μM phenylmethanesulfonyl fluoride, 21 μg/mL Nα-*p*-tosyl-L-lysine chloromethyl ketone, 21 μg/mL tosylphenylalanyl chloromethyl ketone, 0.5 μg/mL aprotinin, 0.2 μg/mL leupeptin, and 1 μg/mL soybean trypsin inhibitor (SBTI)]. The suspended cells were repeatedly (four times) frozen in liquid N_2 and thawed in a 37 °C water bath. Membranes were collected by centrifugation at 40 000 rpm in a Ti60 rotor for 45 min. The membranes were suspended in 50 mM NaHepes, pH 8.0, 50 mM NaCl, 3 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μM GDP and the protease inhibitor cocktail (without SBTI). Cholate (1%) was added for 1 h at 4 °C to extract the membrane associated proteins followed by centrifugation at 100000*g* for 1 h. The supernatant was diluted 5-fold with 20 mM NaHepes, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μM GDP, 0.5% polyoxyethylene 10-laurylsulfate ($C_{12}E_{10}$) and protease inhibitors (without SBTI) and applied to a 4-mL column of nickel-NTA-agarose. Under these conditions, the $\alpha_i\beta_5\gamma_2$ heterotrimer bound to the resin via the 6-His tag on the α subunit. The column was washed (W1) with 100 mL of 20 mM NaHepes, pH 8.0, 300 mM NaCl, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μM GDP, 0.5% $C_{12}E_{10}$, 5 mM imidazole and protease inhibitors (without SBTI), followed by 5 mL (W2) of the same buffer with 1% cholate instead of $C_{12}E_{10}$. $\beta_5\gamma_2$ subunits were eluted in W2 solution containing 30 μM $AlCl_3$, 10 mM NaF and 10 mM $MgCl_2$ (AlF_4^-). Subsequent analysis indicated that the 1% cholate was initiating the elution not the AlF_4^-. Fractions were analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) followed by staining with Coomassie Brilliant Blue or immunoblotting with primary antibodies that recognize β_5 (SGS) and γ_2 (X263) subunits. X263 has been shown to recognize γ_2, γ_3 and γ_7 (*11*).

Elution 1 from the Ni-NTA-agarose column, which contained the greatest amount of β_5 subunit protein, was diluted with 2 volumes of buffer A (20 mM Tris-HCl, pH 8.0, 1 mM DTT, and 1% octylglucoside) and applied to a Mono Q anion-exchange column (Pharmacia) that had been equilibrated with buffer A. The column was washed with 10 mL of buffer A and eluted with a 30-mL linear gradient to 400 mM NaCl in buffer A. Fractions were analyzed by SDS−PAGE followed by staining with Coomassie Brilliant Blue or immunoblotting with a γ subunit-specific antibody (X263). Fractions containing the highest amounts of pure β_5 were aliquoted, frozen in liquid N_2 and stored at −80 °C. Attempts to concentrate the protein resulted in significant protein losses and repeated freeze thawing led to losses in protein activity. β_5 subunit protein concentrations were determined with an Amido Black assay (*12*).

Purification of 6-His-Tagged γ Subunits. Membrane fractions were prepared from 200 mL of Sf9 cell culture, cholate extracted, diluted and loaded onto a 1-mL Ni-NTA column as described above. The column was washed with 25 mL of 20 mM NaHepes, pH 8.0, 300 mM NaCl, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μM GDP, 0.5% $C_{12}E_{10}$, 5 mM imidazole and the protease inhibitor cocktail without SBTI followed by elution with 4 consecutive 1-mL volumes of buffer containing 20 mM NaHepes, pH 8.0, 50 mM NaCl, 1% cholate, 50 mM $MgCl_2$ and 150 mM imidazole. The majority of the protein eluted in the second fraction but the third 1-mL fraction contained the cleanest protein at about 50% purity and was used for all of these studies. The protein was not processed further before use due to problems with protein yields. γ_2 protein concentration was estimated relative to a standard curve generated with known amounts of purified $\beta_1\gamma_2$ protein followed by immublotting with γ-specific antibody (X263) yielding 1 mL of 11 μM γ_2.

Purification of Other G Protein Subunits and PLC. Expression and purification of $\beta_1\gamma_2$ from Sf9 cells has been previously described (*10*, *13*). The final preparation was concentrated and the buffer exchanged by binding to a 0.5-mL hydroxyapatite column and eluting in 20 mM NaHepes pH 8.0, 1 mM DTT, 100 mM NaCl, 1% octyl glucoside and 200 mM KPi pH 8.0. Myristoylated α_{i1} was purified from *Escherichia coli* coexpressing α_{i1} and *N*-myristoyl transferase according to published procedures (*14*) and stored in 50 mM Tris, pH 8.0, 2 mM DTT, 50 μM GDP, 150 mM NaCl. For the experiment in Figure 6B the α_o that was used was purified from bovine brain according to Sternwies and Robishaw (*15*). All other experiments with α_o used protein kindly provided by Maurine Linder. 6-His PLC β2 and 6-His PLC β3 were expressed and purified from Sf9 cells as has been previously described (*13*) except after Ni-NTA chromatography, the PLC was bound to and eluted from a 1-mL HiTrap Heparin Sepharose (Amersham Pharmacia Biotech) column with a gradient from 0 to 800 mM NaCl in 20 mM NaHepes, pH 8.0, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and protease inhibitors without SBTI.

ADP Ribosylation of Gα_{i1} and Gα_o. ADP ribosylation assays were performed as has been described (*16*). Briefly, α_{i1} was mixed with various concentrations of $\beta_1\gamma_2$, β_5 or $\beta_5\gamma_2$ on ice in 15 μL of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.025% $C_{12}E_{10}$. Reactions were initiated by the addition of 25 μL of a starter solution which yielded the following final concentrations: 20 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 2 mM DTT, 100 μM GDP, 2.5 μM NAD, 0.5 mM dimyristoylphosphatidylcholine (Sigma), 5.0 μg/mL PTX, 750 000 cpm/assay [^{32}P]NAD in a total reaction volume of 40 μL. All reactions were transferred to a 30 °C water bath and terminated after 20 min by the addition of 500 μL of ice-cold 30% trichloroacetic acid. The precipitated proteins

were collected on nitrocellulose filters (Schleicher & Schuell, BA85, 25 mm, 0.45-μm pore size) and washed with 14 mL of ice-cold 6% trichloroacetic acid; filters were air-dried and analyzed by liquid scintillation counting.

PLC Assays. Phospholipid vesicles were prepared as previously described (*13*) so that the final reaction (60 μL) contained 50 μM PIP_2 and 200 μM PE and [^{3}H-inositol]-PIP_2 at 6−8000 cpm/assay. 0.8 nM PLC β2 was mixed with $\beta_1\gamma_2$, β_5 and/or 6-His γ_2 or vehicle and incubated on ice for 1 h to allow for $\beta_5\gamma_2$ assembly. The PLC reactions were initiated by addition of 2.8 mM $CaCl_2$ (100 nM free Ca^{2+}) and the samples were transferred to a 30 °C water bath for 10 min. Reactions were terminated by the addition of 200 μL of 10% trichloroacetic acid followed by addition of 100 μL of 10 mg/mL BSA. Precipitated proteins and lipids were centrifuged and 300 μL of supernatant was analyzed by liquid scintillation counting. In all assays, blank solutions corresponding to the storage buffers for each of the proteins were included such that all the reactions had exactly the same solution components. Because different subunits were added in the PLC assays at varying concentrations, the exact solution conditions accounting for the buffer components added with each subunit are not specified for each reaction.

RESULTS

Partial Purification of $\beta_5\gamma_2$ by Binding to 6-His-Tagged α_{i1}. 6-His-tagged-α_{i1}, β_5, and γ_2 were extracted with detergent from the membrane fraction from Sf9 cells expressing these proteins, and the resulting extract was passed through an Ni-NTA-agarose column (see Experimental Procedures for details). In order for $\beta_5\gamma_2$ to associate with the Ni-NTA-agarose it would have to do so through association with α_i. After washing the column extensively with various detergents and high salt to eliminate nonspecific association, $\beta_5\gamma_2$ was eluted as a nearly homogeneous preparation (Figure 1A, top). Analysis of the elutions with antibodies specific for β_5 and γ shows that most of the β_5 and γ_2 protein was retained on the column (bottom panels, Figure 1A). During this step, β_5 is resolved from endogenous Sf9 $\beta\gamma$ with the majority of β_5 eluting in fraction 1 while Sf9 β is enriched in the later fractions.

To determine the requirements for elution and confirm the specificity of binding of $\beta_5\gamma_2$ to α_i, we performed Ni-NTA chromatography on two separate extracts both containing $\beta_5\gamma_2$, but one extract was from cells expressing 6-His-α_i and the other extract was from cells not expressing 6-His-α_i (Figure 1B.). Equal amounts of β_5 subunit were loaded on each column [Figure 1B, bottom panel (Ld)]. The level of β_5 washes to baseline in $C_{12}E_{10}$ (lane $C_{12}E_{10}$ 2) followed by specific elution with 1% cholate (lane cholate 1). While a very small amount of β_5 associated with the column in the absence of 6-His-α_i (not detectable by Coomassie staining, top panel Figure 1B, and barely detectable by ECL Western blotting, bottom panel Figure 1B) a much larger proportion of the applied β_5 associated with the column in the presence of α subunits.

As can be seen in Figure 1A some β_5 protein begins to elute in a cholate wash (W2) that is used to exchange the protein into 1% cholate. This suggests that the addition of cholate could actually be initiating the elution, not the AlF_4^- that is included in the elution buffer. For the experiment in

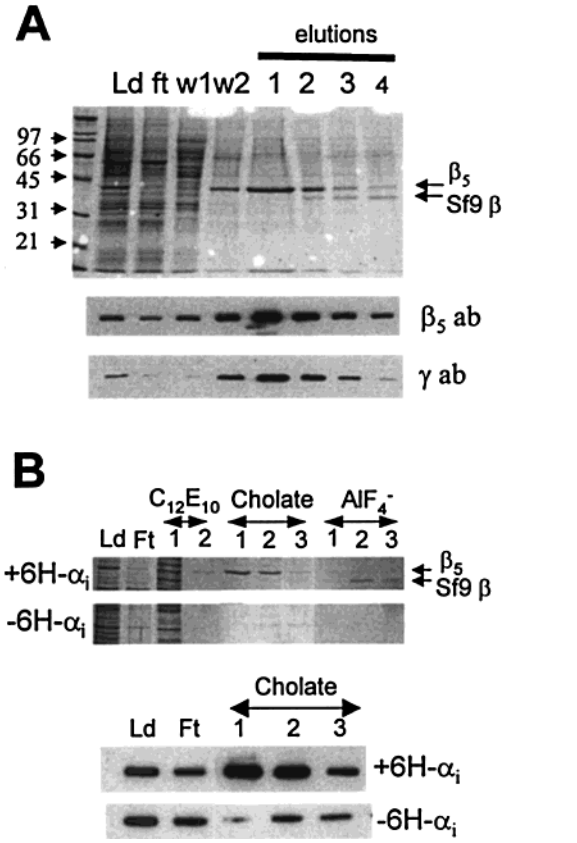

FIGURE 1: Ni-NTA-agarose chromatography of $\beta_5\gamma_2$ bound to 6-His tagged α_{i1}. A. Coomassie stained 12% SDS−PAGE gel of fractions from chromatography on Ni-NTA-agarose: Ld, load prior to chromatography; Ft, sample that flowed through the column and did not bind; W1 and W2, washes as indicated in Experimental Procedures; elutions are sequential fractions of elution buffer. The band just below the β_5 in fractions 2−4 is Sf9 β subunit. γ subunits are in the dye front and cannot be seen. The lower panels are immunoblots of the same fractions of β_5 and γ_2. For resolution of the γ subunit a 17% SDS−PAGE gel was used. This experiment was performed at least 5 times with very similar results. B. Coomassie stained SDS−PAGE gel of fractions from Ni-agarose loaded with extracts of 1 L of Sf9 cells each expressing β_5 and γ_2 with or without 6-His-tagged α_i expressed. The bottom panels show Western blots with anti-β_5 antibody confirming that equal amounts of β_5 were loaded on the two columns. $C_{12}E_{10}$ fractions are sequential 30-mL washes with 0.5% $C_{12}E_{10}$; cholate fractions are sequential 5-mL washes with 1% cholate. AlF_4^- fractions are elutions performed immediately after the cholate washes where AlF_4^- was added to the 1% cholate buffer. Solution compositions were the same as stated for panel A.

Figure 1B the protocol was modified to elute with 1% cholate followed by addition of AlF_4^-. All of the β_5 protein eluted with cholate, while subsequent application of AlF_4^- in the same buffer eluted endogenous Sf9 β but no additional β_5. This procedure routinely elutes $\beta_1\gamma_2$ subunits specifically with AlF_4^- (*10*). Since Sf9 β subunit elutes with AlF_4^- it indicates that our experimental conditions are effective at revealing AlF_4^--dependent dissociation of some G protein subunits. We were unable to establish conditions where addition of AlF_4^- was able to elute the $\beta_5\gamma_2$ complex. Performing the procedure under low-detergent conditions (0.2% cholate) or 0.5% $C_{12}E_{10}$ (at either room temperature or 4 °C) did not allow dissociation in the presence of AlF_4^-, while 1% cholate caused elution in the absence of AlF_4^-. Similar results were obtained when 6-His-α_q was immobilized, although α_q was expressed to a much lower level

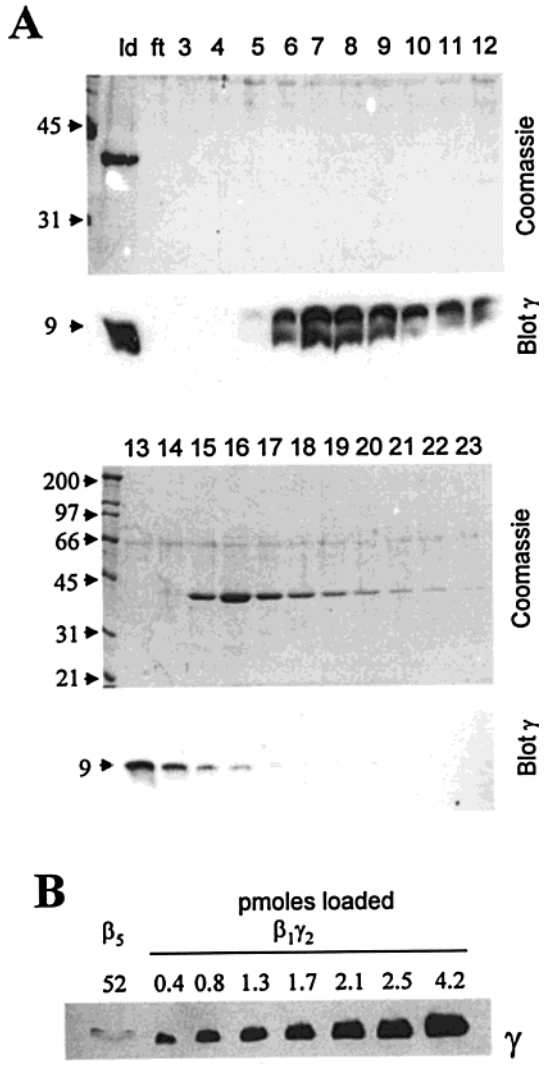

FIGURE 2: Mono Q anion-exchange chromatography leads to resolution of β_5 and γ subunits. A. The Mono Q column was run as in Experimental Procedures and the numbers indicate the numbers of the 1-mL fractions collected from a 30-mL gradient. A 12% SDS−PAGE gel was run for resolution of β_5 and staining, and a separate 17% SDS−PAGE gel was run for resolution of γ and subsequent transfer to nitrocellulose and Western blotting. B. Western blot of the final preparation of β_5 confirming less than 1% contamination with γ subunits. This experiment was performed at least 5 times with similar results.

than 6-His-α_i precluding a direct comparison (data not shown). This lack of dissociation by AlF_4^- could be due to an unusual property of β_5 or just that we did not find the correct specific elution conditions. Nevertheless, $\beta_5\gamma_2$ could be purified to near homogeneity in one step based on its association with 6-His-tagged α_i, in a procedure that is significantly more stringent than coimmunoprecipitation, suggesting that the interaction between $\beta_5\gamma_2$ and α_i is specific.

Separation of β_5 and γ_2 by Mono Q Ion-Exchange Chromatography. β_5 and γ_2 eluted together from the 6-His-α_i/Ni-NTA-agarose column, but the preparation was not completely pure and contained a small amount of endogenous Sf9 β (Figure 1A). Elution 1 (E1) containing the enriched β_5 from the 6-His-α_i/Ni-NTA-agarose column was further purified by Mono Q ion-exchange chromatography (Figure 2A). Fractions eluting from the Mono Q column were analyzed by Western blotting and Coomassie staining. Comparison of the Western blots for γ_2 and Coomassie stain for β_5 show that γ_2 elutes early in the gradient and β_5 elutes later. The apparent doublet of the γ subunit in this figure is due to a gel artifact that sometimes occurs during γ subunit electrophoresis. It is not clear from this data whether the β_5 subunit dissociated from γ_2 during cholate elution from the 6-His-α_i /Ni-NTA-agarose column and was subsequently resolved by ion-exhange chromatography or whether they came apart during ion-exchange chromatography. Separation of β_5 from γ_2 is consistent with observations by others that the $\beta_5\gamma_2$ complex is unstable in some detergents (*17*), but in our studies we were able to extract the $\beta_5\gamma_2$ complex in 1% cholate and purify the complex using 6-His-α_i/Ni-NTA-agarose chromatography in the presence of 0.2% cholate and 0.5% $C_{12}E_{10}$. Analysis of the fractions containing β_5 by Coomassie staining (Figure 2A) showed that it was pure, and immunoblot analysis indicates the β_5 preparation contains less than 1% γ_2 (Figure 2B).

Analysis of β_5 by Trypsin Digestion and Size-Exclusion Chromatography. To characterize the properties of the isolated β_5, we analyzed its folding status as well as its aggregation state. To test whether the β_5 subunit was folded, we assessed its resistance to trypsin digestion. If the protein were unfolded we would expect multiple trypsin cleavage sites to be exposed. If native purified brain $\beta\gamma$ subunits are treated with trypsin they are cleaved only at Arg 129 into 24- and 14-kDa fragments (*18*) indicating that multiple potential trypsin cleavage sites are inaccessible in the folded protein. We reproduced this result with $\beta_1\gamma_2$ (Figure 3). Under the same conditions β_5 was cleaved from a 39-kDa fragment to a 37-kDa fragment that was resistant to further digestion. γ subunits did not influence the sensitivity to trypsin (Figure 3). The amino acid in the position equivalent to Arg 129 of β_1 is methionine in β_5, which could explain some of the difference in the trypsin cleavage patterns between β_5 and β_1. Overall, the result suggests that β_5 is tightly folded and that multiple trypsin cleavage sites are inaccessible to trypsin.

To determine if the β_5 protein was aggregated in the absence of γ subunits, we performed size-exclusion analysis on a Superdex 200 FPLC column. The β_5 protein eluted as a single sharp peak of absorbance at 280 nm that corresponded to the elution position for carbonic anhydrase (29 kDa) (Table 1). No protein eluted in the void volume. The elution position was confirmed by gel electrophoresis of fractions collected from the gel filtration column followed by Coomassie staining to visualize the β subunit (not shown). Based on the M_r of the standards that were resolved, a monomer would be clearly separated from a dimer or any other higher molecular weight oligomer. These results are consistent with β_5 existing as a stably folded monomer in the absence of γ subunits.

β_5 Functionally Assembles with γ_2 in Vitro To Activate PLC β2. To determine if separated β_5 could functionally reassociate with γ, we tested the ability of the combined proteins to activate PLC β2. It has previously been shown that when β_5 and γ_2 are coexpressed in COS-7 cells they are capable of activating PLC β2 (*19*, *20*). As shown in Figure 4, a small activation was observed with β_5 monomer, and there was no effect of partially purified γ, but β_5 and γ_2 together significantly stimulated PLC β2 (Figure 4, top). This indicates that isolated β_5 is properly folded and is capable of assembling with isolated γ_2. The assembled subunits are

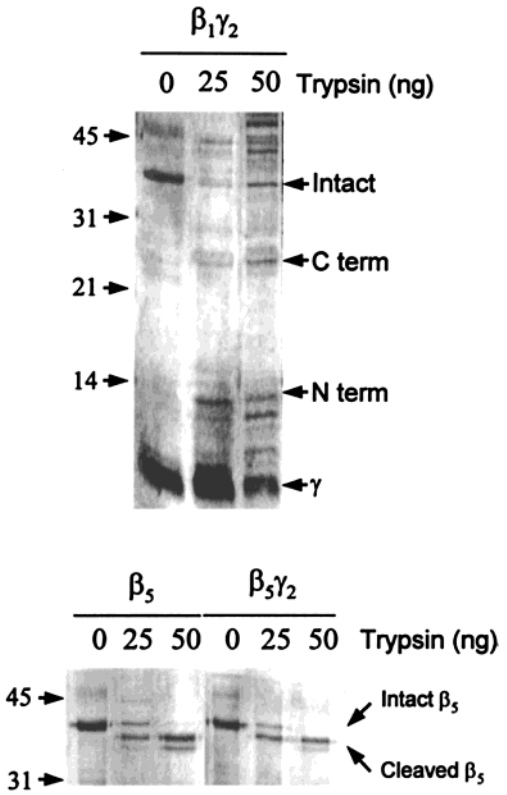

FIGURE 3: Purified β_5 is resistant to trypsin degradation. 500 ng of either $\beta_1\gamma_2$, β_5 alone, or β_5 assembled with γ was incubated for 30 min at 30 °C in the presence of the indicated amounts of trypsin in 18 μL of 10 mM NaHepes, pH 8.0, 30 mM NaCl, 0.3 mM DTT, 0.3% octylglucoside and 70 mM K-phosphate buffer, pH 8.0. Reactions were stopped by addition of PMSF. The resulting proteins were resolved on a 17% polyacrylamide gel and stained with silver. The C-terminal fragment of β_1 is reverse stained by silver on this gel.

Table 1: FPLC Superdex 200 Gel Filtration Chromatography of Purified β_5 Subunit[a]

protein	M_r (kDa)	vol (mL)
β_5	38.7	17.5
standards		
blue dextran	2000	10
bovine serum albumin	66	15.5
carbonic anhydrase	29	17.5
cytochrome *c*	12.4	20.5

[a] Superdex 200 HR10/30 column was equilibrated with 50 mM NaHepes, pH 7.2, 100 mM NaCl and 2 mM DTT at of 0.5 mL/min. Elution positions for all the samples were monitored at 280 nm. The elution position for β_5 was confirmed by gel electrophoresis and Coomassie staining.

slightly less effective than purified $\beta_1\gamma_2$ in activating PLC β2.

We also tested the ability of assembled $\beta_5\gamma_2$ to activate PLC β3. Under conditions where $\beta_1\gamma_2$ gave 100-fold stimulation of PLC activity, $\beta_5\gamma_2$ could not activate PLC β3 (Figure 4, bottom). The basal activity of PLC β3 is much lower than that of PLC β2, but the enzyme is very responsive to $\beta\gamma$ showing at least 100-fold activation by $\beta\gamma$. The scale for the activity of PLC β3 in the presence of β_5 or γ_2 alone was greatly expanded to demonstrate that no significant activation was occurring with these subunits. These assays were performed at the same time as the PLC β2 experiments where the same subunits gave appreciable activation. This

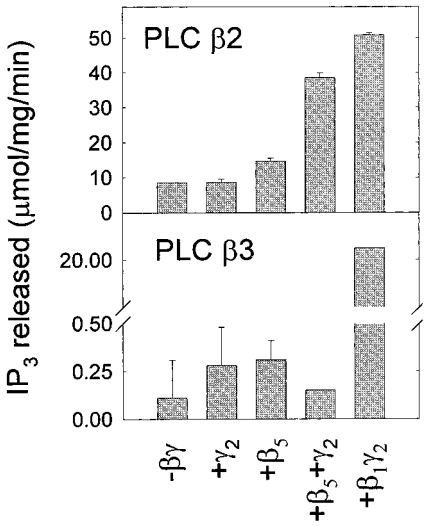

FIGURE 4: Stimulation of PLC activity with assembled β_5 and γ_2. 100 nM β_5 and 300 nM γ_2 were added separately and together to PLC β2 and PLC β3 and assayed as described in Experimental Procedures. $\beta_1\gamma_2$ was also added at 100 nM. Bars are standard errors from duplicate determinations. This experiment was repeated 5 times with similar results.

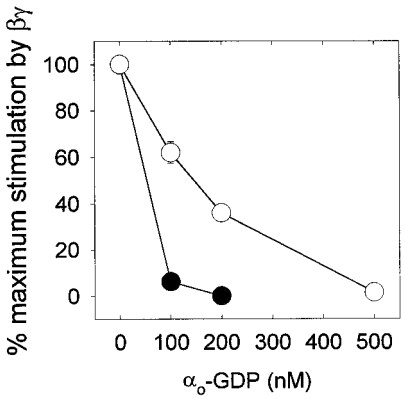

FIGURE 5: $\beta_5\gamma_2$ stimulation of PLC β2 is inhibited by GDP-liganded α subunits. 100 nM β_5 and 300 nM γ_2 (○) or $\beta_1\gamma_2$ (●) were mixed with the indicated concentrations of recombinant myristoylated α_o and used to activate PLC β2 as in Figure 4. The final concentration of GDP was 10 μM in all assays. Bars are standard errors from duplicate determinations. These experiments were performed 3 times with similar results.

result with a native β subunit confirms previous mutational studies suggesting that the binding determinants on $\beta\gamma$ subunits for activation of different effectors are not universal.

α_o Subunits Bind to $\beta_5\gamma_2$ To Prevent Activation of PLC β2. The ability of $\beta\gamma$ subunits to activate effectors is blocked in the presence of near stoichiometric amounts of α-GDP (*21*, *22*). This is thought to occur because α subunits bind to the $\beta\gamma$ subunits and occupy a site that overlaps the effector binding site (*23*). If $\alpha_{i/o}$-GDP blocks the ability of $\beta_5\gamma_2$ to activate effectors, it is further evidence that $\beta_5\gamma_2$ binds to $\alpha_{i/o}$ subunits. The ability of assembled $\beta_5\gamma_2$ to activate PLC β2 was assessed in the presence of myristoylated recombinant α_o-GDP. As shown in Figure 5, increasing stoichiometric ratios of α_o over either $\beta_1\gamma_2$ or $\beta_5\gamma_2$ caused a dramatic decrease in their ability to activate PLC β2. The α subunit is more effective at inhibiting $\beta_1\gamma_2$ stimulation than $\beta_5\gamma_2$ stimulation suggesting that the α subunit may bind more tightly to $\beta_1\gamma_2$. Boiled α_o had no effect on the ability of $\beta_5\gamma_2$ to activate PLC β2, and α_o alone has no effect on the PLC

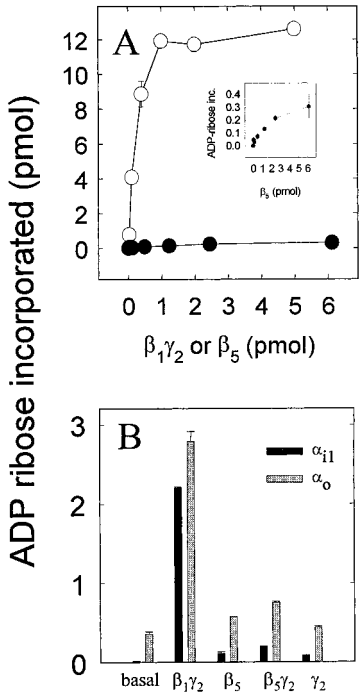

FIGURE 6: Stimulation of ADP ribosylation of α_i by $\beta_1\gamma_2$ or β_5. A. ADP ribosylation of 20 pmol of α_i was performed with the indicated amounts of β_5 (●) or $\beta_1\gamma_2$ (○). The inset is an expansion of the data for β_5 monomer to demonstrate that a small level of stimulation does occur. B. ADP ribosylation of 20 pmol of recombinant mysristoylated α_{i1} or bovine brain α_o with either 1 pmol of $\beta_1\gamma_2$, 5 pmol of β_5, or 5 pmol of β_5 assembled with 18 pmol of γ_2 or 18 pmol of γ_2 alone. The higher basal level of ADP ribosylation of α_o in this experiment is because the α_o preparation from brain may have had a slight contamination with $\beta\gamma$. Symbols are the mean of duplicate determinations and each experiment was repeated 3 times. Error bars represent standard errors from duplicates of a single experiment.

activity in the absence of $\beta\gamma$. These data indicate that α_o-GDP binds to $\beta_5\gamma_2$ and provides further evidence that PTX-sensitive α subunits can bind to $\beta_5\gamma_2$ complexes.

$\beta_5\gamma_2$ and β_5 Poorly Support ADP Ribosylation of $\alpha_{i/o}$ But Inhibit ADP Ribosylation of $\alpha_{i/o}$ Supported by $\beta_1\gamma_2$. To further assess the ability of β_5 to interact with α_i and α_o, we examined the ability of β_5 to support ADP ribosylation by PTX. As seen in Figure 6A, β_5 monomer supported a very small amount of ADP ribosylation of α_{i1} when compared to $\beta_1\gamma_2$. When β_5 was assembled with γ_2 with there was no significant further increased effect over β_5 monomer (Figure 6B) despite the fact that this combination was capable of activating PLC β2. Similar results were observed for α_o; i.e., β_5 or $\beta_5\gamma_2$ did not support significant ADP ribosylation of α_o (Figure 6B).

There are two possible explanations for the poor ability of β_5 or $\beta_5\gamma_2$ to facilitate incorporation of ADP ribose into α subunits. One is that β_5 is not binding to the α subunit, but the results presented in previous sections suggest otherwise. Another possibility is that β_5 binds to $\alpha_{i/o}$ but is not capable of supporting the ADP ribosylation reaction. To test this idea and whether β_5 monomer can interact with α subunits, we examined whether inclusion of β_5 monomer with $\beta_1\gamma_2$ in the ADP ribosylation reaction with PTX would inhibit the ability of $\beta_1\gamma_2$ to support ADP ribosylation by binding to the α subunit (Figure 7). A small stoichiometric excess of β_5 over α subunits inhibited the ability of $\beta_1\gamma_2$ to

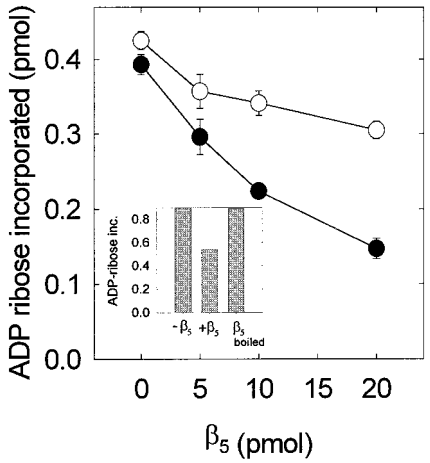

FIGURE 7: Inhibition of $\beta_1\gamma_2$-stimulated ADP ribosylation of α_i and α_o by competition for binding with β_5 monomer. ADP ribosylations contained 5 pmol of α_{i1} (●) or α_o (○) and 0.4 pmol of $\beta_1\gamma_2$. Inset shows the effects of boiling of 20 pmol of β_5 on inhibition of ADP ribosylation of α_{i1}. ADP ribose incorporated into either α_i or α_o alone was 0.013 and 0.016 pmol, respectively. The experiments were repeated 3 times with duplicate determinations for each point.

support ADP ribosylation by PTX of α_i by about 25%. At a 4-fold excess of β_5 over α_i, ADP ribosylation was inhibited by 60%. β_5 also inhibits $\beta\gamma$-stimulated ADP ribosylation of α_o although to a lesser extent. Boiled β_5 had no effect on ADP ribosylation (Figure 7, inset). These data suggest that β_5 monomer can bind to both α_i and α_o to prevent the binding of $\beta_1\gamma_2$. β_5 assembled with γ_2 had similar properties. The apparently selective effect of β_5 on α_i vs α_o indicates that the observed inhibition is not a general nonspecific inhibitory effect. That a stoichiometric excess of the β_5 is required for inhibition is to be expected because $\beta_1\gamma_2$ can act catalytically to support ADP ribosylation so there may not be a 1:1 relationship between the amount of β subunit bound and the amount of ADP ribosylation.

β_5 only slightly inhibits ADP ribosylation of α_o but significantly inhibits ADP ribosylation of α_i. It has been previously observed that factors that affect the ability of $\beta\gamma$ subunits to support ADP ribosylation of α_i may have no effect on the ability of $\beta\gamma$ to support ADP ribosylation of α_o. For example, $\beta_1\gamma_1$ is significantly less effective than $\beta_1\gamma_2$ at supporting PTX-dependent ADP ribosylation of α_i, while $\beta_1\gamma_2$ and $\beta_1\gamma_1$ equally support robust ADP ribosylation of α_o (*11*, *24*). Independent assays indicate that $\beta_1\gamma_1$ has a lower affinity for α_o than $\beta_1\gamma_2$ (*11*). The reason for this discrepancy is unclear, but the mechanism for catalytic support of ADP ribosylation of α subunits by $\beta\gamma$ subunits is complex and not well understood. Thus factors that decrease the interaction of $\beta\gamma$ with α_o seem not to affect $\beta\gamma$-dependent ADP ribosylation of α_o and probably account for the inability of β_5 to strongly inhibit ADP ribosylation of α_o by $\beta_1\gamma_2$.

DISCUSSION

In the experiments presented here we set out to characterize the biochemical properties of the clearly unique β_5 isoform of G protein β subunits. Several key points can be made from these studies. (1) β_5 can form a stable complex with γ_2 that can be purified on the basis of its association with α_i. (2) The GDP-bound forms of α_i and α_o can bind to both $\beta_5\gamma_2$ and β_5 monomers. (3) β_5 can be reassembled with

γ_2 into a functional dimer in vitro that activates PLC β2 but not PLC β3.

Purification of β_5 and γ_2 Subunits. The purification assembled of β_5 and γ_2 contrasts with some reports where stable complexes of β_5 and γ_2 could not be isolated by immunoprecipitation (*5*) but is in agreement with others where β_5 and 6-His/FLAG-tagged γ_2 were coexpressed and purified as a stable complex from Sf9 cells (*25*). It has been previously reported that detergents tend to cause dissociation of β_5 and γ subunits. Surprisingly in our hands most of the $\beta_5\gamma_2$ complex was able to survive extraction from the membrane fraction with cholate, dilution, column association and washes with high salt and detergent. Separation of β_5 from γ_2 only became evident after Mono Q ion-exchange chromatography. This may be the result of previous dissociation of β_5 from γ during cholate elution from the Ni-NTA-agarose, or the ion-exchange chromatography in 1% octylglucoside may have caused the dissociation. This procedure, however, allowed us to study the properties of β_5 in the presence and absence of γ subunits.

We were unable to establish conditions where AlF_4^- could cause dissociation of $\beta_5\gamma_2$ from immobilized 6-His-α_i subunits. It is not clear whether this is due to a unique property of β_5 subunits that allows them to remain associated with activated α_i subunits or just that we could not find the proper conditions for dissociation. The elution conditions were very similar to those previously reported to cause dissociation of α_q from immobilized $\beta_5\gamma_2$ (*25*) and are routinely used in our laboratory to dissociate $\beta_1\gamma_2$ from the same α_i subunits (*10*, *26*). It is well-established that cholate decreases the affinity of $\beta\gamma$ subunits for α subunits and could explain why cholate causes the $\beta_5\gamma_2$ subunits to elute (*27*).

Assembly of β_5 and γ_2 in Vitro and Selective Activation of PLC β2. While it has been previously shown that β and γ subunits can be assembled in vitro (*28*, *29*), this is the first time that γ has been assembled with purified β subunits and in a manner that allows for effector activation. The assembled β_5 and γ_2 subunit was similar to $\beta_1\gamma_2$ in its ability to activate PLC β2 but was unable to activate PLC β3. This is under conditions where an equivalent amount of $\beta_1\gamma_2$ gave a 100-fold activation of PLC β3. The EC_{50}'s for PLC β2 and PLC β3 are very similar (*30*), and excess $\beta_5\gamma_2$ (300 nM) still did not activate PLC β3 indicating that this difference was not due to an overestimate of the amount of assembled $\beta_5\gamma_2$ added to the reaction (not shown). Unusual selectivity of $\beta_5\gamma_2$ for effectors has been previously noted. In particular, purified $\beta_5\gamma_2$ has been shown to activate turkey PLC β with a potency similar to that of $\beta_1\gamma_2$ but was a poor activator of adenylate cyclase type II compared to $\beta_1\gamma_2$ (*9*). In COS cells transfected with adenylate cyclase type II, cotransfected $\beta_5\gamma_2$ inhibited adenylate cyclase while $\beta_1\gamma_2$ activated the same isoform (*31*). Our studies extend these observations to two very closely related isoforms of PLC β and provide further evidence that different binding sites on the surfaces of β subunits are required for activation of even very closely related effectors. While this manuscript was under review, Maier et al. published similar results showing differences in the ability of $\beta_5\gamma_2$ to activate PLC β2 vs PLC β3 (*32*).

Interactions Between β_5 Monomer and $\beta_5\gamma_2$ with α Subunits. Our results suggest association between β_5 and α_i and α_o. The first line of evidence is the ability of $\beta_5\gamma_2$ to associate with a Ni-agarose affinity matrix based on its association with 6-His-α_i. Other evidence for interactions between β_5 and α_i or α_o include competition by β_5 monomer with $\beta_1\gamma_2$ for ADP ribosylation of α_i and α_o and α_o-dependent inhibition of activation of PLC β2 by assembled $\beta_5\gamma_2$. These results are in contrast to a report that indicates that $\beta_5\gamma_2$ does not bind to α_i or α_o using a similar chromatographic method except $\beta_5\gamma_2$ was immobilized and extracts containing α subunits were passed over the columns (*25*). The reason for this discrepancy is unclear, but we have three independent lines of evidence for association of α_i and/or α_o with β_5. One possible difference is that the γ_2 used for their studies is modified with both FLAG and 6-His tags at the N-terminus while we are using native γ_2 subunits. Perhaps this modification interferes with interactions with $\alpha_{i/o}$.

β_5 or $\beta_5\gamma_2$ does not support significant ADP ribosylation of $\alpha_{i/o}$. β_5 and γ_2 can be assembled in vitro to form a functional dimer that can activate PLC β2 indicating that the proteins are functional. β_5 subunit monomer was able to compete for $\beta_1\gamma_2$-dependent ADP ribosylation of $\alpha_{i/o}$ suggesting that β_5 monomer can interact with $\alpha_{i/o}$. Taken together the inability to support ADP ribosylation of α_{i1} or α_o must be due to some unique structural properties of β_5 that do not allow PTX binding to the $\beta_5\alpha_i$ or $\beta_5\gamma_2\alpha_i$ complex. Alternatively, binding of β_5 or $\beta_5\gamma_2$ to $\alpha_{i/o}$ may not induce a conformational change required for ADP ribosylation.

The association of α_i and α_o with β_5 has significance with respect to the newly discovered RGS protein association with β_5. In one report β5/RGS11 was shown to have GTPase activating (GAP) activity toward α_o but not α_i or other G protein α subunits. A question that remains is what governs this specificity: is it the RGS or β_5 component of the complex? Our data suggest that β_5 can bind to the GDP form of α_i or α_o whether the γ subunit is present. This raises the possibility that the β_5/RGS complex could associate with $\alpha_{i/o}$ in its GDP-bound state and possibly be released upon G protein activation. In the report by Snow et al. (*5*) they indicate that they could not detect any interaction with GDP-bound α subunits. Our data indicate that the β_5 subunit can bind to GDP α_i and α_o, but it is possible that the RGS protein occludes the α subunit binding site. Another report shows that β_5 prevents the interaction between RGS7 and α_o and suggests that the role of β_5 is to inhibit interactions between RGS and α subunits (*6*). Our data suggest that β_5 monomer and $\beta_5\gamma_2$ are able to bind α_o-GDP (Figures 5 and 7). In order for β_5 to inhibit RGS binding it would have to block an α subunit binding site on RGS7 and RGS7 would have to block the α subunit binding site on β_5.

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