The G Protein β₅ Subunit Interacts Selectively with the G₉ α Subunit*1

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The diversity in the heterotrimeric G protein α, β, and γ subunits may allow selective protein-protein interactions and provide specificity for signaling pathways. We examined the ability of five α subunits (α₁, α₁α, α₅, α₆, and α₉) to associate with three β subunits (β₁, β₂, and β₅) dimerized to a γ subunit containing an amino-terminal hexahistidine-FLAG affinity tag (γ₂HF). Sf9 insect cells were used to overexpress recombinant proteins. The hexahistidine-FLAG sequence does not hinder the function of the β₁γ₂HF dimer as it can be specifically eluted from an α₁-agarose column with GDP and GTPγS, and purified β₁γ₂HF dimer stimulates type II adenyl cyclase. The β₁γ₂HF and β₂γ₂HF dimers immobilized on an anti-FLAG affinity column bound all five α subunits tested, whereas the β₅γ₂HF dimer bound only α₉. The ability of other α subunits to compete with the α₁ subunit for binding to the β₅γ₂HF dimer was tested. Addition of increasing amounts of purified, recombinant α₁ subunit to a Sf9 cell extract did not decrease the amount of α₁ bound to the β₅γ₂HF column. When G proteins in an extract of brain membranes were activated with GDP and GTPγS and deactivated in the presence of equal amounts of the β₂γ₂HF or β₅γ₂HF dimers, only α₁ bound to the β₅γ₂HF dimer. The α₁, β₁γ₂HF interaction on the column was functional as GDP, and GTPγS specifically eluted α₁ from the column. These results indicate that although the β₁ and β₂ subunits interact with α subunits from the α₁, α₅, α₆, and α₉ families, the structurally divergent β₅ subunit only interacts with α₉.

All cells possess multiple signaling pathways that transmit signals from the hormones, autacoids, neurotransmitters, and growth factors in their environment. Complex biochemical mechanisms exist to discriminate, integrate, and modulate a cell’s response to this constantly changing set of stimuli. One of the best characterized signal transduction systems is the pathway used by receptors coupled to heterotrimeric G proteins (1, 2). Our current understanding of this signaling path-

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1 The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; Sf9 cells, Spodoptera frugiperda cells (ATCC number CRL 1711); DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; C₁₁E₁₀, polyoxyethylene 10 lauryl ether; Genapol C-100, polyoxyethylene 10 dodecyl ether; ECL®, enhanced chemiluminescence; TLCK, N-p-tosyl-l-lysine chloromethyl ketone; FLAG antibody, anti-FLAG® M2 antibody; GTPγS, guanosine 5’-3-O-(thio)triphosphate; GDP-AMP, a mixture of GDP, MgCl₂, NaF, and AlCl₃, at the indicated concentrations; MAP kinase, mitogen-activated protein kinase; PLC, phospholipase C.

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insertions within the WD repeat regions of the molecule. The β2 subunit is expressed predominantly in the brain, with only trace amounts detected by Northern analysis in the kidney. The β3,1 subunit appears to be expressed only in retina. Both the β2 and β3,1 subunits can stimulate PLC-β2 activity when transiently transfected into COS-7 cells with the γ2 subunit (20, 21). However, the β2γ5 dimer fails to activate the MAP kinase pathway when transfected into these cells (22). This observation suggests that dimers containing the β2 subunit may have different functions from those containing other β subunits. In the experiments reported here, we have tested the ability of several α subunits to interact with βγ dimers containing the β1, β2, or β3 subunit to determine if the variations in amino acid sequence observed for the β subunits are manifested as differences in affinity for α subunits. SF9 cells were co-infected with an affinity tagged γ2 subunit and various β subunits. The resulting βγ5 subunits were immobilized via the affinity tag and allowed to interact with a variety of recombinant α subunits expressed in SF9 cells. The results show that the β1γ5 and β2γ5 subunits interact with five different α subunits from three families, whereas the β3γ5 subunit only interacts with the α6 subunit.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses for the γ5 Subunits—The polymerase chain reaction was used to modify the cDNA encoding the γ2 subunit (23) by adding XbaI and BamHI restriction sites to the 5’ and 3’ ends of the γ2 coding region, respectively. The primers used were Sense primer: 5’-AATCTCTAGATGGCCAGCAACAACACAGC-3’ and Antisense primer: 5’-CCTGGATCTTTAAGGATACGACAGAAACTC-3’ BamHI. The products of the polymerase chain reaction were digested with XbaI and BamHI and ligated into the pDoubleTrouble (pDT) vector (24) to add the nucleotide sequences for the hexahistidine and FLAG affinity tags to the 5’ end of the γ2 coding region. To construct useful restriction sites for subcloning into the baculovirus transfer vector pVL1393, the γ2 coding sequence was excised from pDT with KpnI and BamHI and subcloned into the pCNTR shuttle vector using the Prime Efficiency Blunt-End DNA Ligation Kit (5’ Prime – 3’ Prime). The γ5 coding region was excised from pCNTR with BamHI and ligated into the BamHI site of pVL1393 to place the ATG of the hexahistidine and FLAG sequence in-frame with the polyhedron promoter. The mouse βγ2 cDNA in a Bluescript SKII vector was kindly provided by Dr. Melvin I. Simon of the California Institute of Technology. The 1803-base pair BamHI-XbaI fragment of the βγ2 cDNA was subcloned into the BamHI-XbaI sites of pVL1393. To ensure fidelity, both completed transfer vectors were sequenced in the forward and reverse directions using dye terminator sequencing on an automated sequencer (Applied Biosystems, model 377). Recombinant baculoviruses were isolated following co-transfection of the transfer vector and linearized BaculoGold viral DNA into Sf9 cells overexpressing the desired α subunit with a βγ5 affinity column. The results show that the α1, α2, and α5 subunits interacted with the β1γ5 and β2γ5 subunits, whereas the α3, α4, and α6 subunits did not interact with either of these dimers.

Expression and Purification of Recombinant G Protein α and βγ Subunits—Recombinant G protein subunits were expressed in insect cells (26, 27, 30). In most experiments, the recombinant α and βγ subunits were extracted from cell pellets using the detergent Genapol C-100 at a concentration of 0.1% (w/v). All steps were performed at 4 °C. Frozen pellets were thawed in 15 × their wet weight in lysis buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 17 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml benazinate, and 2 μg/ml each of aprotinin, leupeptin, and pepstatin, and burst by nitrogen cavitation (600 p.s.i. for 20 min) at 0 °C. The crude lysate was mixed with an equal volume of lysis buffer supplemented with 0.2% (w/v) Genapol C-100 and stirred for 1 h. The Genapol extract was centrifuged at 100,000 × g for 60 min and the supernatant decanted, and aliquots were frozen in liquid N2. The α subunit and the adenyl cyclase assays were performed as described (26). The α5 subunit was used in the adenylyl cyclase and γ5 subunit binding assays. SF9 cell pellets overexpressing the α5 subunit were extracted without detergent; a 100,000 × g supernatant was prepared and the protein purified on a DEAE column exactly as described (26). The preparation of α5 subunit is approximately 95% pure, as determined by quantitation of a silver-stained gel.

Preparation of the βγ-Anti-FLAG Affinity Column—All column steps were carried out at 4 °C. Typically, 1 ml of the Genapol C-100 extract of SF9 cells overexpressing the desired βγ5 subunit was applied to a 0.5-ml anti-FLAG M2 affinity gel column equilibrated with column buffer (lysis buffer containing 0.1% Genapol C-100 and 1 mM β-mercaptoethanol) at a flow rate of 0.2 ml/min. The resulting βγ5-anti-FLAG affinity gel column (βγ5 affinity column) was washed three times with 3 ml of column buffer. This procedure resulted in a highly pure preparation of βγ dimers immobilized on the column (see Fig. 1). The amount of βγ dimer immobilized on the column was about 6 μg/ml of resin as judged by silver staining of the βγ dimer eluted from the column with 0.1 M glycine, pH 3.5. In the experiment presented in Fig. 5A, the procedure was modified such that 2 ml of α5 subunit extract and 2 ml of α5 subunit extract were mixed and applied to the βγ5 affinity column. In the experiment presented in Fig. 6, a range of 17.5–525 μg of partially purified α5 was mixed with 2 ml of α5 extract and applied to the βγ5 affinity column.

Specific Elution of a Subunits from βγ5-Anti-FLAG Affinity Gel—To demonstrate a functional interaction between α subunits and the immobilized βγ5 subunits, a βγ5-anti-FLAG affinity column (0.5 ml) was prepared at 4 °C as described above, washed three times with 3 ml of column buffer, and then equilibrated in α subunit binding buffer containing 300 mM NaCl and 0.2% (w/v) C12E10, 10 mM β-mercaptoethanol, and 0.5 μM GDP. Then, 17.5 μg of purified α5 subunit diluted in 1 ml of α subunit binding buffer was applied to the βγ5 affinity column at a flow rate of 0.2 ml/min. The α5 subunit affinity column was washed 4 times with 4 ml of α subunit binding buffer and twice with 2 ml of α subunit binding buffer containing 300 mM NaCl. The column was incubated at room temperature for 15 min. The α5 subunit was eluted with 4 × 0.5 ml of 20 mM Hepes, pH 8.0, 50 mM NaCl, 1 mM MgCl2, 0.3% (w/v) C12E10, 10 μM β-mercaptoethanol, and 5 μM GDP. Then, 17.5 μg of purified α5 subunit diluted in 1 ml of α subunit binding buffer was applied to the βγ5 affinity column at a flow rate of 0.2 ml/min. The α5 subunit affinity column was washed 4 times with 4 ml of α subunit binding buffer and twice with 2 ml of α subunit binding buffer containing 300 mM NaCl. The column was incubated at room temperature for 15 min. The α5 subunit was eluted with 4 × 0.5 ml of 20 mM Hepes, pH 8.0, 50 mM NaCl, 1 mM EDTA, 1.0% cholate, 10 mM β-mercaptoethanol, 10 mM MgCl2, and 100 μM GTPγS, also at room temperature. The column was washed twice with 4 ml of α subunit binding buffer containing 300 mM NaCl, before final elution with 0.1 M glycine, pH 3.5.

Slight modifications of the above procedure were used to examine the interaction of the α5 subunit with the β5γ5 subunit. The 0.5 ml β5γ5 affinity column was prepared and washed as described in the previous section, except that the GDP concentration was increased to 50 μM in the α subunit binding buffer and the column buffer. Two ml of a Genapol C-100 extract of SF9 cells expressing the α5 subunit was applied at a flow rate of 0.2 ml/min. The β5γ5 affinity column was washed twice with 1 ml of column buffer, four times with 1 ml of α subunit binding buffer with 0.2% (w/v) C12E10, and finally twice with 2 ml of α subunit binding buffer containing 300 mM NaCl and 0.2% (w/v) C12E10. The column was brought to room temperature for 15 min and α5 was specifically eluted with 20 mM Hepes, pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.05% cholate, 10 mM β-mercaptoethanol, 10 mM MgCl2, 10 mM NaF, 30 μM AlCl3, and 50 μM GDP. The cholate concentration was reduced to 0.05% in this buffer because higher cholate concentrations dissociated β5 from γ5.

Extraction of G Proteins from Bovine Brain Membranes and Activation with GDP-AMF—Frozen bovine brains were obtained from Pel-Freez Biologicals, Inc. (Rogers, AR) according to the protocol of Stowe and Robishaw (31), with the addition of 0.2 μg/ml aprotinin to all buffers. The membrane preparations were stored at ~80 °C. Membranes were thawed, washed once with ice-cold 20 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, and pelleted at 40,000 × g for 30 min at 4 °C. The washed membrane pellet (1 g of protein) was extracted for 1 h at 4 °C with 200 ml of 0.5% (w/v) C12E10 in 50 mM Tris, pH 8.0. The extract
was clarified by centrifugation at 143,000 × g for 60 min and stored at −80 °C. To examine the interaction of the G proteins in the membrane extracts with the various β subunits, the extracts were thawed and mixed with the different βγ1Hb Genaprol extracts from Sf9 cells and activated by addition of concentrated stocks to give final concentrations of 0.5 mM GTPγS, 5 mM NaF, 15 μM AlF3, and 2.5 μM GDP. The mixture was incubated at 30 °C for 45 min (32). The α subunits were deactivated by addition of 500 mM EDTA to a final concentration of 20 mM EDTA and incubated for an additional 30 min at 30 °C. Typically, 4 ml of the deactivated mixture was applied to 0.5 ml of anti-FLAG affinity gel equilibrated with 20 mM Hepes, pH 8, 150 mM NaCl, 5 mM MgSO4, 1 mM DTT, 1 mM EDTA, 0.1% CHAPS, as described previously (13). The first elution fraction, containing 400 mM NaCl and eluted with 1.0 ml of 0.1 M glycine, pH 3.5, was described above.

Silver Staining, Immunoblotting, and Quantitation—Samples were prepared for electrophoresis, loaded on 0.75 mm, 12% acrylamide gels, and the gels stained with silver according to the method of Bloom et al. (33). Purified bovine brain Gαi/o heterotrimer (31) was used as a standard. The objective of this study was to determine if selectivity in α-βγ interactions could be observed in vitro with recombinant G protein subunits isolated from baculovirus-infected Sf9 cells. We first constructed a recombinant baculovirus encoding a single FLAG epitope-tagged rat type II adenyl cyclase cyclophilin A (unpublished). The silver-stained SDS-polyacrylamide gel in Fig. 1 illustrates the steps involved in the preparation of the αβγ subunits. SF9 cells were co-infected with recombinant baculovirus encoding for the β1 and γ2Hb subunits, and the recombinant β1γ2Hb protein was extracted from membranes of cells harvested 48 h post-infection. Crude detergent extracts were applied to anti-FLAG M2 affinity gel columns and washed with 5–10 column volumes. The resulting product of this one-step purification is shown in lane 3 of Fig. 1. The FLAG epitope tag on the γ2Hb subunit is available for binding to the anti-FLAG antibody and produces a dramatic increase in purity in a single step. The presence of the hexahistidine tag and FLAG epitope results in reduced electrophoretic mobility of the γ2Hb subunit relative to γα (lane 1 versus 3). Approximately 12 μg of β1γ2Hb were captured per ml of anti-FLAG M2 affinity gel suspension, as determined by quantitation of the eluted β1 subunit on a silver-stained gel. In subsequent experiments

RESULTS

The objective of this study was to determine if selectivity in α-βγ interactions could be observed in vitro with recombinant G protein subunits isolated from baculovirus-infected Sf9 cells. We first constructed a recombinant baculovirus encoding sequential affinity tags on the amino terminus of the γ subunit, a hexahistidine tag followed by a FLAG epitope tag (24). When used in conjunction with an anti-FLAG antibody covalently linked to agarose beads, the FLAG epitope tag provides a convenient method for separating α subunits bound to βγ subunits from α subunits free in solution. Previous work has shown that addition of a hexahistidine or FLAG affinity tag to the amino terminus of the γ subunit does not prohibit association with the β subunit (37–39) or the subsequent association of the βγ dimer with α subunits (37). The heterotrimeric G protein crystal structure also suggests that an extension of the amino terminus of the γ subunit would be unlikely to interfere with α-βγ interactions (40, 41).

The silver-stained SDS-polyacrylamide gel in Fig. 1 illustrates the steps involved in the preparation of the βγ and α-βγ affinity columns. SF9 cells were co-infected with recombinant baculovirus encoding the β1 and γ2Hb subunits, and the recombinant β1γ2Hb protein was extracted from membranes of cells harvested 48 h post-infection. Crude detergent extracts were applied to anti-FLAG M2 affinity gel columns and washed with 5–10 column volumes. The resulting product of this one-step purification is shown in lane 3 of Fig. 1. The FLAG epitope tag on the γ2Hb subunit is available for binding to the anti-FLAG antibody and produces a dramatic increase in purity in a single step. The presence of the hexahistidine tag and FLAG epitope results in reduced electrophoretic mobility of the γ2Hb subunit relative to γα (lane 1 versus 3). Approximately 12 μg of β1γ2Hb were captured per ml of anti-FLAG M2 affinity gel suspension, as determined by quantitation of the eluted β1 subunit on a silver-stained gel. In subsequent experiments
The β5 Subunit Interacts Selectively with αq

Stimulation of type II adenyl cyclase by native and affinity tagged βγ subunits

| cAMP produced | nM | pmol/μl/min |
|---------------|----|-------------|
| βγ | β1γ2 | β1γ2HF |
| 0   | 5.0 ± 0.3 | 5.0 ± 0.3 |
| 5   | 37.3 ± 3.4 | 12.7 ± 0.7 |
| 10  | 55.0 ± 5.1 | 22.8 ± 2.3 |
| 25  | 80.1 ± 6.7 | 38.6 ± 3.8 |
| 50  | 109.9 ± 9.0 | 52.4 ± 6.0 |
| 100 | 129.8 ± 7.0 | 75.3 ± 6.2 |

Sf9 cells were infected with a recombinant baculovirus encoding for type II adenyl cyclase. Membranes prepared, the membranes stimulated with GTPγS-αq, and the indicated concentration of βγ subunit for 7 min, and the cyclic AMP produced measured using a radioimmunoassay. The βγ subunits were purified on a DEAE and αq affinity column. The basal rate of cAMP production without GTPγS-αq was 1.0 pmol/μl/min. See “Experimental Procedures” for details. The data are averages of 2–3 duplicate determinations.

2A shows a Western blot, developed with an anti-β, antibody, of the DEAE pool (PL) applied to the αq column and the αq column pass-through (PT). Comparison of lanes 1 and 2 shows a typical result for β1γ2. A very high proportion of the β1γ2 present in the DEAE pool binds to the αq-affinity column. Lanes 3 and 4 show a very similar result obtained when a DEAE pool containing β1γ2HF was applied to the αq-affinity column. This observation is consistent with the result obtained with immobilized β1γ2HF and αq free in solution, as described above. To further evidence of functional αqβ1γ2HF interaction, we treated the β1γ2HF-loaded αq-affinity column with GDP-AMF. Fig. 2B shows a silver-stained SDS-polyacrylamide gel of the αq-affinity column pass-through (lane 2), wash fractions (lanes 3–5), and subsequent elution of β1γ2HF by treatment with GDP-AMF (lanes 6–8). Thus, β1γ2HF binds tightly to immobilized αq1 and elutes upon activation of αq1 with GDP-AMF.

We next tested the ability of β1γ2HF to stimulate one effector, type II adenyl cyclase. It is known that β1γ2 is a potent activator of type II cyclase in the presence of an αq subunit activated with GTPγS (44). The data in Table I compare the stimulation of type II cyclase by β1γ2 and β1γ2HF in the concentration range 0–100 nM. At 100 nM, the β1γ2HF dimer is capable of a 15-fold stimulation of cyclase over the effect of GTPγS-αq alone. However, at each concentration tested, the β1γ2 dimer activates adenyl cyclase to a significantly greater extent than does the β1γ2HF dimer. This reduced stimulation could be due to a decreased effective concentration of β1γ2HF relative to β1γ2 at the adenyl cyclase-containing membrane surface, or to a specific interference between the affinity tags on the γ subunit’s amino terminus and type II cyclase. This matter is under further investigation. We conclude that the presence of the hexahistidine and FLAG epitopes on the amino terminus of the γ subunit does not abrogate interaction of β1γ2HF with at least one effector, type II adenyl cyclase.

Previous work with the adenosine A1 receptor showed little difference between the ability of αq1, αq2, and αq3 to support high affinity binding of agonist in the presence of β1γ2 (13, 45). Since this observation implies similar affinity of the three αq subunits for β1γ2, we tested the ability of another αq isomorph, the αq2 subunit, to bind to a β1γ2HF affinity column. Two ml of a crude detergent extract of Sf9 cells infected with recombinant baculovirus encoding the αq2 subunit was applied to a 0.5-ml β1γ2HF affinity column as described above, washed extensively, and the bound αq2 and β1γ2HF eluted with glycine. A silver-stained polyacrylamide gel of the product is shown in Fig. 3A, lane 2. Thus, the immobilized β1γ2HF was also able to bind αq2, and a 2-ml volume of crude extract containing αq2 subunit was

Fig. 2. The β1γ2HF dimer associates with an αq-agarose affinity column. A, extracts of Sf9 cells overexpressing β1γ2 and β1γ2HF dimers were partially purified on a DEAE ion-exchange column, and the β1γ2 and β1γ2HF dimers were applied to αq-agarose affinity columns. Aliquots of the pooled DEAE fractions (PL) and α-column pass-throughs (PT) were resolved on a 12% SDS-acrylamide gel and transferred to nitrocellulose. Lanes 1–4 were probed with an anti-β-common primary antibody detected using a horseradish peroxidase-conjugated secondary antibody. The migration position of the β1 subunit is indicated on the left; the βγ combinations are indicated above the appropriate lanes. B, purification of the β1γ1 subunit on an αq-agarose column. The β1γ1 column was specifically eluted from the αq-agarose support with GDP-AMF. Proteins in the pass-through (PT), washes (W1–W3), and eluates (E1–E3) were resolved on a 12% SDS-acrylamide gel and stained with silver. Lane 1, bovine brain standard; lane 2, pass-through (PT) after application of the β1γ1 column onto the αq-agarose column; lanes 3–5, wash fractions before application of GDP-AMF; lanes 6–8, the β1γ1 subunit eluted from αq-agarose column by treatment with GDP-AMF. The migration positions of the bovine brain α, β, and γ subunits are indicated on the left. Migration positions of the β1 and γ subunits are indicated on the right.

The β1γ2HF affinity column was specifically bound to the αq column and eluted with GTPγS. The αq eluted specifically, in a volume of 0.5 ml, as shown in lane 6. Lane 7 shows the elution of the remaining β1γ2HF dimer with glycine. Thus, the αq subunit can be dissociated from the β1γ2HF subunit with GTPγS treatment, analogous to activation of the native heterotrimer in solution (42). Heterotrimeric G proteins can also be activated with GDP-AMF resulting in dissociation of the βγ dimer (45). When an αqβ1γ2HF affinity column similar to that described above was activated with GDP-AMF, the αq subunit was specifically eluted (data not shown).

To test the functionality of the αqβ1γ2HF interaction in another way, we subjected a detergent extract of β1γ2HF to our normal βγ purification strategy. DEAE ion-exchange chromatography followed by αq-agarose affinity chromatography (30). The αq-agarose affinity chromatography exploits the ability of GDP-AMF to dissociate the βγ subunit from the α subunit. Fig.

designed to monitor α-βγ interaction, the β1γ2HF was captured as before and the resulting βγ2HF affinity column used to specifically bind partially purified αq subunits. To determine first the amount of αq necessary for stoichiometric binding to immobilized β1γ2HF, replicate β1γ2HF affinity columns were prepared and then varying amounts of αq subunit applied. Stoichiometric binding was achieved at a 3–7-fold excess (w/w) of αq over immobilized β1γ2HF (data not shown). Lane 4 shows the αq preparation used for these experiments. Lane 5 shows the resulting αqβ1γ2HF eluted with 0.1 μl glycine after an excess of αq was applied to the β1γ2HF affinity column.

To demonstrate that the immobilized β1γ2HF was properly folded and functional, a 0.5-ml αqβ1γ2HF affinity column, prepared identically to that shown in lane 5, was treated with 100 μM GTPγS. The αq eluted specifically, in a volume of 0.5 ml, as shown in lane 6. Lane 7 shows the elution of the remaining β1γ2HF dimer with glycine. Thus, the αq subunit can be dissociated from the β1γ2HF subunit with GTPγS treatment, analogous to activation of the native heterotrimer in solution (42). Heterotrimeric G proteins can also be activated with GDP-AMF resulting in dissociation of the βγ dimer (45). When an αqβ1γ2HF affinity column similar to that described above was activated with GDP-AMF, the αq subunit was specifically eluted (data not shown).

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sufficient excess to obtain stoichiometric binding of α2 to immobilized β1γ2HF.

Having demonstrated functional activity for β1γ2HF and its ability to bind both purified α2 and a crude detergent extract of the α2 subunit, we tested the ability of other βγ2HF dimers to bind α2. The β1γ, β2γ, and β3γ2HF columns were first constructed by application of appropriate crude cell extracts to anti-FLAG M2 affinity gel. Pilot experiments were performed to ensure that equivalent amounts of β1γ, β2γ, and β3γ2HF were bound to anti-FLAG columns by applying a sufficient excess of each βγ2HF detergent extract to saturate the available FLAG binding sites. Equal volumes of a crude detergent extract of Sf9 cells infected with recombinant baculovirus encoding the α2 subunit were then applied to each βγ2HF affinity column; the eluate from the βγ2HF affinity column was used for SDS-PAGE analysis. The primary antibody was detected using an alkaline phosphatase-conjugated secondary antibody. The migration position of the α2 subunit is indicated on the left.

FIG. 3. The α2 subunit does not associate with β2γ2HF. A, a detergent extract of the α2 subunit overexpressed in Sf9 cells was applied to β1γ, β2γ, and β3γ2HF affinity columns. After extensive washing, the αβγ2HF heterotrimers were eluted from the anti-FLAG M2 affinity gel with 0.1 M glycine, pH 8. A β2γ5HF affinity column to which no α2 extract was applied was also eluted with 0.1 M glycine (lane 5). Proteins in each eluate were resolved on a 12% SDS-acrylamide gel and stained with silver. Lane 1, bovine brain standard; lane 2, elute from the β1γ2HF affinity column; lane 3, elute from the β2γ5HF affinity column; lane 4, elute from the β3γ2HF affinity column; lane 5, β2γ5HF standard. B, immunoblot of the samples in A probed with an anti-α common primary antibody. The primary antibody was detected using an alkaline phosphatase-conjugated secondary antibody. The migration position of the α2 subunit is indicated on the left.

2. The concentration of β2γ5HF in the DEAE pool applied to the α1γ-agarose column was compared with the concentration of β1γ2HF in the column pass-through. Both the DEAE pool and the α1γ column pass-through gave similar intensity when developed with an anti-β subunit antibody (data not shown), indicating little or no binding. Furthermore, no β2γ5HF product was detected on silver-stained acrylamide gels after treatment of the α1γ-agarose with GDP-AMF. Therefore, the low affinity of β2γ5HF for α1 is not due to immobilization of the βγ2HF.

We then selected representatives of three α subfamilies, α1γ, α2, and α3 (46), to investigate the ability of β2γ to interact with other α subunits. Crude detergent extracts of Sf9 cells infected with recombinant baculovirus encoding the appropriate α subunit isoform were applied to three βγ2HF affinity columns constructed as before. After extensive washing, the specifically bound α subunits were eluted, along with their βγ2HF counterparts, by treatment with 0.1 M glycine. The resulting products were analyzed by Western blotting with either an anti-α common antibody in the case of α2, α3, and α4 or with an anti-α specific antibody for α3. The results are shown in Fig. 4. As observed earlier using α2 (Fig. 3), β1γ2HF and β2γ5HF bind easily detectable amounts of α2 under conditions where β3γ2HF does not (Fig. 4, lanes 1–3). This pattern is repeated with respect to binding α3 and α4 (lanes 4–9). However, when detergent extracts containing recombinant α2 were applied to each βγ2HF column, β1γ2HF, β2γ5HF, and β3γ2HF affinity columns bound α2 (lanes 10–12). Thus, β2γ5HF appears to bind the α2 subunit selectively. To determine if the β2γ5HF dimer was able to interact with other members of the Gα family, we have performed pilot experiments with a recombinant, avian α11 subunit (29). This protein is 96% identical in amino acid composition to the mouse α11 subunit (47), and 100% identical in the 20 amino acids shown to contact the β subunit in the X-ray structure of the heterotrimer (41). Preliminary results indicate that crude detergent extracts containing α11 bind equally well to all three βγ2HF dimers (data not shown). Thus, at least one other member of the Gα family binds to the β2γ5HF dimer. The interaction of the other members of the family (the Gα1, α13, α15 subunits) with the β2γ5HF subunit is currently under investigation.

The observation of selective α1β2γ5HF interaction raises the
Fig. 5. The β5γ2HF dimer selectively associates with the αq subunit in the presence of recombinant and native G proteins. A, a mixture of αi and αq subunits overexpressed in SF9 cells was prepared as described under “Experimental Procedures” and applied to β1γ2HF and β2γ2HF affinity columns. After extensive washing, specifically bound αβγ2HF heterotrimers were eluted from the anti-FLAG M2 resin with 0.1 m glycine. The proteins in the load (L) and eluate (E) were resolved on a 12% SDS-acrylamide gel and transferred to nitrocellulose. B, the G proteins in an extract of bovine brain membranes were activated with GDP-AMF, mixed with equal aliquots of SF9 cell extracts expressing the β1γ2HF or the β2γ2HF dimers, incubated for 45 min, and quenched with EDTA as described under “Experimental Procedures.” Each mixture was applied to a separate anti-FLAG M2 affinity gel column. After extensive washing, specifically bound αβγ2HF heterotrimers were eluted with 0.1 m glycine, pH 3.5. The proteins in the load (L) and eluate (E) were resolved on a 12% SDS-acrylamide gel and transferred to nitrocellulose. In both A and B, lanes 1, 2, 5, and 6 were probed with an anti-α-common primary antibody. Lanes 3, 4, 7, and 8 were probed with an anti-αq primary antibody. Both primary antibodies were detected using an alkaline phosphatase-conjugated secondary antibody. The migration positions of the αi and αq subunits are indicated on the left.

issue of relative affinities. One approach to this question is to determine the ability of other α subunits to compete with αq for binding to β5γ2HF. Since the αq preparation was from a crude cell extract, we first selected a similar preparation of the αi2 subunit for competition experiments. The β1γ2HF and β2γ2HF columns were constructed as before, and then a mixture of equal volumes of αi2 and αq detergent extracts were applied to each. After extensive washing, the αβγ2HF complex was eluted with 0.1 m glycine. The αi2/αq mixture applied to the columns (L) was compared with the glycine elution fractions (E) by Western blot using an anti-α common or anti-αq antibodies. Lanes 1–4 of Fig. 5A show the result obtained with β1γ2HF. As expected from the individual α subunit experiments, the β1γ2HF affinity column captured both αi and αq subunits (lane 2 versus 4). The β2γ2HF affinity column also bound αq (lane 8) at a level roughly comparable to that bound by β1γ2HF (lane 4 versus 8). However, β2γ2HF bound no detectable αi2 subunit (lane 2 versus 6).

Since all the above experiments were performed with recombinant proteins, we tested the α subunit selectivity of the βγ affinity columns with native α subunits. As bovine brain membranes are known to contain a complex mixture of α subunits, including αi, αo, and αg (3, 48), we used an extract of bovine brain membranes as a starting material for these experiments. Crude detergent extracts of SF9 cells infected with recombinant baculovirus encoding for the β1γ2HF or β2γ2HF dimers were mixed with brain membrane extract and the α subunits activated by treatment with GDP-AMF as described under “Experimental Procedures.” The mixtures were deactivated by addition of excess EDTA, resulting in the association of a fraction of the α5 α subunits with the recombinant βγ dimer. The deactivated mixture was applied to an anti-FLAG affinity column and the αβγ2HF complexes eluted. The proteins in the mixtures applied to the affinity columns and the glycine elution fractions were resolved on acrylamide gels, transferred to nitrocellulose, and probed with anti-α subunit antibodies. The β1γ2HF dimer bound α subunits which gave positive signals with anti-α common antibodies and anti-αq/11 antibodies (Fig. 5B, lanes 2 and 4). However, the β2γ2HF dimer only associated with α subunits detected by the anti-αq/11 antibody (lane 6 versus 8), in agreement with the selectivity observed with recombinant α subunits (Fig. 5A). Interestingly, the β2γ2HF dimer binds a clearly resolved doublet from the brain extract (Fig. 5B, lane 8). The anti-αq/11 antibody used in these experiments does not cross-react with αo subunits, and therefore this doublet is most likely αg and/or αi1. Thus, when the β2γ2HF dimer was presented with a complex mixture of native heterotrimeric G proteins, it selectively bound the αq/11 subunits. Moreover, it did not appear to interact with the αi or αo subunits which are present at high concentrations in brain membranes.

Because the αq, αi2, and bovine brain preparations are all crude detergent extracts, it is not possible to estimate the molar ratio of competing α subunit to αq subunit applied to the immobilized β5γ2HF. To address this issue in part, we employed the purified αi1 preparation described previously. The αq subunit in this preparation represents approximately 95% of the intensity on a silver-stained gel. Increasing amounts of this αi1 stock were diluted into a fixed, larger volume of αq crude extract. Based on the amount of β5 subunit immobilized on the column as estimated from silver-stained gels, a 3–100-fold excess of αi1 was added to the αq extract. These mixtures were applied to immobilized β5γ2HF, washed, and eluted with 0.1 m glycine. The loading mixture, the last wash, and the elution fractions were examined by Western blot using an anti-αq/11 antibody (Fig. 6A) and an anti-α common antibody (Fig. 6B).

Even at the largest excess of αi1 over the immobilized β5γ2HF, there was no detectable competition by αi1 for αq binding to β5γ2HF. The ECL signal representing bound αq in Fig. 6A was quantitated on a scanning densitometer. Fig. 6C shows a plot of this integrated intensity versus excess αi1 present. Note that there is no apparent diminution of αq binding at ratios of αi1 to β5γ2HF far in excess of the ratio required for stoichiometric binding of αi1 by β5γ2HF (about 3:1).

To demonstrate that the α5β5γ2HF interaction was functional, we constructed an α5β5γ2HF affinity column as described in Fig. 4 and treated the immobilized heterotrimer with GDP-AMF to activate and thereby dissociate αq. Fig. 7A presents a silver-stained gel of the GDP-AMF elution product (E1–E3-AMF, lanes 4–6). Because αq and β5 co-migrate under these electrophoresis conditions, we verified the identity of the GDP-AMF and glycine elution products by Western blot with an anti-αq/11 antibody (Fig. 7B). Comparison of lanes 4 and 7 in Fig. 7B shows that the majority of the αq bound to β5γ2HF eluted specifically with GDP-AMF. Thus, the αq subunit is associating with the immobilized β5γ2HF in a manner that permits the αq subunit to be activated and to dissociate from the β5γ2HF.
FIG. 6. The β52HF dimer selectively associates with the αq subunit in the presence of an excess of partially purified αi1 subunit. A, 2 ml of an extract of Sf9 cells overexpressing the αi1 subunit was combined with increasing amounts of partially purified αq (17–525 μg, a 3–100-fold excess of αq, over β5 (w/w)) and applied to separate β52HF affinity columns. After extensive washing, specifically bound αi1β52HF heterotrimers were eluted from the anti-FLAG M2 affinity gel with 0.1 μg glycine. Proteins in the load (L), wash (W), and eluate (E1, E2, and E3) were resolved on a 12% SDS-acrylamide gel and transferred to nitrocellulose. Samples were probed with an anti-αi1 primary antibody and detected using a horseradish peroxidase-conjugated secondary antibody. The migration position of the αi1 subunit is indicated on the left. B, immunoblot of the same column samples as in A but probed with an anti-β-common antibody. The migration position of the αi1 subunit is indicated on the left. Lanes 1–5 contained no αi1; lanes 6–10, 3 × αi1; lanes 11–15, 10 × αi1; lanes 16–20, 30 × αi1; lanes 21–25, 100 × αi1. C, a plot of the integrated intensity of the αi1 subunit signal from fractions E1–E3 shown in A versus 3–100-fold excess of the αi1 subunit over the immobilized β5 (w/w).

DISCUSSION

The data presented in this report provide clear evidence that the diversity of the subunits in the G protein heterotrimer can have important functional consequences for the interaction of certain α and β subunits. Although all the α subunits examined interact with the β1 or β3 subunit, the structurally different β2 subunit interacts selectively with the αq subunit and the nearly identical αi1 subunit. We inspected two heterotrimeric crystal structures for sites of intersubunit contact which might be responsible for the observed selectivity (40, 41). These structures show that nine locations involving 16 amino acids on the β2 subunit are primarily responsible for interacting with the Switch I, Switch II, and the amino-terminal regions of the αi1 subunit. Of these 16 amino acids, only 3 are different in the β2 subunit (Leu84 → Gly, Tyr299 → Leu, and Ser298 → Thr, based on the β2 sequence). Although the essential residues necessary for a WD repeat (20, 49) are conserved in the β2 subunit, the overall amino acid sequence of the protein is only 52% identical and 62% similar to that of the β3 subunit. Thus, there are amino acid differences in the sequences surrounding the direct αi1 subunit contact sites and other regions of dissimilarity distributed throughout the entire β subunit sequence. Similarly, examination of the sequences of the α subunits shows multiple differences in the amino acids contacting the β subunits in the αi1, αx, αy, and αq subunits, but there is only one site where the αq subunit is unique (41). The αx, αy, and αq subunits have a Phe at position 195 in the beginning of the Switch II region, and the αq subunit share a Val at this position (41). Since there are multiple differences in sequence in both the α and β isoforms under consideration relative to the isoforms that have been crystallized, it is not possible to suggest a molecular basis for the selective interaction of the β52HF dimer with the αq subunit. However, the net effect of the various differences in α–β contacts must be substantial, as we have found that a large excess of the αi1 subunit does not measurably compete with the αx subunit for binding to the β2 subunit (see Fig. 6).

In evaluating the selectivity of the β52HF dimer for α subunits in the Gq family, it is important to consider the fidelity with which Sf9 cells modify recombinant proteins. The α subunits of most G proteins are modified with myristoyl and/or palmitoyl groups at their amino terminus, and the γ subunits are modified with a prenyl group at their carboxy terminus (50). These modifications markedly affect the affinity of the α subunits for the βγ dimers (51). The available evidence suggests that the proteins used in this work are properly modified. Recombinant Gi and Gq α subunits have been shown to be myristoylated (26), and the Gq and Gq1 subunits are able to activate phospholipase C–β equally with native proteins (52). The Gi α subunit produced in Sf9 cells fully activates adenyl cyclase and is 500-fold more potent than the protein expressed in Escherichia coli but is not as potent as αi1 purified from liver (53). The carboxy terminus of the γq subunit expressed in Sf9 cells appears to be properly and fully processed (54). Thus the available experimental evidence supports the hypothesis that recombinant proteins isolated from Sf9 cells are properly modified, and therefore the interactions reported here with recombinant proteins mimic those in intact cells. Most importantly, the major result of the study is considerably strengthened by the data shown in Fig. 5d demonstrating that the β52HF dimer also selectively associates with the αqsub1 subunits in a mixture of native G proteins extracted from brain membranes.

Little is known about the biological role of the six different β subunits in determining the specificity of cellular signaling.
The β1–β2 subunits are widely expressed, each contain 340 amino acids and are 80–90% identical in sequence (3). In contrast, Northern analysis of various murine tissues shows the β3 subunit to be expressed predominantly in the brain (20), but more recently β3 subunit expression has been detected in rat portal vein (55). Expression of the similar β3a subunit which has a 42-amino acid amino-terminal extension appears restricted to certain areas of the retina (21). These two β subunits do appear to be localized to the membrane (21) and are thus presumed to be involved in G protein-mediated signaling in sensory and nervous tissue. The data in this report suggest that the β3 subunit (and possibly the β3a subunit) participates in signaling via αR-linked receptors. Interestingly, treatment of rod outer segment membranes with GTPγS failed to release the β3a subunit (21). Because members of the αR family are slow to exchange guanine nucleotides and are more readily activated by AMP (52), thisobservation is consistent with our finding of a specific interaction between the β3 and αR subunits.

The biological implications of the restricted tissue distribution and the divergent sequences of the two β3 subunits are not fully understood. The β3 dimer has multiple roles in G protein-mediated signaling. In addition to being required for the αR subunit to couple to receptors (9, 10, 14), the dimer can regulate the activity of multiple effectors including certain isoforms of PLC-β, K+ channels, phosphatidylinositol 3-kinase, adenylyl cyclase, the MAP kinase pathway and can help localize receptor kinases to the plasma membrane (4). The functional roles of the two β3 subunits have not been fully explored, but they have been demonstrated to form functional dimers with the γ subunits, the γ subunits (20, 21). Analysis of the interaction of the β and γ subunits using the yeast two-hybrid technique also shows an interaction between the β3 subunit and multiple γ subunits (56). Moreover, the β3γ dimer markedly increases inositol phosphate breakdown in COS-7 cells transfected with the cDNAs for either β3 subunit, the γ subunit, and PLC-β2 (20–22). Although the β3γ dimer can activate PLC-β3 in transfected COS cells, it does not activate the MAP kinase or JNK kinase pathways in these cells (22). In contrast, transfection of the β3γ dimer is able to activate both PLC-β and the kinases (22, 57, 58). Our preliminary experiments show that the β3γ subunit is not able to activate type II adenylyl cyclase. Thus, the β3 subunit (and possibly the β3a subunit) may not interact with certain important effectors.

The data described above combined with the data in this report suggest a number of possibilities for the biological role of the β3 subunit in signaling. First, heterotrimers containing the β3 subunit are most likely to couple to the αR subunit, and thus only αR-linked receptors may generate a β3γ subunit to regulate effectors. The ability of other members of the G family to couple to the β3 subunit needs to be explored. Second, β3γ dimers containing the β3 subunit may only be capable of interacting with a subset of the effectors regulated by other βγ dimers. In the retina, the αR-linked pathways have been assumed to play a minor role in visual signal transduction (59), but recent studies of mouse retina using immunological techniques have demonstrated the presence of the αR subunit and PLC-β (60). Thus, a function for this signaling pathway may emerge. A wide variety of αR-linked receptors exist in neural tissue (61). One interesting pathway regulated by m1 or bradykinin receptors via the αR subunit involves inhibition of M-type potassium currents (62, 63). The known ability of the β3 dimer to regulate K+ channels via multiple mechanisms (4, 61, 64) suggests interesting potential roles for dimers containing the β3 subunit in the regulation of ion channel activity. As multiple G protein-mediated signals are often integrated by a single neuron (61, 64), selective inputs by different βγ dimers may allow distinct cellular responses. The observation that the β3γ subunit does not appear to activate the MAP kinase pathway (22) reinforces this possibility and indicates that dimers containing the β3 subunit may regulate a limited range of effectors. Thus, there may be an advantage to a more restricted βγ signal in retina and neurons. Since recombinant βγ dimers of defined composition have not been tested against all the known effectors regulated in this manner, it will be important to determine which effectors are regulated by dimers containing the β3 subunit. This information may help explain the restricted tissue distribution of these proteins.

In summary, the data in this report provide partial understanding for the large diversity of the proteins comprising the G protein heterotrimer. The finding that the β3 subunit interacts selectively with the αR subunit suggests that it will be important to examine this issue in a number of signaling systems using recombinant proteins.

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