Differential Sensitivity of Phosphatidylinositol 3-Kinase p110γ to Isoforms of G Protein βγ Dimers*

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The ability of G protein α and βγ subunits to activate the p110γ isoform of phosphatidylinositol 3-kinase (PtdIns 3-kinase) was examined using pure, recombinant G proteins and the p101/p110γ form of PtdIns 3-kinase reconstituted into synthetic lipid vesicles. GTP-activated Gαs, Gαi, Gαq, or Gαo activated PtdIns 3-kinase. Dimers containing Gβ2γ2 complexed with γ2-stimulated PtdIns 3-kinase activity about 26-fold with EC50 values ranging from 4 to 7 nM. Gβ2γ2 was not able to stimulate PtdIns 3-kinase despite producing a 10-fold activation of avian phospholipase Cβ. A series of dimers with β subunits containing point mutations in the amino acids that undergo a conformational change upon interaction of βγ with phosphoducin (β1H311Aγ2, β1R314Aγ2, and β1W332Aγ2) was tested, and only β1W332Aγ2 inhibited the ability of the dimer to stimulate PtdIns 3-kinase. Dimers containing the βγ subunit complexed with a panel of different Gγ subunits displayed variation in their ability to stimulate PtdIns 3-kinase. The β1γ12, β1γ10, β1γ12, and β1γ13 dimers all activated PtdIns 3-kinase about 26-fold with 4–25 nM EC50 values. The β1γ11 dimer, which contains the farnesyl isoprenoid group and is highly expressed in tissues containing the p101/p110γ form of PtdIns 3-kinase, was ineffective. The role of the prenyl group on the γ subunit in determining the activation of PtdIns 3-kinase was examined using γ subunits with altered CAAX boxes directing the addition of farnesyl to the γ subunit and geranylgeranyl to the γ1 and γ11 subunits. Replacement of the geranylgeranyl group of the γ subunit with farnesyl inhibited the activity of β1γ2 on PtdIns 3-kinase. Conversely, replacement of the farnesyl group on the γ1 and γ11 subunit with geranylgeranyl restored almost full activity. These findings suggest that all β subunits, with the exception of β2, interact equally well with PtdIns 3-kinase. In contrast, the composition of the γ subunit and its prenyl group markedly affects the ability of the βγ dimer to stimulate PtdIns 3-kinase.

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The generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the inner leaflet of the plasma membrane is critical to the regulation of cell function (1–3). The phosphorylated inositol head group provides a docking site for proteins containing pleckstrin homology domains (PH domains) and leads to activation of many enzymes including the phosphoinositide-dependent protein kinase and protein kinase B (Akt). Activation of protein kinase B regulates multiple cellular functions including differentiation, regulation of metabolic events, cell survival, and motility (1–3). In keeping with this central role, the level of PIP3 is tightly regulated, it can be elevated by multiple classes of receptors (1, 2), and there are specific phosphatidylinositol 5-phosphatases (SHIP and PTEN) that degrade the signal (4–8).

A large family of phosphatidylinositol 4,5-bisphosphate (PIP2) (3,4,5-trisphosphate) (PIP3) in the inner leaflet of the plasma membrane is critical to the regulation of cell function (1–3). The phosphorylated inositol head group provides a docking site for proteins containing pleckstrin homology domains (PH domains) and leads to activation of many enzymes including the phosphoinositide-dependent protein kinase and protein kinase B (Akt). Activation of protein kinase B regulates multiple cellular functions including differentiation, regulation of metabolic events, cell survival, and motility (1–3). In keeping with this central role, the level of PIP3 is tightly regulated, it can be elevated by multiple classes of receptors (1, 2), and there are specific phosphatidylinositol 5-phosphatases (SHIP and PTEN) that degrade the signal (4–8).

The Class 1B catalytic subunit also has a molecular mass of 110 kDa; its catalytic subunit is termed p110γ. The regulatory subunit for this isoform has a molecular mass of 101 kDa, has no recognizable protein-protein interaction domains, and dimerizes with the p110γ catalytic moiety (2, 9, 10). The p110γ form of the enzyme is highly expressed in cells of hematopoietic origin and is markedly activated by interaction with the Gγ dimer released after activation of receptors (9–12). Thus, ligands working through G protein-coupled receptors regulate the p110γ form of PtdIns 3-kinase and, hence, affect cell survival, reorganization of the cytoskeleton, cell shape changes, and cell migration, which are of central importance to the

1 The abbreviations used are: PIP3, phosphatidylinositol 3,4,5-trisphosphate; G proteins, guanine nucleotide-binding regulatory proteins; PtdIns 3-kinase, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; Sf9 cells, S. frugiperda cells; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; Genapol C-100, polyoxyethylene (10) dodecyl ether; HF, the hexahistidine-FLAG epitope tag; GTP•S, guanosine 5′-3-O-(thiodiphosphate); Ni2+-NTA, nickel nitritriacetic acid; PLC phospholipase C.
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biology of hematopoietic cells (13–16). Based on experiments performed with mice in which the p110γ isoform of PtdIns 3-kinase has been genetically ablated, this effector has multiple roles in the function of platelets, neutrophils, macrophages, mast cells, and monocytes (17–19). For example, neutrophils p110γ−/− mice show no increase in PIP3 levels upon stimulation of the fMet-Leu-Phe receptor, no activation of protein kinase B, and clear defects in the respiratory burst, cell shape changes, and migration (17–19).

The G protein-signaling pathway is surprisingly complex with large families of molecules comprising the receptors, G proteins, and effectors (20–22). Most cell types express multiple isoforms of each category, raising questions regarding the signaling specificity inherent in the multiple isoforms of these proteins (20–22). The isoforms of the G protein α and β subunits contribute extensively to the specificity of this signaling system. Receptors couple selectively to certain α subunits, and most effectors interact selectively with the different isoforms of the α and β subunits (20, 21, 23, 24). Thus, an important issue in cell signaling is identification of the protein-protein interactions underlying responses to G protein-coupled receptors.

Given the primary role of the G protein βγ subunit in the activation of the p110γ/p110γ form of PtdIns 3-kinase (9, 11, 12, 25), we investigated the ability of a complete spectrum of G protein α subunits and β subunits to activate the p110γ isoform of PtdIns 3-kinase. Using pure, recombinant proteins reconstituted into synthetic lipid vesicles, we find that the p110γ isoform responds selectively to the different βγ subunits and does not respond to activated α subunits. Moreover, the specific form of the γ subunit in the βγ dimer and the prenyl group on its C terminus play major roles in the activation of PtdIns 3-kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents used for SF9 cell culture and purification of α and βγ dimers have been described (26, 27). GDP, imidazole, and HEPES were from Sigma. Chaps and GTPγS were from Roche Applied Science. Genapol C-100 was from Calbiochem. Ni2+/NTA base buffer containing 50 mM MgCl2, 10 mM NaF, and 30 mM GTPγS, and the p101 subunit were kindly donated by Dr. Leonard R. Stephens, University of North Carolina (35). The baculoviruses expressing the G protein βγ subunits when reconstituted into Sf9 cell membranes (29, 31, 36). SDS pol, the extracts were applied to a Ni2+/NTA column. The Ni2+/NTA dimer was purified as described (29). Verification of the protein isolated was accomplished using matrix-assisted laser desorption ionization mass spectrometry as described (38). In keeping with our previous experience (34) most subunits in the dimers used in this study are phosphorylated and properly modified with the geranylgeranyl (γγγγγ) or farnesyl (γγγγγ) isoprenoid groups. Moreover, the γ subunits engineered to have altered CAAX sequences (e.g. γγγγγ/L71S) were shown to have the expected change in the prenyl group at the C terminus of the γ subunit (i.e. farnesyl on γγγγ/L71S) (34).

**Purification of G Protein βγ Subunits**—The βγ dimers were purified using a Gα γ affinity chromatography procedure that uses a His6-tagged α subunit to select properly modified G protein βγ subunits (37). SF9 insect cells were co-infected with the His6Gα γ baculovirus along with the specific β γ baculoviruses of interest, and the cell membranes were prepared as described and extracted with a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 0.1% Chaps, GTPγS, the National Center for Biotechnology Information (NCBI). The baculovirus for Gα γ proteins and the p110γ subunit were kindly donated by Dr. Leonard R. Stephens, University of North Carolina (35). The baculoviruses used in this study have been constructing the G protein baculoviruses used in this study have been constructed based on its ability to couple to recombinant receptors and the recombinant virus prepared as described (31). Sf9 cell membranes containing the G protein βγ subunits were extracted with 80 °C before G protein purification (see below). Pellets that were to be used for purification of PtdIns 3-kinase were processed immediately as freezing and thawing cell pellets released proteases active on the p110γ subunit (see below).

**Purification of G Protein γ Subunits**—The purification procedures used for purification of the α subunits of Gα γ were very similar; our methods for purification of Gα γ and GαGβγ have been described in detail (29, 31). Briefly, the desired Gα γ subunit was overexpressed in SF9 insect cells, with βγ subunits prepared by this procedure are published elsewhere (29, 31).

**Construction of Recombinant Baculoviruses**—The methods for constructing the G protein baculoviruses used in this study have been published as follows: Gα, Gα, Gα (28), Gα (29), βγγγ (30, 31), βγγγ (32), γγγγγγ (30), γγγγγγ (31). The baculovirus for γγγγγγ was the kind gift of Dr. David Siderovski, University of North Carolina (35). The baculoviruses encoding the “conformational change” mutants (H111A, R134A, W322A) and the “prenyl pocket” mutants (T299K, S331A, W339A, V315A, F335A, K373A) in the βγ subunit were prepared as described (36). The cDNAs encoding the porcine PtdIns 3-kinase p110γ subunit and the p101 subunit were kindly donated by Dr. Leonard R. Stephens, Cambridge University, Cambridge, UK (9), and used to construct new recombinant baculoviruses. The cDNA for the p110γ subunit was used to prepare a p110γ baculovirus with a FLAG epitope on the N terminus using complimentary synthetic oligonucleotides to create the flag sequence. The new cDNA was subcloned into the Xhol and EcoRI sites in the Sf9 transfer vector pAcSG2, and sequenced, and the recombinant virus was prepared via our standard techniques (26, 27). The cDNA for p110γ was modified to contain a hexahistidine tag on the N terminus using complimentary synthetic oligonucleotides, subcloned into the EcoRI and NotI sites in the Sf9 transfer vector pAcSG2, and sequenced, and the recombinant virus prepared as described (26, 27).

**Culture and Infection of SF9 Cells**—Spodoptera frugiperda cells (SF9 insect cells) were cultured and maintained at 27 °C as described (26, 27). Cell culture, viral amplification, and baculovirus infections for protein production were performed in the presence of 1.0% antibiotic/antimycotic (10,000 units/ml penicillin G, 10,000 mg/ml streptomycin, and 25 mg/ml amphotericin B). SF9 insect cells were infected at a multiplicity of infection of 3 with recombinant baculoviruses expressing G protein α and/or β subunits or with viruses for the p101 and the p110γ subunits of PtdIns 3-kinase prepared as described above. The incubations were allowed to proceed for 24–60–80 h or until cell viability fell to 80%, and the cells were harvested by centrifugation at 100 × g.

For the purification of G protein α and βγ subunits, the cell pellet was washed three times in insect cell phosphate-buffered saline (7.3 mM NaH2PO4, pH 6.2, 58 mM KCl, 47 mM NaCl, 5.0 mM CaCl2) and suspended in an ice-cold buffer composed of 25 mM HEPES, pH 7.5, 1 mM MgCl2, 120 mM NaCl, 1 mM 2-mercaptoethanol supplemented with a mixture of freshly prepared protease inhibitors (aprotinin, leupeptin, and pepstatin (at 2 mg/ml), benzamidine at 20 mg/ml, and Pefabloc SC Plus at 100 mg/ml) and frozen. Cell pellets were stored at this buffer at -80 °C before G protein purification (see below). Pellets that were to be used for purification of PtdIns 3-kinase were processed immediately as freezing and thawing cell pellets released proteases active on the p110γ subunit (see below).
Amicon Ultra 100 concentrator. A typical preparation yielded about 100 ng of pure, recombinant turkey PLCβ multilamellar vesicles. The large multilamellar vesicles were extruded through a 3 ml anti-FLAG-affinity gel column, and the column was washed with 30 ml (10 bed volumes) of 25 mM HEPES, pH 7.5, 3 mM MgCl₂, 120 mM NaCl, 1 mM EGTA, 0.1% (v/v) Triton X-100, 1.0% (w/v) betaine. The protein eluted from the Ni²⁺ column was immediately loaded onto a 3 ml anti-FLAG-affinity gel column, and the column was washed with 30 ml (10 bed volumes) of 25 mM HEPES, pH 7.5, 3 mM MgCl₂, 120 mM NaCl, 1 mM EGTA, 0.1% (v/v) Triton X-100, 1.0% (w/v) betaine plus the above protease inhibitors. The column was washed with 30 ml of the above buffer containing 1% (v/v) Triton X-100 then 30 ml of the buffer with 280 mM NaCl. The column was eluted with 10 ml of the above buffer containing 0.5 mg/ml FLAG peptide, and 1-ml fractions were collected. To determine the yield and purity of each preparation, fractions collected from the FLAG column were resolved on an 8% SDS gel and stained with Coomassie Blue (Invitrogen, “Simply Blue”). Fractions containing PtdIns 3-kinase were pooled and concentrated to ~300 ng/µl using an Amicon Ultra 100 concentrator. A typical preparation yielded about 100 µg of protein. The pure protein was stored at ~80 °C in the elution buffer.

**Assay of Phosphatidylinositol 4,5-Bisphosphate 3-Kinase—The activity of PtdIns 3-kinase was assayed using G protein α subunits or βγ dimers reconstituted into synthetic phospholipid vesicles.** Phospholipid vesicles were prepared at a molar ratio of 4:1 PE to PIP₂ (1 mM PE, 250 mM PIP₂) in a buffer containing 50 mM Hepes, pH 8.0, 80 mM KCl, 3 mM MgCl₂, 1 mM dithiothreitol. The phospholipids retained in the organic phase were resolved by thin layer chromatography on a potassium oxide-pretreated silica TLC plate (12). The mobile phase was 65 ml of i-propanol, 4 ml of glacial acetic acid, and 31 ml of deionized water. After development for 2-3 h, the plate was dried and subjected to autoradiography with Amersham Biosciences Hyperfilm MP. The silica plates were scraped using the autoradiograph as a guide to identify the [³²P]PIP₃, and the radioactivity was quantified by liquid scintillation counting.

**Assay of Phospholipase Cβ—**The activity of PLCβ was measured via the ability to produce Inositol trisphosphate from a PIP₂-containing reaction mixture using [³²P]ATP to monitor the extent of the reaction. The purified G protein α and βγ dimers were diluted in 20 mM Hepes, pH 7.5, and reconstituted at final concentrations of 0.1–300 nM into large unilamellar vesicles on ice for 30 min in a buffer containing 20 mM Hepes, pH 7.5, 1 mM MgCl₂, 150 mM NaCl, and 0.04% Chaps. The final Chaps concentration in the assay was kept below 0.04% because the activity of PtdIns 3-kinase is inhibited by concentrations of Chaps above 0.04%. The assay was started by the addition of 10 ng of PtdIns 3-kinase to a 50-µl reaction mixture containing 20 mM Hepes, pH 7.5, 1 mM dithiothreitol, 50 mM ATP, 3 mM MgCl₂, 1 mg/ml bovine serum albumin, and 10 µCi of [³²P]ATP and incubated for 15 min at 30°C. During the development of this assay it was found that bovine serum albumin concentrations ranging from 0 to 1.0 mg/ml did not affect the basal or Gβγ-stimulated activity of PtdIns 3-kinase. The rate of PIP₃ production was linear for 30 min. The reaction was stopped by the addition of ice-cold 1 N HCl, and the lipids were extracted with CHCl₃/MeOH (2:1 v:v). The samples were vortexed and centrifuged at 4 °C at 1500 × g for 2 min, the aqueous phase was aspirated off, and the organic phase was retained. The organic phase was washed with ice-cold 1 N HCl followed by a vortex/centrifugation cycle. Samples of the phospholipids retained in the organic phase were resolved by thin layer chromatography on a potassium oxide-pretreated silica TLC plate (12). The mobile phase was 65 ml of l-propanol, 4 ml of glacial acetic acid, and 31 ml of deionized water. After development for 2-3 h, the plate was dried and subjected to autoradiography with Amersham Biosciences Hyperfilm MP. The silica plates were scraped using the autoradiograph as a guide to identify the [³²P]PIP₃, and the radioactivity was quantified by liquid scintillation counting.

**Activation of G Protein α Subunits—Activation of the Ga subunits before reconstitution into the phospholipid vesicles used in the PLCβ and PtdIns 3-kinase assays was achieved by diluting the various α subunits to a final concentration of 1000 nM in a buffer containing 50 mM Hepes, pH 8.0, 1 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 1% sodium cholate, 1 mM dithiothreitol, and 1 mM GTP·S. The α subunits were incubated in this solution at 30 °C for 30 min before the addition to the unilamellar phospholipid vesicles. The final concentration of the α subunits reconstituted into the vesicles was 0.1–300 nM.** The protocols for PtdIns 3-kinase and PLCβ assays as described above were followed with the exception that a final concentration of 10 mM NaF and 20 µM AICl₃ was added to each incubation at the time the PtdIns 3-kinase or avian PLCβ was added to the vesicles.

**Gal Electrophoresis and Immunoblotting—**The identity and purity of protein samples were confirmed by gel electrophoresis followed by silver staining and/or by Western blot analysis. Preparations were resolved on 8 or 12% SDS gels, and the gels were stained with Coomassie Blue or silver transferred to nitrocellulose membranes. Visualization of proteins on Western blots with specific antisera was performed using the ECL chemiluminescence system (Amersham Biosciences).

**Calculations, Statistics, and Expression of Results—**The experiments presented under “Results” are representative of four or more experiments. The EC₅₀ values presented in Table 1 were obtained by fitting the points to sigmoidal curves using the routines provided in the Graph Pad Prism software. The statistical significance of the results of different data sets was determined on normalized data using the F statistic (41). Data were normalized because the V₅₀ of PtdIns 3-kinase varied. Differences in the maximal effect of βγ dimers on PtdIns 3-kinase activity were determined by the paired t test.

**RESULTS—**

Pure, recombinant G proteins, and PtdIns 3-kinase were reconstituted into unilamellar synthetic lipid vesicles to investigate the ability of various G protein α subunits and βγ isoforms to stimulate PtdIns 3-kinase activity. All proteins were expressed in Sf9 insect cells infected with recombinant baculoviruses and purified as described under “Experimental Procedures.” The data in Fig. 1 demonstrate the purity of these proteins and the ability of the βγ dimer to markedly activate PtdIns 3-kinase. Fig. 1A presents a silver-stained polyacrylamide gel resolving typical preparations of Gβ₁γ₂ and Gβ₁γ₁₁, and Fig. 1B demonstrates the purity of the p101/p110γ form of PtdIns 3-kinase. Fig. 1C presents a section of an autoradiograph demonstrating the resolution of the [³²P]-labeled PIP₃ from other components in the vesicle assay used to monitor PtdIns 3-kinase activity. Fig. 1D shows the low background activity in the assay and shows that β₁γ₂ can activate PtdIns 3-kinase more than 20-fold with an EC₅₀ of ~5 nM. This result agrees with the literature (9, 12).

It has been recognized that the βγ dimer markedly activates the p110γ form of PtdIns 3-kinase (9, 11, 12); however, there are differing reports about the effect of G protein α subunits on the activity of this enzyme. The original report describing the cloning of the p110γ isoform reported that the Gα and Gα subunits purified from bovine brain and retina, respectively, caused a stimulation of PtdIns 3-kinase (42); however, subsequent work has suggested that Gα subunits do not activate the enzyme (9–11). To examine this issue with pure, recombinant proteins, we prepared four different α subunits from the Gα, Gα and Gα families by purifying them from a βγ affinity column and examined their ability to activate the enzyme. The α subunits were activated using a 30-min incubation with GTP·S and reconstituted into synthetic lipid vesicles, and the activity of PtdIns 3-kinase activity was measured in the presence of AIF. Fig. 2A compares the ability of pure, recombinant Gα, Gα, and Gα α subunits to activate PtdIns 3-kinase with that of β₁γ₂. None of the α subunits tested activated PtdIns 3-kinase, whereas β₁γ₂ stimulated the enzyme more than 20-fold. To ensure that the protocols used to activate the α subunits were effective, we examined the ability of an aliquot of the activated Gα to stimulate its effector, PLCβ, in a lipid vesicle assay using avian PLCβ (34). This assay was performed at the...
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FIG. 1. Purification of PtdIns 3-kinase and Gβγ from SF9 cells. A, silver-stained 12% SDS-polyacrylamide gel demonstrating the purity of two representative βγ dimers, βγ2 and βγ71. All dimers used in this study had similar purity. The proteins were purified as described under “Experimental Procedures.” Molecular mass calibration in kDa (MW) is shown at the right. B, silver-stained 8% SDS-polyacrylamide gel demonstrating the purity of the p110/p110γ form of PtdIns 3-kinase (PI3K). The enzyme was purified from the extracts of SF9 cells as described under “Experimental Procedures.” Molecular mass calibration in kDa (MW) is shown at the right. The p101 and p110γ subunits migrate slightly higher than their indicated molecular masses because of the N-terminal epitope tags. C, section of autoradiograph made with Amersham Biosciences Hyperfilm MP from the potassium oxalate-pre-treated silica TLC plate resolving [32P]PIP3 from other components in the PtdIns 3-kinase assay. The enzyme was stimulated with increasing concentrations of the βγ2 dimer. The migration position of [32P]PIP3 is shown by the band, and the migration positions of PE, phosphatidylinositol (PI), and PIP2 standards are indicated by arrows. The TLC plate was loaded and developed as described under “Experimental Procedures.” D, activation of PtdIns 3-kinase by Gβγ reconstituted into synthetic lipid vesicles. Large unilamellar phospholipid vesicles were prepared at a molar ratio of 4:1 PE to PIP2 (1 mM PE, 250 μM PIP2) as described under “Experimental Procedures.” The βγ71 dimer (open circles) was reconstituted into the vesicles at the indicated concentrations, and the activity of PtdIns 3-kinase was measured as the ability to produce PIP3 from PIP2 using [32P]ATP to monitor production of radio-active PIP3.

same time as the PtdIns 3-kinase assay. Fig. 2B indicates that the activated Gq α subunit can stimulate avian PLCβ activity about 10-fold and equally with the βγ2 dimer. Activation of Gq, α provides a stringent control, as the purified form of this α subunit slowly exchanges GTP and is difficult to activate (43). As expected, the Gγ, Gq, and Gz α subunits did not activate PLCβ (44). Each of the preparations of G α subunit used in these experiments was tested for its ability to couple to the appropriate recombinant receptors expressed in SF9 cell membranes (31, 45). All of the Gz subunits were active proteins in this assay. The Gq α subunit was also able to stimulate type I and type II adenyl cyclase (31).

To explore the question of whether the many isoforms of the Gβγ dimer differentially activate PtdIns 3-kinase, we prepared a panel of 10 recombinant βγ dimers. The 7 known β subunits fall into two subfamilies, one comprised of β1–4 and one containing β5 and β11. The γ subunits separate into 5 subfamilies, one with γ1,8,11, one containing γ3,4,9, one comprised of γ7,12, and another containing γ6,10 (22, 46). Gγ13 is quite divergent and forms its own subfamily (22, 46) but seems to have functional activities comparable with γ2 (35). To minimize the complexity of exploring all 84 possible combinations of the existing β and γ subunits, we chose subunits known to be effective activators of PtdIns 3-kinase such as β1 and γ2 and selected β or γ isoforms from each of the subfamilies to compare with these benchmarks. Thus, dimers were prepared containing the β1γ1 subunits, each complexed with γ2 as the partner, and a panel containing different γ subunits was prepared with the β1 subunit.

The effects of these 10 different βγ dimers on PtdIns 3-kinase activity are presented beginning in Fig. 3. In agreement with previous experiments (47), Fig. 3 illustrates that dimers containing the β1γ3 subunits complexed with the γ2 subunit all activate PtdIns 3-kinase with EC50 values ranging from 4 to 7 nm. Interestingly, the β1γ2 dimer activates PtdIns 3-kinase activity to a greater extent than the β1γ2 dimer; this difference has also been seen with type II adenyl cyclase (31). As expected (47), the β1γ2HF dimer did not activate PtdIns 3-kinase at concentrations up to 100 nM. In experiments not shown, 300–400 nM β1γ2HF did not activate the enzyme. Importantly, this particular preparation of β1γ2HF was an active molecule, as indicated by its ability to activate PLCβ 10-fold (not shown), an activation equivalent to that seen with β1γ2 in Fig. 2B. The EC50 values of the five βγ dimers for activation of PtdIns 3-kinase are presented in Table I. The values range from 4 to 7 nM and are not statistically different from each other.

To investigate the regions of the β subunit that might be important for activating PtdIns 3-kinase, we made two types of mutations in the β1 subunit. These mutations have been termed the conformational change and prenyl pocket mutations (36). Based on the two x-ray structures of βγ, complexed with phosducin, three amino acids (His-311, Arg-314, and Trp-332) in the β subunit undergo significant conformational changes when the dimer binds to phosducin (48, 49). Two Gγ1 subunits containing point mutations in which these amino acids were changed to alanine (H311A and W332A) bind phosducin and phosducin-like-protein less well than the native β1γ2 activity used...
vesicles, and the activity of PtdIns 3-kinase measured as described under "Experimental Procedures." Ten ng of pure PtdIns 3-kinase was added to each assay. Open circles, β1γ2; closed squares, β2γ2; closed triangles, β1γ2; open diamonds, β1γ2; open inverted triangles, β1γ2.

TABLE I

EC50 values for activation of PtdIns 3-kinase by isoforms of Gβγ

The various forms of recombinant Gβγ were purified from baculovi-

rus-infected Sf9 cells as described under "Experimental Procedures." PtdIns 3-kinase activity was measured by reconstituting pure Gβγ isoforms into unilamellar, synthetic lipid vesicles containing PIP2, and γ-32P-labeled ATP, adding 10 ng of pure PtdIns 3-kinase, and generating [32P]PiP2 as described under "Experimental Procedures." The EC50 values were calculated with the Graph Pad Prism curve-fitting program as described under "Experimental Procedures." The EC50 value for each different βγ dimer was compared against that of β1γ2. Statistical differences between the fitted curves were determined using the F-statistic. NA, not applicable.

| Dimer         | EC50 | Range  |
|---------------|------|--------|
| β Series      |      |        |
| β1γ2          | 5.6  | 4.6–6.8|
| β2γ2          | 6.8  | 4.1–7.5|
| β1γ3          | 5.3  | 3.0–9.3|
| β2γ3          | 4.2  | 2.7–6.5|
| β1γ2·HF       | ~310 | NA     |
| γ Series      |      |        |
| β1γ1          | 16   | 9.4–27 |
| β2γ1          | 5.6  | 4.6–6.8|
| β1γ10         | 24.9 | 13–49  |
| β2γ10         | 12   | NA     |
| β1γ2          | 3.26 | 2.2–4.6|
| β2γ2          | 8.0  | 5.8–11 |
| β-Mutants (conformational change) |      |        |
| β1H331Aγ2     | 7.4  | 5.9–9.0|
| β2R314Aγ2     | 3.5  | 2.1–5.9|
| β1W332Aγ2     | 11.9 | 8–18   |
| Prenyl switch mutants |      |        |
| β1γ2          | 5.6  | 4.6–6.8|
| β1γ2S71L      | 35.9 | 27–46  |
| β1γ1          | 16   | 9.4–27 |
| β1γ2S74L      | 52.3 | 39–68  |
| β2γ11         | 12   | NA     |
| β1γ2S74L      | 53.3 | 37–75  |
| Prenyl pocket mutants |      |        |
| β1γ2          | 5.6  | 4.6–6.8|
| β2T298Yγ2     | 4.5  | 3.7–5.4|
| β2S331Aγ2     | 2.4  | 1.2–5.2|
| β2W339Aγ2     | 8.7  | 2.8–26 |
| β2V315Aγ2     | 33.7 | 17–65  |
| β2F354Aγ2     | 13.8 | 9.3–21 |
| β2K337Aγ2     | 17.1 | 11–26  |

*Significant difference in the EC50 value (p < 0.001).

or β1γ2 dimers (50). In addition, dimers with Gβ subunits containing mutations in any of these three residues are 25–100-fold less potent than native β1γ2 dimers at activating type II adenyl cyclase or PLCβ (36). In contrast, dimers containing point mutations at any of these three residues are equally effective as the β1γ2 dimer in supporting coupling of the adenosine A1 receptor to the Gα subunit (36). This result and the fact that the dimers are purified from a Go subunit column suggest that the β subunits with these point mutations are properly folded.

Dimers containing β2 subunits harboring these three mutations were purified in a complex with the γ2 subunit and examined for their ability to activate PtdIns 3-kinase. The EC50 value for the Trp-332 mutation is shifted to the right about 4-fold, to 12 nm (Table I). Interestingly, dimers with β2 subunits harboring the Trp-332 mutation are uniformly less active in assays of multiple effectors including PLCβ, type II adenyl cyclase, K+ channels, Ca2+ channels, and binding to phosducin (50–53). However, even though this residue is also important for binding of the α subunit to the β subunit (54), the W332A mutation does not appear to inhibit the A1 adenosine receptor Gαβγ interaction to a significant extent (38).

Representative βγ dimers containing members of the 5 families of γ subunits had much larger differences in their activity on PtdIns 3-kinase. The data in Fig. 5 indicate that dimers containing the γ2, γ12, and γ18 subunits were about equally effective in activating PtdIns 3-kinase with EC50 values ranging from 4 to 7 nm. Dimers containing the γ16 subunit were statistically less effective, and dimers containing γ11 did not effectively activate the enzyme. The EC50 values for all combinations tested are shown in Table I. Note that the weak activation of PtdIns 3-kinase caused by βγ11 had an EC50 value of about 12 nm, suggesting that it bound to PtdIns 3-kinase with reasonable affinity but could not cause the conformational change needed for increased activity.

In addition to differences in amino acid sequence, a major difference between γ11 and the other γ subunits used in these experiments is that γ11 contains a C15 farnesyl group on its C terminus (34). Multiple studies indicate that the composition of the prenyl group on the C terminus of the γ subunit is very important for the interaction of the βγ dimer with receptors and effectors (34, 55–58). For this reason we tested the role of the prenyl group in the activation of PtdIns 3-kinase by using γ subunits that contained altered CAAX boxes to direct the
addition of a C15 farnesyl group to \( \gamma_2 \) (\( \gamma_2\)-L718) or a C20 geranylgeranyl group to \( \gamma_1 \) (\( \gamma_1\)-S74L) or \( \gamma_1\)-S74L. Thus, the amino acid sequence of the \( \gamma \) subunit is not altered. Dimers containing \( \gamma \) subunits with these mutations have been tested for their activity on PLC\( \beta \) and type II adenyl cyclase with the result that dimers containing the C15 farnesyl group are less active than those containing the geranylgeranyl group (34).

The data presented in Fig. 6A indicate that switching the prenyl group on \( \gamma_2 \) from geranylgeranyl to farnesyl shifts the activation of PtdIns 3-kinase about 1 log to the right (EC\(_{50}\) from 5.6 to 35 nM, see Table I). As expected (47), Fig. 6B indicates that the \( \beta_1\gamma_1 \) dimer, like the similar \( \beta_1\gamma_2 \) dimer, poorly activates PtdIns 3-kinase. More importantly, switching the farnesyl group on \( \gamma_1 \) (or \( \gamma_2 \)) to the geranylgeranyl group produces dimers that are nearly as active as the native \( \beta_1\gamma_2 \). Note from Fig. 6B that \( \beta_1\gamma_1\)-S74L and \( \beta_1\gamma_1\)-S74L can activate PtdIns 3-kinase to about the same extent as \( \beta_1\gamma_2 \). However, they are still less potent at activating the enzyme (EC\(_{50}\) values of about 53 nM). The requirement of the geranylgeranyl group for proper activation of PtdIns 3-kinase is very similar to the near absolute requirement for the geranylgeranyl group in the stimulation of type II adenyl cyclase by \( \beta \) dimers (34). Importantly, previous studies show that dimers containing \( \gamma \) subunits modified with either the farnesyl or geranylgeranyl groups intercalate into phospholipid vesicles equally well (40), suggesting that the type of prenyl group on the \( \gamma \) subunit is very important for the interaction with and activation of PtdIns 3-kinase.

One x-ray structure of the \( \beta_1\gamma_1 \) dimer bound to phosducin indicates that the prenyl group may fold into a pocket in the seventh blade on the surface of the \( \beta_1 \) subunit (48). Mutations of the residues in this area designed to destabilize the binding of the prenyl group in its pocket (F335A, K337A, V315A, S331A, W339A, and T329K in \( \beta_1 \)) reduce the ability of \( \beta_1\gamma_2 \) to activate PtdIns 3-kinase and PLC\( \beta \), leading to the development of \( \beta_1\gamma_1\)-S74L (47). Point mutations at Val-315, Thr-329, and Trp-339 in \( \beta_1 \) also reduce the affinity of the interaction between \( \beta_1\gamma_1 \) or \( \beta_1\gamma_2 \) and phosducin (50). Accordingly, \( \beta_1\gamma_2 \) dimers containing mutations in this area of the \( \beta \) subunit were tested for their ability to activate PtdIns 3-kinase. The data in Fig. 7 indicate that the effects of these prenyl pocket mutations fall into two groups. Dimers containing \( \beta_1\)T329K\( \gamma_2 \) and \( \beta_1\)S331A\( \gamma_2 \) and \( \beta_1\)W339A\( \gamma_2 \) activate PtdIns 3-kinase with EC\(_{50}\) values equal to that observed with the \( \beta_1\gamma_2 \) dimer. Dimers containing the F335A, K337A, and V315A point mutations shift the EC\(_{50}\) for activation of PtdIns 3-kinase about 4–5-fold to the right (see Table I). A slightly larger rightward shift in EC\(_{50}\) was observed in the ability of dimers harboring these mutations to activate type II adenyl cyclase and PLC\( \beta \) (36). The observation that they cause a right shift in the ability of the dimer to activate PtdIns 3-kinase suggests that the prenyl group also plays an important role in the interaction of the \( \gamma \) subunit with this enzyme.

**DISCUSSION**

The family of heterotrimeric G proteins is composed of \( \alpha \), \( \beta \), and \( \gamma \) subunits with many isoforms of each subunit expressed in most cells. The \( \alpha \) subunits are expressed from 17 different genes and, through the splice variants that exist in cells, form 22 different \( \alpha \) subunits. These subunits separate into four major families (21, 22, 46). The multiple \( \alpha \) subunits couple to the superfamily of seven transmembrane domain receptors, which initiate signaling from light, odorants, hormones, neurotransmitters, and autacoids (21, 23). The \( \beta \) and \( \gamma \) subunits form a dimer that is an integral and important part of the G protein-signaling mechanism. The \( \beta \gamma \) dimer is required for the receptor to form a high affinity complex with agonists (59–61),
for the receptor to efficiently catalyze the exchange of GTP for GTP on the $\alpha$ subunit, and as the mediator of signaling to multiple downstream targets (20, 21). The $\beta$ subunits are expressed from 5 genes (with 2 splice variants, $\beta_{38}$ and $\beta_{37}$), and the $\gamma$ subunits are expressed from 12 genes. The $\beta$ subunits separate into 2 families, one containing $\beta_{1,2}$, which are about 90% identical, and one containing $\beta_{3,4}$, which are only 50% similar to $\beta_1$ (22, 46). The $\gamma$ subunits can be divided into 5 subfamilies, one subfamily consists of $\gamma$ subunits that are modified with farnesyl (C15) at their C terminus ($\gamma$ subunits 1,8, and 11) and three other subfamilies containing geranylgeranyl (C20) at their C terminus. The second subfamily is comprised of $\gamma$ subunits 2,3,4,9, whereas the third and fourth subfamilies only consist of two members each, $\gamma$ subunits 7 and 12, and $\gamma$ subunits 5 and 10, respectively. $G_{\gamma 13}$ is the most divergent $\gamma$ and forms its own subfamily (22, 46) but has signaling properties similar to $\gamma_2$ (35).

The $\beta\gamma$ dimer has been demonstrated to regulate more than 20 effectors including ion channels, adenyl cyclase, phospholipase C, tyrosine kinases, and PtdIns 3-kinase (20, 21). The large number of targets for the $\beta\gamma$ dimer, and the possibility that the many different isoforms of the dimer represent selective signals make it important to determine the signaling specificity inherent in these molecules. The generation of PIP3 in the membrane of hematopoietic cells such as neutrophils, mast cells, and macrophages is markedly increased by $G_i$-linked receptors (20, 62). This finding is consistent with the idea that $G_i$-linked receptors, $G_{\alpha}$ subunit, or $G_\text{q}\alpha$ subunits can activate PtdIns 3-kinase equally. As expected (47), the $G_{\alpha}$ subunit was not able to activate the enzyme (Fig. 2B), yet $G_{\alpha}$-linked receptors such as the fMet-Leu-Phe or C5a receptors markedly activate the p110/p110$\gamma$ form of PtdIns 3-kinase in mast cells, neutrophils, and macrophages (3, 17–19, 63, 64). Thus, it seems highly unlikely that dimers containing the $G_{\gamma 11}$ subunit are released after activation of fMet-Leu-Phe or C5a receptors. Activation of these receptors must release a dimer such as $G_{\beta 2}$, which is able to activate PtdIns 3-kinase 20–40-fold. Interestingly, dimers containing $G_{\gamma 11}$ are not very effective at activating type II adenyl cyclase (34) or regulating ion channels (68). Because this $\gamma$ subunit is widely expressed (69), these observations suggest that cells containing $G_{\gamma 11}$ could use dimers containing $G_{\gamma 11}$ to couple $\alpha$ subunits to receptors, allowing receptors to activate $\alpha$ subunits without inducing effects on downstream $\beta\gamma$ targets. Taken further, this argues that receptor activation may release specific $\beta\gamma$ dimers, a concept that gains experimental support from biochemical experiments (31, 71–73), antisense mRNA experiments (74, 75), small interfering RNA experiments (76), and data obtained with knockout mice (77).

Interestingly, activation of receptors coupled to the $G_{\alpha}$ subunit are able to inhibit the activation of neutrophils, mast cells, eosinophils, and platelets (78–81). The inhibition is due in part to a reduction in the PIP3 signal (82, 83). Thus, in hematopoietic cells, receptors coupled to $G_i$ provide stimulatory inputs, and receptors coupled to $G_{\alpha}$ provide negative inputs into a major biological process. However, activation of $G_{\alpha}$ must also release some isoform of the $\beta\gamma$ dimer, posing a potential question, Why would an inhibitory pathway release a signaling molecule (the $\beta\gamma$ dimer) that can markedly activate the very pathway to be inhibited? Thus, it seems logical that the $\beta\gamma$ dimer released by activation of $G_{\alpha}$-coupled receptors would not be able to stimulate PtdIns 3-kinase.

There are many possible reasons that $\beta\gamma$ dimers released from $G_{\alpha}$ may not be able to stimulate PtdIns 3-kinase. The dimer might be compartmentalized (84), it could be released in too low a concentration to activate the effectors, or it might be part of a scaffolding complex that restricts its activity (85). Another interesting possibility is that the dimer which couples to $G_{\alpha}$ in these cells may contain $\beta$ or $\gamma$ subunits that do not interact well with the effectors. A significant amount of data suggests that distinct $\beta\gamma$ isoforms can selectively regulate effectors (22, 86). For example, the $\beta_5\gamma_2$ dimer is able to inhibit the T-type Ca$^{2+}$ channel, whereas the $\beta_1\gamma_2$ dimer is ineffective.

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$^{2}$ W. E. McIntire and J. C. Garrison, unpublished data.
(68), the \( \beta_{7}\gamma_{7} \) dimer inhibits the GIRK 1.4 K^+ channel, whereas dimers containing the other four \( \beta \) subunits activate the channel (87), and \( \beta_{5}\gamma_{7} \) dimers appear to be important for stimulating PLC\( \beta \) and producing Ca\(^{2+}\) transients in mouse macrophages whereas dimers containing the \( \beta_{1} \) subunit are not effective (76).

Another body of literature indicates that specific \( \beta \) dimers may be released after activation of specific receptors. Examples include the original antisense experiments performed in GH3 cells, suggesting that specific \( \beta \) isoforms couple to muscarinic and somatostatin receptors (74, 75, 88), results from mice in which the \( \gamma_{11} \) gene has been ablated, showing that loss of \( \gamma_{11} \) causes loss of the Golf \( \alpha \) subunit, resulting in specific changes in cyclase activity in the striatum (77), reconstitution studies showing a preference of certain receptors for specific \( \beta \) dimers (31, 70–73), and results using small interfering RNA to knock down the \( \beta_{2} \) subunit in the J774.A1 macrophage line, showing loss of this subunit blunts the ability of the C5a receptor to stimulate a rise in intracellular Ca\(^{2+}\) (76). Our own data show that dimers containing \( \gamma_{11} \) support coupling of \( \alpha \) subunits to receptors very well (70), yet these dimers are very poor at regulating effectors such as PtdIns 3-kinase (Figs. 5 and 6), type II adenyl cyclase (34), PLC\( \beta \) (34), and T-type Ca\(^{2+}\) channels (68). Taken together these results suggest the possibility that activation of specific receptors may release specific \( \beta \) dimers. Based on our results, if the \( \beta_{2} \) dimer released by receptor activation is \( \beta_{2}\gamma_{11} \), it is not likely to activate effectors. This hypothesis suggests that receptor activation may not always release a \( \beta \) dimer that acts on downstream signaling pathways. This possibility would restrict receptor activation of effectors to those able to interact with that specific subunit coupled to the receptor and provides interesting avenues for future experiments in intact cells.

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