A Chemical Biology Approach Demonstrates G Protein βγ Subunits Are Sufficient to Mediate Directional Neutrophil Chemotaxis*

Received for publication, April 25, 2014, and in revised form, May 6, 2014 Published, JBC Papers in Press, May 7, 2014, DOI 10.1074/jbc.M114.576827

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Background: G protein βγ (Gβγ) subunits are required for chemokine-dependent directional chemotaxis.

Results: A chemical activator of Gβγ signaling activated Gβγ signaling and induced directional chemotaxis of neutrophils.

Conclusion: Gβγ signaling is sufficient to induce directional chemotaxis of neutrophils.

Significance: Demonstrates that G protein-coupled receptor signals other than Gβγ are not required for directional migration of neutrophils in response to a gradient.

Our laboratory has identified a number of small molecules that bind to G protein βγ subunits (Gβγ) by competing for peptide binding to the Gβγ “hot spot.” M119/Gallein were identified as inhibitors of Gβγ subunit signaling. Here we examine the activity of another molecule identified in this screen, 12155, which we show that in contrast to M119/Gallein had no effect on Gβγ-mediated phospholipase C or phosphoinositide 3-kinase (PI3K) γ activation in vitro. Also in direct contrast to M119/Gallein, 12155 caused receptor-independent Ca2+ release, and activated other downstream targets of Gβγ including extracellular signal regulated kinase (ERK), protein kinase B (Akt) in HL60 cells differentiated to neutrophils. We show that 12155 releases Gβγ in vitro from Gαi,βγ, heterotrimeric by causing its dissociation from GoGDP without inducing nucleotide exchange in the Gα subunit. We used this novel probe to examine the hypothesis that Gβγ release is sufficient to direct chemotaxis of neutrophils in the absence of receptor or G protein α subunit activation. 12155 directed chemotaxis of HL60 cells and primary neutrophils in a transwell migration assay with responses similar to those seen for the natural chemotactic peptide n-formyl-Met-Leu-Phe. These data indicate that release of free Gβγ is sufficient to drive directional chemotaxis in a G protein-coupled receptor signaling-independent manner.

G protein-coupled receptors (GPCR) are transmembrane proteins that regulate a variety of cellular and physiological processes. Upon activation GPCRs bind G protein heterotrimers, consisting of GoGDP and Gβγ subunits, and catalyze nucleotide exchange and GTP binding to the Gα subunit (1). This results in functional dissociation of the GTP-bound Go subunits from Gβγ subunits. The free G protein subunits bind directly to various target proteins initiating activation of downstream signaling pathways. Gβγ subunits bind most effectors at a common surface that is obscured by GoGDP in the inactive state known as the “hot spot” (2, 3). Although effectors tend to share this binding surface, each interacts with specific subsets of amino acids on Gβγ (3–5). These differential binding modes for effectors have been effectively exploited in the discovery of peptides and small molecules that prevent activation of some effectors by Gβγ, without affecting the regulation of others (4, 6).

Directional migration of cells toward a chemotactic stimulus is required for tissue formation, wound healing, and immune responses. Chemokine receptors are a large family of GPCRs that respond to gradients of chemokine chemoattractants to mediate directional migration of cells (7). The majority of chemokine receptors are coupled to the G12/13 family of G proteins and transduce signals through Gβγ released from Gi heterotrimers (8). Multiple pathways are activated by Gβγ including phospholipase Cβ (PLCβ) and phosphoinositide 3-kinase γ (PI3Kγ) (9, 10). PI3Kγ activation at the leading edge of immune cells downstream is an important determinant of cell polarity and directional migration through generation of a gradient of phosphatidylinositol 3,4,5-trisphosphate (PIP3). Gβγ also directly activates cdc42 by binding to PAK, causing F-actin localization and inhibition of phosphatase and tensin homolog at the leading edge (11).

In addition to Gβγ signals there are also reports that Go13–mediated Rho activation leads to migration of MEFs and cancer cells and that Go13 plays a role in neutrophil migration at the trailing edge of migrating cells (12, 13). Along with G protein subunits themselves, regulators of G protein signaling (RGS proteins) were also shown to be mediators of cell migration in B lymphocytes (23). Recently, β-arrestin and GRK, which are mediators of the receptor desensitization pathway, have also been shown to play a role in cell migration (14–17). All these proteins are part of the GPCR signaling system, and may play a part in regulating chemoattractant-directed cell migration.

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* This work was supported, in whole or in part, by National Institutes of Health Grant R01GM081772 (to A. V. S.).

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‡ The abbreviations used are: GPCR, G protein-coupled receptor; PLC, phospholipase C; PTK, pertussis toxin; PIPγ, phosphatidylinositol 3,4,5-trisphosphate; RGS, regulators of G protein signaling; bGβγ, biotinylated Gβγ subunits; fMLP, n-formyl-Met-Leu-Phe; ELISA, enzyme-linked immunosorbent assay; SIGK, peptide SIGKAFKILGYPDYD; GTPγS, guanosine 5′-γ-thio)triphosphate; SPR, surface plasmon resonance; DMSO, dimethyl sulfoxide; HBSS, Hanks’ balanced salt solution.
Although it is clear that Gβγ is required for directed migration in response to chemotactic peptides, chemotactic GPCR signaling is complex and it is unclear to what extent Gβγ signaling on its own is sufficient drive directional migration of cells, independently of the GPCR, GPCR modulating proteins, or parallel GPCR-driven signaling pathways.

Here we used a chemical biology approach to address these questions. The small molecule 12155 (NSC12155) was previously identified in a screen of the National Cancer Institute diversity library for Gβγ-binding molecules (4). Here we show that 12155 binds to Gβγ, but does not inhibit signaling from Gβγ, rather it displaces GoGDP thereby activating Gβγ-mediated signal transduction without activating GPCRs, Ga subunits, or associated pathways. Using this approach we demonstrate that Gβγ signaling in neutrophils is sufficient to mediate directional chemotaxis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Small molecules were obtained from the National Cancer Institute/DTP Open Chemical Repository. Molecules were abbreviated as follows: 12155 (NSC12155) (1,3-bis(4-amino-2-methylquinolin-6-yl)urea) and M119 (NSC119910) (2-(4,5,6-trihydroxy-3-oxo-3H-xanthan-9-yl)-cyclohexane-1-carboxylic acid). The molecules were prepared as 50 mM stocks in dimethyl sulfoxide (DMSO). Wortmannin was obtained from Acros Organics (Geel, Belgium). Pertussis toxin (PTX), formyl-Met-Leu-Ph (fMLP), DMSO, and GTPγS were purchased from Sigma. U73122 and U73343 were obtained from Calbiochem. [d-Ala²,N-Me-Phe³,Gly⁵-ol]-enkephalin (DAMGO) was purchased from Bachem (Torrance, CA). Brain phosphatidylinositol 4,5-bisphosphate (PIP₂), liver phosphatidylinositol (PI), and liver phosphatidylethanolamine were from Avanti Polar Lipids (Alabaster, AL). [³⁵S]GTPγS and [³²P]-inositol]PIP₂ were purchased from PerkinElmer Life Sciences.

**Cell Culture**—HL60 cells were cultured in RPMI 1640 medium (Invitrogen) containing 10% FBS and 1% penicillin/streptomycin and differentiated at 0.2 x 10⁶ cells/ml for 4–6 days with 1.2% DMSO.

**Isolation of Mouse Neutrophils**—Neutrophils were obtained from the bone marrow of adult C57 Bl6 mice. All procedures were carried on ice using ice-cold buffers. Bone marrow was flushed with PBS, pH 7.4, and red blood cells (RBCs) were lysed using ACK lysis buffer. The white blood cells were separated from the lysed RBCs by centrifugation at 1400 x g for 3 min. The cells were counted and 10⁸ cells/ml were used for further isolation. Pure neutrophils were isolated using the neutrophil negative selection kit (StemCell).

**Purification of Wild Type or Biotin-tagged Gβγ**—Purification of Gβ₁γ₂ and in vivo biotinylated Gβ₁γ₂ (bGβγ) was done as described by coexpressing Gβγ with His₆ Goα₁ in High Five insect cells and nickel-agarose chromatography (18, 19).

**Purification of Goα₁-GFP—GFP-Goα₁ was expressed and purified from High Five cells by modification of a previously described method (19).** High Five cells at 1.5 x 10⁶ cells/ml were infected with viruses encoding Gβ₁, His₆ Gγ₂, and Goα₁-GFP, and grown for 60 h with continuous shaking at 27 °C. GFP was inserted between amino acids 122 and 123 of Goα₁. The cells were harvested and membrane extracts were loaded on an nickel-nitritoltriacetic acid column. After washing, Goα₁-GFP was eluted using AlF₄⁻ and MgCl₂. The eluted fractions were analyzed by SDS-PAGE, Coomassie Blue staining, Western blotting, and fluorimetric detection of the GFP signal, and fractions containing 80% pure GFP-Goα₁ were pooled, snap frozen in liquid N₂, and stored at −80 °C.

**SIGK Competition Assay—**Compounds were preincubated with 20 nM bGβ₁γ₂ in a 384-well plate. F88 phage displaying the peptide SIGKAFKILGYPDYD was added. After 15 min, anti-M13 antibody was added and incubated 1 h. The complexes were then bound to streptavidin-coated AlphaScreen donor beads (PerkinElmer Life Sciences) and Protein A AlphaScreen acceptor beads and AlphaScreen signal was read after 1.5 h on a Wallac Envision Multilabel Reader (PerkinElmer Life Sciences).

Alternatively (Table 1) an ELISA-based assay was performed as previously described (4, 20). Briefly bGβ₁γ₂ was immobilized in a streptavidin-coated 96-well plate followed by incubation with compound and F88 phage displaying the peptide SIGKAFKILGYPDYD for 1 h at room temperature. After washing bound phage was detected with a horseradish peroxidase-conjugated anti-M13 antibody followed by color development with ABTS.

**Surface Plasmon Resonance (SPR)**—SPR was performed as described in Ref. 21, with modifications. bGβγ was immobilized on the surface of streptavidin-coated sensorchips (GE Healthcare) by injecting 500 nM bGβγ in 50 mM HEPES, pH 7.6, 1 mM EDTA, 50 mM NaCl, 100 mM KCl, 0.1% poloxamethylene 10 lauryl ether (C₁₂E₁₀), and 1 mM dithiothreitol. Unbound streptavidin was blocked with 1 μM biotin and buffer was changed to include 0.1% DMSO. Small molecule(s) were then injected and binding was observed for 1 min followed by dissociation for 5 min, at a flow-rate of 50 μl/min. After dissociation, the Gβγ surface was regenerated using 610 mM MgCl₂, 205 mM urea, and 610 mM guanidine HCl, followed by the second injection.

For Goα binding to Gβγ, 10 mM MgCl₂ and 10 μM GDP were added to the above mentioned buffer, about 500 micro-refractive index units Gβγ was immobilized to the chip and binding of Goα was observed at a flow rate of 15 μl/min for 5 min, followed by dissociation for 5 min. After the dissociation phase AlF₄⁻ was added to the buffer and injected over the surface for complete dissociation of Goα from Gβγ. To determine the role of 12155 in inhibition of Goα binding to Gβγ, 12155 (10 μM) was added to all buffers, the baseline was allowed to stabilize, and the binding of Goα was determined as described above. The data were analyzed using BIA evaluation software (Biacore), and KD was determined by globally fitting the binding curves determined at several concentrations of compound with a 1:1 binding model.

**HL60 Cell Ca²⁺ Release (Fluorimeter)**—Differentiated HL60 cells were harvested by centrifugation and resuspended in 1 ml of Hanks’ balanced salt solution (HBSS) buffer with 1 mM Ca²⁺ and 10 mM HEPES, pH 7.4. Cells were incubated with 1 μM Fura 2-AM (Molecular Probes) for 45 min at 37 °C, excess dye was removed by centrifugation and resuspension in HBSS with Ca²⁺ and 10 mM HEPES, pH 7.4, at 2 x 10⁷ cells/ml. Cells were diluted 10-fold into 1.8 ml of HEPES-buffered HBSS without
Ca\(^{2+}\), and release of Ca\(^{2+}\) was monitored with 340 and 380 nm excitation and emission detection at 510 nm in a PTI fluorimeter.

**Ca\(^{2+}\) Release Assay (Flexstation)**—For experiments in Fig. 3, A and C, the release of Ca\(^{2+}\) was monitored real-time in 96-well plates in a Flex station fluorescence plate reader (Molecular Devices) at 340 and 380 nm excitation and emission detection at 510 nm. Differentiated HL60 cells were suspended in 1 ml of HBSS buffer with 1 mM Ca\(^{2+}\) and 10 mM HEPES, pH 7.4. Cells were incubated with 1 µM Fura 2-AM (Molecular Probes) for 45 min at 37 °C, excess dye was removed by centrifugation, washed, and re-suspension in HBSS without Ca\(^{2+}\) and 10 mM HEPES, pH 7.4, at 1.5 × 10\(^{5}\) cells/ml. For the assay 150,000 cells were used per well and 95 µl were loaded into each well. For inhibitors cells were pre-treated with M119 or U73122 or U73343, for 15 min prior to the analysis. PTX (100 ng/ml) treatment was for 5 h prior to the experiment. The wells were injected with 5 µl of either fMLP (50 nM) or 12155 (10 µM), unless stated otherwise.

**Ca\(^{2+}\) Calibration**—The 340/380 Fura-2 ratio was calibrated as a function of Ca\(^{2+}\) concentration in both the PTI fluorimeter and the Flexstation using a calcium calibration buffer kit (Invitrogen) containing 10 mM EGTA and 1 mM Mg\(^{2+}\). Ca\(^{2+}\) was titrated from 0 to 39 µM free Ca\(^{2+}\) with 1 µM Fura-2 free acid to generate a standard curve from which Ca\(^{2+}\) concentrations were calculated from the 340/380 ratios obtained from Fura-2-loaded cells.

**Western Blotting**—Differentiated HL60 cells were incubated overnight in RPMI 1640 medium without serum at 37 °C. Cells were harvested and treated with DMSO (solvent), 12155 (10 µM), M119 (10 µM), or fMLP (250 nM) for 5 min. The reaction was stopped by centrifugation of the cells at 13,000 × g for 1 min at 4 °C followed by suctioning of the media and lysis in SDS sample buffer. The samples were heated at 95 °C for 10 min and loaded on a 12% polyacrylamide gel. The gel was transferred overnight and immunoblotted for ERK p44/42 (Cell Signaling), pERK P-p44/42 (Cell Signaling), or pAKT Ser-473 (Cell Signaling). The secondary antibody was anti-rabbit Li-Cor Ab (Odyssey) and was detected using a Li-Cor imaging system (Odyssey).

**Phospholipase C Activity Assay**—The assay was done as previously described (22). Briefly, lipids vesicles containing ~4000 cpm of \[^{3}H\]-inositol]PIP2, 25 µM PIP2, and 100 µM phosphatidylethanolamine were mixed with 0.5 ng of PLCβ2. 100 nM Gβγ was preincubated with DMSO or compounds for 10 min, followed by addition to the PLC and lipid mixture. The reaction was set at 30 °C for 30 min and quenched with 5% BSA and 10% TCA. Released soluble \[^{3}H\]IP3 was measured by liquid scintillating counting.

**Flow Cytometry**—The assay was done as described in Refs. 23 and 24, with some modifications. Briefly, 10 nM Gβγ was immobilized on streptavidin beads (Spherotech) in 20 mM HEPES, pH 8.0, 1 mM DTT, 100 mM KCl, 20 mM NaCl, 0.2 mM free Mg\(^{2+}\), 0.1% C12E10, 0.1% bovine serum albumin (BSA), and 10 µM GDP, for 1 h at 4 °C. For the competition assay 12155 was incubated with the Gβγ-bound beads (10 nM Gβγ) for 30 min at room temperature with shaking prior to addition and incubation with GFP-labeled Ga1 GDP (20 nM) in buffer containing 10 µM GDP and no GTP for 30 min at room temperature. To determine whether 12155 causes dissociation, a complex of 20 nM GFP-Ga1 with 10 nM immobilized bGβγ was pre-formed by incubating GFP-Ga1 for 1 h and excess unlabeled Ga1 (1 µM) was added to determine the intrinsic off rate of GFP-Ga1 from Gβγ. To determine whether 12155 enhanced the GFP-Ga1 off rate, 10 µM 12155 or 10 µM SIGK for comparison were used to initiate the dissociation reaction. The amount of GFP-Ga1 bound to immobilized Gβγ was assayed using a FACSScan flow cytometer (BD Biosciences) exciting at 488 nm and detecting the emission at 510 nm associated with each bead. In the dissociation assay, after addition of competitor to the preassembled GFP/Ga1/βγ mixture, aliquots were removed at various intervals and analyzed by FACS to determine the amount of remaining bead-bound GFP-Ga1 fluorescence.

**[35S]GTPγS Binding Assay**—The assay was done as described in Ref. 25.

**PI3 Kinase Activity Assay**—Activation of PI3Kγ was performed as described (26). 10 ng of purified PI3Kγ was assayed with or without 10 nM Gβγ and injected in the presence or absence of compounds. The assay (60 µl final volume) contained sonicated micelles of 600 µM bovine liver phosphatidylethanolamine and 300 µM bovine liver PI substrate in 40 mM NaHEPES, pH 7.4, 2 mM EGTA, 1 mM DTT, 0.2 mM EDTA, 120 mM NaCl, 5 mM MgCl2, 1 mM β-glycerophosphate, 50 µM sodium orthovanadate, 1 mg/ml of BSA. Reactions were initiated by the addition of 10 µM ATP with [γ-32P]-ATP (5 µCi assay) and by transfer from 4 to 30 °C. Reactions were terminated by addition of 500 µl of 2:1 methanol/chloroform solution. A stable two-phase system was generated by addition of 100 µl of 2.4 M HCl with 5 mM tetrabutylammonium hydrogen sulfate following by 440 µl of CHCl3. Samples were centrifuged, and the lower organic phase containing the lipids was extracted three times with a solution containing 48% methanol, 3% chloroform, 0.5 M HCl, 1 mM EDTA, 10 mM tetrabutylammonium hydrogen sulfate. The lower organic phase was removed and dried under nitrogen, and Cerenkov counting was used to quantitate radioactive 32P incorporation into the lipid fraction using a counting efficiency of 50%. Previous experiments with thin layer chromatography established that in this assay system with purified protein and lipids, greater than 95% of the label migrates in the PI-3-P position. The experiment was performed twice with quadruplicate determinations in each and the data averaged as shown.

**Transwell Migration of HL60 Cells and Neutrophils (Boyden Chamber)**—The assay was done as described previously in Ref. 27. Differentiated HL60 cells or primary mouse neutrophils were used as indicated. For PTX treatment, HL60 cells were treated with PTX (100 ng/ml) overnight before the day of the experiment. For wortmannin, the HL60 cells were treated with wortmannin (1 µM) for 20 min prior to the experiment. Cells were suspended to 10 × 106 cells/ml in HBSS containing Ca\(^{2+}\), 10 mM HEPES, pH 7.4, and 0.1% BSA. Chemoattractant 12155 (10 µM) or fMLP (250 nM) or vehicle control DMSO were added in the lower chamber and 50 µl of cells (106 cells/ml) were in the upper chamber. Cells were either pretreated with Gallein or DMSO for 15 min, or 12155 or fMLP were added to the upper chamber just prior to the chemokinesis assay. The assay used a filter with a 3-µm pore size (Neuroprobe), and was performed...
**Gβγ Signaling Is Sufficient for Chemotaxis**

for 1 h at 37 °C, 5% CO₂. Cells that had migrated and adhered to the bottom surface of the filter were stained and 3 fields each were counted using a light microscope. The data are shown as chemotaxis index, which is the ratio of the number of cells migrated in the presence of the chemoattractant versus those migrated in presence of DMSO.

**Statistical Analysis**—All statistical analyses were performed by one-way analysis of variance using the Tukey post-test or a paired t test in figures with only two columns. The value of the analysis is as follows: *, p < 0.05; **, p < 0.01; ***, p < 0.001 and N.S., not significant.

**RESULTS**

12155 Binds to Gβγ and Prevents Binding of SIGK Peptide to Gβγ—Using a chemical screening approach we previously identified small molecules that bind to Gβγ subunits and modulate the activity of Gβγ subunits in cells, in vitro, and in animals (4, 27–29). The primary screen was based on computationally docking small molecules from the National Cancer Institute diversity library to a peptide/effector/Gα subunit-binding surface on Gβγ (4). Binding of selected molecules to Gβγ was confirmed in a competition assay for SIGK peptide binding to Gβγ (4). The small molecule 12155 (Fig. 1A) was identified in this screen. 12155 inhibited binding of phage displayed SIGK to Gβγ in a concentration-dependent manner in a competition AlphaScreen assay with an IC₅₀ of 5 μM and a Hill slope of −1.7 (Fig. 1B). Direct binding of 12155 to Gβγ was confirmed using SPR yielding a dissociation constant of 1.5 μM (Fig. 1C).

To understand the requirements for 12155 binding to Gβγ a series of related molecules were obtained from the National Cancer Institute and tested in a competition ELISA (Table 1). 12155 (1,3-bis(4-amino-2-methylquinolin-6-yl)urea) is composed of two aminomethylquinoline moieties connected by a urea linkage. Aminomethylquinoline alone did not bind, nor did 1,3-di-6-quinolylurea (71881) lacking the amino and methyl substitutions on the linked quinoline moieties. Substitution of the urea linkage with longer or bulkier linkers also reduced binding. These data indicate a specific binding mode for 12155 binding to Gβγ requiring two covalently connected amino quinoline moieties with a defined spacing between them. 12155 Has No Effect on Gβγ-mediated PLCβ or PI3Kγ Activation in Vitro—12155 was tested for its ability to inhibit PLCβ and PI3K in vitro (Fig. 1D). Surprisingly 12155 had no effect on Gβγ-mediated PLCβ (Fig. 1D), PLCβ3 (data not shown), or PI3Kγ activation (Fig. 1E) at concentrations up to 100 μM. M119 and Gallein are well characterized small molecule inhibitors of Gβγ that were also identified in this screen, and have previously been shown to inhibit Gβγ-dependent PLCβ and PI3Kγ activation (4, 27). In the same assays 10 μM M119 strongly inhibited Gβγ-dependent regulation.

12155 Causes Gβγ-dependent Ca²⁺ Release—The contrast between the properties of 12155 and M119 with respect to the ability to inhibit activation of downstream effectors by Gβγ is striking, and is consistent with our previous observations that unique Gβγ-binding modes of different small molecules have distinct effects on Gβγ signaling properties. To further explore the activity of 12155 we examined its action on Gβγ signaling pathways in HL60 cells differentiated to neutrophils. In neutrophils, chemoattractant peptide receptors such as the receptor for fMLP, FPR1, regulate multiple cellular processes such as cell migration and inflammatory mediator release through Gβγ-dependent signaling pathways. These pathways include PLCβ-dependent Ca²⁺ signaling, PI3Kγ-dependent PI(3,4,5)P₃ formation and Akt activation, and PAK and Cdc42 regulation.

To examine Gβγ-dependent PLC regulation we monitored fMLP-dependent Ca²⁺ release in cells loaded with the Ca²⁺ indicator dye Fura-2 in media with low extracellular Ca²⁺ to ensure that the primary source of Ca²⁺ is IP₃-dependent release from internal stores. fMLP stimulated a rapid and robust Ca²⁺ increase that decayed to baseline over the course of 3–4 min (Fig. 2A). Surprisingly addition of 12155 alone stimulated a rapid and robust release of Ca²⁺ in the absence of fMLP (Fig. 2B). The dose (data not shown). Although U73122 has been reported to have off target effects, many cases of bona fide inhibition of PLC signaling by U73122 in cells have been reported, and the results are consistent with what would be expected in these cells. To show that the activity of 12155 was dependent on Gβγ, cells were treated with the Gβγ inhibitor M119 prior to addition of 12155. M119 pretreatment inhibited 12155-mediated Ca²⁺ release in a concentration-dependent manner (Fig. 3B). These data support the idea that 12155 causes release of Ca²⁺ from intracellular stores in a Gβγ- and PLC-dependent manner.

To determine whether the activity of 12155 was independent of the receptor, cells were treated with PTX to prevent activation of G proteins by Go-coupled GPCRs including FPR1. In PTX-treated cells, 12155 robustly stimulated release of Ca²⁺ (Fig. 3C), whereas the same treatment completely eliminated fMLP-dependent Ca²⁺ signals, suggesting the activity of 12155 to be independent of Go-coupled receptors. PTX did cause some inhibition of the 12155-dependent response. This could suggest some receptor-dependent component of 12155-mediated Ca²⁺ release, but the inhibition also could be due to adverse effects of PTX on the cells or could influence the sensitivity of Goγ-Gβγ interactions to dissociation by 12155.
cant PTX-insensitive component is consistent with a mechanism involving direct action of 12155 at Gβγ subunits to promote PLC signaling.

To further confirm the action of 12155 on Gβγ signaling in HL60 cells we examined ERK and Akt pathways known to be regulated by Gβγ signaling in these cells. Cells were treated with DMSO, 12155, M119, or fMLP. 12155 but not M119 increased the levels of phosphorylated ERK (Fig. 4A) and AKT (Fig. 4B) although not to the same level as fMLP. PTX treatment completely eliminated Akt activation by fMLP but not by 12155 (Fig. 4C). Activation of Akt is consistent with the well-established role of Gβγ in regulation of PI3K in neutrophils. Together these data support that 12155 activates Gβγ signaling in a receptor-independent manner in neutrophil-like differentiated HL60 cells.

**FIGURE 1.** 12155 binds to Gβγ and has no effect on Gβγ-mediated PLCβ2, β3, and PI3Kγ activation, in vitro. A, chemical structures of 12155 (NSC12155) and M119 (NSC19910). B, 12155 competes with SIGK for binding to Gβγ. bGβγ was incubated with 12155 followed by addition of SIGK peptide expressing phage. The amount of bound SIGK was analyzed using an AlphaScreen based assay. Data shown are combined from two experiments with triplicate determinations. C, 12155 binding to bGβγ measured by SPR. bGβγ was immobilized on a streptavidin-coated sensor chip. 12155 was injected over the surface of the chip and binding was measured as a change in refractive index (n = 3). Upper panel is a representative set of SPR traces (black) globally fit with a 1:1 binding model (colored traces) with the corresponding residuals in the lower panel. D, 12155 has no effect of Gβγ-mediated PLCβ2 activation. The assay contained as indicated Gβγ (30 nM) and PLCβ2 and the indicated concentrations of 12155 or M119. [3H]PIP2 was used as the substrate for PLC and the amount of [3H]IP3 produced was detected. The representative data shown are the mean ± S.E. of duplicate determinations (n = 3 separate experiments). E, PI3K γ activation. Assay included ± Gβγ (10 nM), 10 ng of PI3Kγ with, as indicated, 10 µM M119 or 100 µM 12155 results are combined data from 2 independent experiments.

12155 Causes Dissociation of Gβγ from Ga—GPCRs stimulate Gβγ signaling by catalyzing GDP release from, and GTP binding to, the Ga subunit resulting in functional dissociation of Ga from Gβγ subunits. We hypothesized that 12155 activates Gβγ signaling by binding directly to Gβγ in G protein heterotrimers and causing dissociation of GaGDP from Gβγ, or by binding to free Gβγ and disrupting the Ga-Gβγ binding
**TABLE 1**
Structure activity analysis of 12155 related compounds

| Structure | NSC number | Relative binding |
|-----------|------------|------------------|
| ![Structure](image1) | 12155 | 5 μM |
| ![Structure](image2) | 60281 | Non-binder |
| ![Structure](image3) | 12158 | Non-binder |
| ![Structure](image4) | 71881 | Weak binder |
| ![Structure](image5) | 12156 | 25 μM |
| ![Structure](image6) | 12153 | Non-binder |
| ![Structure](image7) | 12157 | Non-binder |
| ![Structure](image8) | 22906 | Weak binder |
| ![Structure](image9) | 22904 | Weak binder |

**FIGURE 2.** 12155 induces Ca^{2+} release independent of agonist stimulation. A, fMLP-induced Ca^{2+} release in HL60 cells. HL60 cells were differentiated, loaded with Fura-2, suspended in buffer with no added extracellular Ca^{2+} (to emphasize release from intracellular stores), and treated with fMLP (50 nM) (a representative trace is shown of n = 3 individual measurements in a fluorimeter). B, 12155 induces Ca^{2+} release in HL60 cells. 10 μM 12155 was added where indicated, and a representative trace is shown of n = 3 individual measurements in a fluorimeter. C, M119 does not stimulate Ca^{2+} release and inhibits fMLP-induced stimulation of Ca^{2+} release. Differentiated HL60 cells were treated with M119 (10 μM, 1st arrow), followed by fMLP (50 nM, 2nd arrow) (a representative trace is shown of n = 3 individual measurements in a fluorimeter). D, differentiated HL60 cells were treated with 12155 (0.3 to 30 μM) and the peak height was measured. Data are pooled from three independent experiments. For all panels all responses were measured as the ratio of 510 nm emission with 340 nm/380 nm excitation. Ionomycin (5 μM) was added to normalize for cell viability and dye loading after all treatments (not shown).

The text continues with a discussion of the experimental results, particularly focusing on the ability of 12155 to induce Ca^{2+} release independent of agonist stimulation and its apparent affinity for Gβγ. The text notes that 12155 was added to Gβγ prior to GFP-Gαi1 and incubated to allow the binding to come to equilibrium prior to measurement of GFP-Gαi1 binding to Gβγ by flow cytometry. 12155 inhibited binding of GFP-Gαi1 to Gβγ in a concentration-dependent manner (Fig. 5C) consistent with its apparent affinity for Gβγ and the SPR data. This experiment establishes that 12155 binding to Gβγ is sufficient to compete for Gβγ binding to GoGDP. In cells 12155 could activate signaling by preventing rebinding of GoGDP to Gβγ after basal Gα subunit dissociation, or 12155 could bind to Gβγ in the context of the heterotrimer and promote Gβγ dissociation from GαGDP as previously reported for to complications from interpreting the binding curves that include 12155 binding and intrinsic Gα dissociation. We previously employed an assay (24) to examine association and dissociation of fluorescent Gα subunits from bGβγ subunits bound to beads using flow cytometry (31). This method allows for measurement of protein-protein interactions at concentrations near the Kd of interactions in the low nanomolar to high picomolar range and allows for the kinetics of association and dissociation of G protein subunits to be measured (24, 31). First an equilibrium competition analysis was performed where 12155 was added to Gβγ prior to GFP-Gαi1 and incubated to allow the binding to come to equilibrium prior to measurement of GFP-Gαi1 binding to Gβγ by flow cytometry. 12155 inhibited binding of GFP-Gαi1 to Gβγ in a concentration-dependent manner (Fig. 5C) consistent with its apparent affinity for Gβγ and the SPR data.

This experiment establishes that 12155 binding to Gβγ is sufficient to compete for Gβγ binding to GoGDP. In cells 12155 could activate signaling by preventing rebinding of GoGDP to Gβγ after basal Gα subunit dissociation, or 12155 could bind to Gβγ in the context of the heterotrimer and promote Gβγ dissociation from GαGDP as previously reported for Gβγ Signaling Is Sufficient for Chemotaxis.
12155 does not cause nucleotide exchange in the Gaα subunit. When 12155 was added along with DAMGO it decreased the DAMGO-dependent increase in $[^{35}S]$GTPγS binding, consistent with disruption of the heterotrimer by 12155, leading to less heterotrimeric complex being available for activation by DAMGO.

12155 Acts as a Chemoattractant for HL60 Neutrophils—Development of an agonist that activates Gβγ, independently of the receptor, and Ga subunit nucleotide exchange, provides a powerful tool to study the role of free Gβγ in an acute cellular setting. Gβγ plays an important role in chemoattractant receptor-dependent cell migration (27, 32). Although Gβγ is important for migration of cells, it is unclear if release of free Gβγ is sufficient to mediate directional chemotaxis in the absence of activation of Ga subunits or other GPCR-mediated pathways.

To determine whether Gβγ release from GaαGDP is sufficient to drive neutrophil chemotaxis we asked whether 12155...
could cause directional HL60 cell migration in a transwell migration assay. In this assay chemoattractants are placed in a lower chamber, which is separated from cells in the upper chamber by a membrane with pores that allow migration of cells from the upper chamber to the lower chamber in response to the gradient of chemoattractant established between the upper and lower chambers. Thus this method measures the ability of cells to sense and migrate directionally in response to chemotactic gradients. fMLP stimulated robust migration of HL60 cells into the lower chamber whereas gallein, a Gβγ inhibitor, does not. Interestingly, 12155 robustly stimulated cell migration to an extent equivalent to fMLP (Fig. 6A).

To examine chemotaxis of primary cells, neutrophils were isolated from mouse bone marrow and tested in the transwell...
assay for their ability to respond to a gradient of 12155 (Fig. 6B). 12155 potently, and effectively, stimulated primary neutrophil chemotaxis with an EC_{50} of ~5–10 μM, although the response did not saturate at 30 μM. These data are consistent with its concentration dependence for activation of Ca^{2+} release, although the concentrations required are significantly higher than for binding the purified protein in vitro. This is not surprising because the compound has to cross cell membranes and interfere with subunit association in cells.

Accumulation of cells in the lower chamber could be the result of stimulation of random migration that lacks directionality (chemokinesis) or directional migration in response to a gradient (chemotaxis). To distinguish between directional and random migration chemotactant is placed in both the upper and lower chambers at equal concentrations thereby eliminating gradient formation. When 12155 or fMLP were added as a uniform stimulus, there was no significant migration of differentiated HL60 cells to the lower chamber (Fig. 6C). This indicates that a gradient of 12155 is sufficient to act as a chemotactant and direct chemotaxis of HL60 cells and primary neutrophils.

12155 Causes Receptor-independent Gβγ-mediated PI3K-dependent Cell Migration—To show the migration induced by 12155 to be independent of GPCR activation, HL60 cells were treated with PTX prior to 12155 or fMLP. PTX completely inhibited fMLP-dependent chemotaxis as expected but only partially inhibited 12155-stimulated cell migration (Fig. 7A). To provide evidence that 12155 mediates chemotaxis through Gβγ, cells were treated M119. We have previously shown that M119 inhibits PI3K and Gβγ-mediated cell migration in HL60 cells and neutrophils (27). M119 completely blocked both fMLP- and 12155-mediated cell migration (Fig. 7B).

PI3Kγ is a central molecule in Gβγ-mediated cell migration (9) and 12155 activates Gβγ-mediated Akt phosphorylation (Fig. 4B). Cells were pretreated with wortmannin to inhibit PI3K and chemotaxis was measured in response to gradients of 12155 and fMLP. Wortmannin inhibited 12155-mediated cell migration, supporting the idea that 12155 activates PI3K-dependent cell migration (Fig. 7, C and D). Together these data indicate that a gradient of 12155 serves as a chemotactic stimulus to drive directional cell migration by directly driving dissociation of Gβγ from GoGDP indicating that chemotaxis does not require GPCR activation and can be promoted solely by the activation of Gβγ-dependent signaling pathways.
**Gβγ Signaling Is Sufficient for Chemotaxis**

**DISCUSSION**

Here we show that a small molecule 12155 activates Gβγ signaling and is a sufficient stimulus to drive directional chemotaxis of neutrophils. The data shows that 12155 activates Gβγ signaling by binding directly to Gβγ and causing release of GaGDP without causing nucleotide exchange. 12155 was found in a competition screen for binding of the SIGK-phage displayed peptide to Gβγ. A cell permeable version of SIGK, myristoyl-SIGK, was shown by our laboratory to stimulate Gβγ-dependent ERK phosphorylation in smooth muscle and other cell types (23). SIGK binds at a surface on Gβγ that corresponds to the binding site for the Gα subunit switch II helix and competes for binding of effectors such as phospholipase Cβ and PI3Kγ but not type I adenyl cyclase or N-type Ca2+ channels. Our data supported a model that explained the ability of cell-permeable SIGK to stimulate ERK activation: SIGK binding to Gβγ in Gβγ-GaGDP heterotrimer in cells enhances the release of GaGDP, leaving Gβγ bound to SIGK. This released SIGK-bound Gβγ could then signal to ERK because bound SIGK did not interfere with effector surfaces on Gβγ required for activation of this pathway. SIGK did not activate PLCβ or PI3Kγ because with SIGK bound to the released Gβγ the binding sites for PLCβ and PI3Kγ were occupied with SIGK. 12155 is one of several molecules that were found in the screen for binding to Gβγ in a competition assay for SIGK binding. Unlike other molecules identified in this screen such as M119, 12155 was not inhibited by Gβγ-dependent regulation of PLCβ or PI3Kγ. Thus, in contrast to SIGK, when 12155-bound Gβγ is free from GaGDP it can signal downstream to multiple Gβγ-dependent signaling pathways.

Our data do not clearly discriminate between a cellular mechanism where 12155 binds to heterotrimer and drives Gβγ dissociation or where 12155 binds to free Gβγ to prevent rebinding to GaGDP. 12155 enhances the GaGDP dissociation rate from GβγαGDP heterotrimers by greater than 2-fold *in vitro*, indicating that it can bind to heterotrimer. How this rate enhancement *in vitro* relates to the rate of free Gβγ release in cells, and whether this is sufficient to drive the observed rapid Ca2+ increase is not clear. Ca2+ responses are highly amplified so a small but rapid increase free Gβγ could be sufficient to drive a rapid Ca2+ increase. Regardless of the specific mechanism, the data support a model where 12155 treatment of cells leads to an increase in free Gβγ without activation of Gα.

The chemical biology based approach used here has several advantages, one of the most important of which is the ability to rapidly perturb native systems without requiring overexpression of proteins. Protein overexpression can upset the balance of natural stoichiometries and requires 24 to 48 h during which time the system can adapt to the intended perturbation. In this particular system the use of a chemical activator also allowed for establishment of a gradient with which a directional chemotaxis assay could be established. A possible concern is the potential for off-target effects complicating interpretation of the experiments. 12155 is also known as surfen and is known to bind to, and antagonize, interactions with heparin sulfate (33, 34) as well as bind to and inhibit anthrax lethal factor (35). Our data are highly consistent with the idea that the chemotaxis mediated by 12155 is due to its ability to bind to Gβγ including the observation that M119 inhibition of Gβγ signaling inhibits 12155 directed migration, whereas PTX is only weakly effective.

Previous studies have identified Gβγ to be important in inducing cell migration, with inhibition of its signaling leading to inhibition of migration (27, 32). Here we show that Gβγ release from GaGDP alone is sufficient to induce chemotaxis in solution. This indicates that the GPCRs, activated Gαi or Gαi13 subunits, other GPCR accessory proteins, such as GRK2 and β-arrestin, or the regulators of G proteins signaling (RGS proteins) Gαi13, are not required to mediate directional chemotaxis of neutrophils in solution. Numerous reports have suggested Gα subunits along with RGS, GRK2, and β-arrestin to play a role in migration (12–17), but these proteins may serve to modulate Gβγ-mediated chemotaxis. A recent report suggested that there is a role for GaGDP in directing cell migration that could be involved in 12155-mediated signaling and could be the subject for further investigation (36). All of the migration assays in this work were conducted in a transwell migration chamber in the absence of extracellular matrix. This allowed us to assess the role of Gβγ in setting the compass and driving directional chemotaxis but does not mimic neutrophil migration during an infection that includes adhesion to endothelial cells, rolling, trans-endothelial migration, and interstitial migration where other aspects of GPCR signaling may play important roles.

Other studies have examined the relative roles of Gα and Gβγ signaling downstream of the receptor to discern their relative roles in directing the chemotaxis. One study used HEK293 cells transfected with CXCR1 and a Gαiγ chimera that couples to CXCR1 but does not regulate Gαi effectors such as adenyl cyclase (8). In these cells IL-8 was able to act as a chemotactic stimulus even though Gαi was not activated implying that Gαi signaling is not required for directional chemotaxis of HEK293 cells. Another study used the rapamycin-dependent FKBP-FRB dimerization system to bypass the G protein signaling system in HL60 cells and directly activate PI3K activity to produce PIP3. They demonstrate that uniform application of rapamycin causes polarization of the cells and increased migration, although directional movement of cells in a gradient was not tested (30). Both of these systems although providing valuable information are somewhat artificial either using transfected HEK293 or HL60 cells. In our study we show that in primary neutrophils a gradient of compound that releases free Gβγ from GaGDP can act as a chemoattractant.

In this study we demonstrated the utility of a small molecule Gβγ activator in a cellular setting, to activate Gβγ-mediated signaling pathways. This compound will be helpful in elucidating more pathways and functions related to Gβγ or GPCR-dependent signaling. Understanding the details of where 12155 binds and how it modulates release of Gβγ from GaGDP is a question of interest. We propose the existence of a key residue(s) on Gβγ, on binding to which, 12155 induces dissociation of Gβγ from Gα. Finding the binding site of 12155 by a mutational analysis of the binding site or through solving the three-
dimensional co-crystal structure of 12155 with Gβγ can further illuminate this hypothesis.

Acknowledgments—We thank Dr. Jean Bidlack for providing CHO cell membranes and Brain Knapp for help with the GTPγS binding assay.

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