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Dynamic regulation of neutrophil polarity and migration by the heterotrimeric G protein subunits $G\alpha_i$ -GTP and $G\beta\gamma$

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Abstract

Activation of the G_i family of heterotrimeric guanine nucleotide-binding proteins (G proteins) releases $\beta\gamma$ subunits, which are the major transducers of chemotactic G protein-coupled receptor (GPCR)-dependent cell migration. The small molecule 12155 binds directly to $G\beta\gamma$ and activates $G\beta\gamma$ signaling without activating the $G\alpha_i$ subunit in the G_i heterotrimer. We used 12155 to examine the relative roles of $G\alpha_i$ and $G\beta\gamma$ activation in the migration of neutrophils on surfaces coated with the integrin ligand intercellular adhesion molecule-1 (ICAM-1). We found that 12155 suppressed basal migration by inhibiting the polarization of neutrophils and increasing their adhesion to ICAM-1-coated surfaces. GPCR-independent activation of endogenous $G\alpha_i$ and $G\beta\gamma$ with the mastoparan analog Mas7 resulted in normal migration. Furthermore, 12155-treated cells expressing a constitutively active form of $G\alpha_{i1}$ became polarized and migrated. The extent and duration of signaling by the second messenger cyclic adenosine monophosphate (cAMP) were enhanced by 12155. Inhibiting the activity of cAMP-dependent protein kinase (PKA) restored the polarity of 12155-treated cells but did not decrease their adhesion to ICAM-1 and failed to restore migration. Together, these data provide evidence for a direct role of activated $G\alpha_i$ in promoting cell polarization through a cAMP-dependent mechanism and in inhibiting adhesion through a cAMP-independent mechanism.

INTRODUCTION

Cell migration is responsible for multiple processes, including tissue formation, wound healing, and immune responses. Directed cell migration, or chemotaxis, is defined as the

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movement of a cell toward a chemotactic stimulus, and it involves various environmental cues that activate multiple signaling pathways, which lead to coordination and assembly of multicomponent structures and physical regulation both spatially and temporally (1). These pathways drive cell polarization, which results from the protrusion of the leading edge in the direction of the chemotactic gradient, integrin-mediated adhesion, and retraction of the tail at the back of the cell (2). Cells achieve polarization and directional movement in gradients as shallow as 5% across the length of the cell. Indeed, cells can become polarized and migrate in the absence of a chemotactic gradient, although they do so in random directions. Extensive studies of neutrophils and Dictyostelium discoideum indicate that the receptors for chemoattractants are uniformly distributed on the cell surface, and that polarization occurs because of localized activation of downstream signaling components, which result from self-amplifying positive feedback loops at the leading edge coupled with global inhibitory signals that suppress activation at the trailing edge, key features of the local excitation, global inhibition (LEGI) model for directed cell migration (3, 4).

Chemoattractant receptors are heterotrimeric guanine nucleotide binding protein (G protein)-coupled receptors (GPCRs) that activate G proteins (consisting of $G\alpha_i$ and $G\beta\gamma$ subunits) of the G_i family (Fig. 1A). Active coupling of GPCRs to G proteins induces a conformational change in the $G\alpha$ subunit, which leads to its exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) (5, 6). GTP binding induces a conformational change in the G subunit, which releases the bound $G\beta\gamma$ subunit. Dissociated $G\alpha_i$ -GTP and $G\beta\gamma$ are the active forms of the proteins and they signal independently of each other. Inactivation occurs through the hydrolysis of GTP to GDP by the $G\alpha$ subunit and the rebinding of $G\beta\gamma$ to $G\alpha$ -GDP. Both the chemoattractant receptors and the G proteins are uniformly distributed in the plasma membrane of polarized cells (7).

In response to GPCR activation, $G\beta\gamma$ subunits play a prominent role in immune cell migration through the direct activation of phosphatidylinositol 3-kinase γ (PI3K γ) (8–11) and guanine nucleotide exchange factors, such as phosphatidylinositol 3,4,5-trisphosphate (PIP₃)-dependent Rac exchanger protein (pREX) and ELMO (engulfment and cell motility)/DOCK (dedicator of cytokinesis), which leads to the subsequent activation of the small GTPases RhoG, Rac, and Cdc42 (12–15). The $G\beta\gamma$ -dependent activation of PI3K γ and the subsequent generation of PIP₃ set up a positive feedback loop involving Rac and Cdc42 that ultimately results in the polarized accumulation of PIP₃, actin polarization, and formation of the leading edge of the cell (16–18). The roles of $G\alpha_i$ signaling in these processes have not been well explored, and it has been suggested that the only role of $G\alpha_i$ is to regulate the release of $G\beta\gamma$ subunits (19, 20). Any determination of specific roles for $G\alpha_i$ in chemotaxis is hampered by the fact that perturbations that inhibit $G\alpha_i$ signaling also inactivate obligatory $G\beta\gamma$ signaling. For example, the modification of $G\alpha_i$ by pertussis toxin blocks any interactions between the $G\alpha_i$ -bg heterotrimer and GPCRs, thereby inhibiting both $G\alpha$ and $G\beta\gamma$ signaling. Similarly, knockout of specific G protein α subunits, either in mice or with specific short inhibitory RNAs in cell culture, prevents signaling by both $G\alpha$ and its associated $G\beta\gamma$ subunits.

We previously identified a small molecule (12155) that acutely activates $G\beta\gamma$ subunit signaling by displacing $G\alpha$ -GDP from $G\beta\gamma$ without activating $G\alpha$, which provides a

powerful tool with which to determine the specific functions of the $G\beta\gamma$ and α subunits (Fig. 1B). In experiments with this molecule, we previously demonstrated that activation of $G\beta\gamma$ was sufficient to induce directional neutrophil chemotaxis in a Transwell, suspension-based assay (21), supporting the notion that activated $G\alpha_i$ is not needed for directional chemotaxis in cell suspensions in a steep chemotactic gradient. In vivo, the migration of neutrophils is a more complex process, requiring forward protrusion, adhesion to the endothelial substrata, deadhesion, and diapedesis. To determine the role of signaling by individual G protein subunits in this complex process, we examined stimulated migration in response to uniform application of 12155 on a two-dimensional surface to mimic endothelial adhesion. In this context, we showed that $G\alpha_i$ -GTP signaling was critical for neutrophil polarization and chemotaxis. We present evidence that when it is present in large quantities, cyclic adenosine monophosphate (cAMP) acts as a global inhibitor of cell polarization and that $G\alpha_i$ -GTP is required to dynamically inhibit cAMP production and to suppress cell adhesion.

RESULTS

Uniform $G\beta\gamma$ stimulation inhibits neutrophil motility on ICAM-1-coated surfaces

To understand how $G\beta\gamma$ signaling regulates cell polarization and migration in the absence of $G\alpha$ -GTP signaling, we compared the migration of primary mouse neutrophils treated with the chemoattractant N-formyl-Met-Leu-Phe (*f*MLP) or 12155, a molecule that releases free $G\beta\gamma$ from G protein heterotrimers without activating $G\alpha$ (Fig. 1, A and B). Mouse neutrophils were imaged on slides coated with the integrin ligand intercellular adhesion molecule-1 (ICAM-1), treated uniformly with vehicle [dimethyl sulfoxide (DMSO)], *f*MLP, or 12155 (without any gradient), and tracked by differential interference contrast (DIC) microscopy. Basal polarization and migration of primary mouse neutrophils on ICAM-1 is a well-established phenomenon that is important for immune surveillance in vivo. Consistent with this, basal mouse neutrophil migration on ICAM-1-coated slides was unaffected by treatment with vehicle (Fig. 1C). This basal polarization of cells was not observed on glass slides coated with bovine serum albumin (fig. S1), indicating that integrin engagement enhances basal cell polarization in the absence of an applied chemoattractant. Application of *f*MLP increased the velocity and the distance traveled by these cells (Fig. 1, C and D). In contrast, 12155 completely suppressed basal migration (Fig. 1, C to E, fig. S2, and movie S1). This finding contrasts sharply with what was observed in a Transwell assay in which 12155 stimulated directional chemotaxis (21). Thus, activation of $G\beta\gamma$ in the absence of receptor or $G\alpha_i$ activation actively suppressed cell migration on a surface coated with a cell adhesion molecule.

Uniform $G\beta\gamma$ activation promotes the formation of nonpolarized lamellipodia and increases cell adhesion

Inhibition of cell migration could reflect either decreased polarization of cells, increased adhesion, or simply a decreased ability to respond to chemoattractants. In the absence of chemoattractants, neutrophils plated on ICAM-1 exhibited basal polarity with distinct leading and trailing edges (Fig. 2, A and B, and fig. S3), and stimulation with *f*MLP increased the percentage of polarized cells (Fig. 2, A and B, and fig. S3). Application of 12155, on the other hand, completely suppressed basal cell polarity, resulting in cells that

had no distinct trailing edge and had actin-based protrusions uniformly surrounding the cell, resembling a “fried egg” (Fig. 2, A and B, and fig. S3). The percentage of cells displaying this morphology increased with increasing concentrations of 12155 and we calculated a median effective concentration (EC_{50}) of ~ 10 mM, which is consistent with the affinity of 12155 for $G\beta\gamma$ in vitro being ~ 3 μ M (Fig. 2C) (21). To measure adhesion, neutrophils were plated on ICAM-1–coated plates, treated with vehicle (DMSO), 12155, or *f*MPLP for 5 min, and then washed, and the numbers of cells that detached from the ICAM-1–coated surface were counted. Treatment with 12155, but not *f*MPLP, decreased the number of cells that detached with washing, which suggested that there was an increase in their adhesion (Fig. 2D). Thus, on an ICAM-1–coated surface, uniform activation of $G\beta\gamma$ with 12155 suppressed cell polarity and increased cell adhesion, both of which likely contributed to the lack of motility of these cells.

The effects of 12155 on polarity and adhesion require free $G\beta\gamma$ subunit signaling

To demonstrate the specificity of 12155 for $G\beta\gamma$, we expressed a well characterized protein-based $G\beta\gamma$ signaling inhibitor, the C terminus of GPCR kinase 2 (GRK2ct) (22), in an HL-60 human promyelocytic leukemia cell line that can be differentiated into neutrophil-like cells. This cell line has the advantage that exogenous proteins can be expressed by the introduction of mammalian complementary DNA expression constructs by nucleofection. The transfection efficiency of these cells was 20 to 40%, so individual cells expressing GRK2ct were identified by cotransfection with a plasmid encoding yellow fluorescent protein (YFP) before analysis. Untransfected cells and transfected cells expressing YFP alone (35 of 41 cells) responded to 12155 by adopting a circular, flattened morphology as described earlier (Fig. 3 and fig. S4). Cells transfected with plasmid encoding GRK2ct did not respond to 12155 (only 4 of 41 cells responded; Fig. 3 and fig. S4). It is likely that this blockade required large amounts of GRK2ct because cotransfected cells with low amounts of YFP (weakly fluorescent), which were assumed to also have decreased amounts of GRK2ct, showed an increased response to 12155 (15 of 17 weakly fluorescent cells responded to 12155; Fig. 3 and fig. S4). These data support the idea that the ability of 12155 to increase adhesion and decrease cell polarity is directly due to its specific ability to release $G\beta\gamma$ subunits.

Receptor-independent activation of both $G\alpha_i$ and $G\beta\gamma$ stimulates polarity and migration

$G\beta\gamma$ subunits are the primary mediators of signals downstream of chemotactic peptide receptors in immune cells. The observation that the activation of $G\beta\gamma$ alone by 12155 suppressed polarity and migration indicates that signals beyond $G\beta\gamma$ activation are necessary to establish polarity and migratory capacity. Chemokine receptors and chemoattractant receptors, such as formyl peptide receptor 1 (FPR1), have the potential to activate $G\alpha_i$, $G\alpha_{12/13}$, GRKs, arrestins, and other receptor-associated signaling molecules. On the other hand, 12155 releases free $G\beta\gamma$ subunits without nucleotide cycling or activating other GPCR-directed pathways (21). We hypothesized that the key difference between the GPCR-dependent and the 12155-dependent activation of $G\beta\gamma$ subunits was the generation of GTP-loaded $G\alpha_i$. To test this, we used the mastoparan analog Mas7, which specifically and directly activates G_i proteins by binding to the G_i heterotrimer and catalyzing the binding of GTP to the $G\alpha_i$ subunit independently of receptor activation (Fig.

4A) (23). Stimulation of neutrophils with Mas7 led to polarization and migration that was indistinguishable from that observed in response to *f*MLP (Fig. 4, B and C, and movie S2). Cells treated with either *f*MLP or Mas7 displayed enhanced polarization, and the number of cells displaying polarity was greater than that under basal conditions, whereas cells treated with 12155 had no distinguishable polarity (Fig. 4, D and E, and fig. S5). These data suggest that $G\alpha_i$ activation is both required and sufficient to complement $G\beta\gamma$ -directed signaling to promote cell polarity and migration.

Active $G\alpha_i$ prevents the 12155-dependent effects on cell polarization and adhesion

To more directly demonstrate a role for active $G\alpha_i$ in cooperating with $G\beta\gamma$ to enable proper cell polarization and chemotaxis, we transfected differentiated HL-60 cells with plasmids encoding wild-type $G\alpha_{i1}$ or a constitutively active form of $G\alpha_i$, $G\alpha_{i1}Q204L$. In each case, the cells were cotransfected with plasmid encoding YFP, and individual fluorescent cells were analyzed (Fig. 5). Neither $G\alpha_{i1}Q204L$ nor wild-type $G\alpha_{i1}$ had a noticeable effect on the behavior of unstimulated cells (Fig. 5, fig. S6, and movies S3 to S5). Furthermore, cells expressing $G\alpha_{i1}Q204L$ apparently responded normally to *f*MLP (movie S3). On the other hand, when $G\alpha_{i1}Q204L$ -expressing cells were treated with 12155, they de-adhered from the ICAM-1-coated surface, polarized, projected pseudopods in specific directions, and migrated slowly (Fig. 5, fig. S6B, and movies S4 to S6). Cells transfected with plasmid encoding wild-type $G\alpha_{i1}$ responded to 12155 with a nonpolarized adhesive phenotype that was indistinguishable from that of untransfected cells (Fig. 5, fig. S6A, and movie S7). The marked change in the 12155-dependent phenotype upon introduction of the $G\alpha_{i1}Q204L$ mutant was variable, but none of the cells displayed the characteristic 12155-induced fried egg morphology. $G\alpha_{i1}Q204L$ did not fully recapitulate the behavior of cells expressing wild-type $G\alpha_{i1}$, in that the cells moved more slowly and had a somewhat elongated phenotype; however, full restoration of the wild-type phenotype was not expected because global $G\alpha_{i1}Q204L$ overexpression would not be expected to restore the spatiotemporal regulation of $G\alpha_i$ signaling by receptors. Overall, these experiments provide evidence for a direct and active role of $G\alpha_i$ -GTP in conjunction with $G\beta\gamma$ to signal downstream and regulate cell migration in a manner that is independent of the participation of $G\alpha_i$ in the G protein cycle and regulating $G\beta\gamma$ release.

$G\alpha_i$ -GTP inhibits cAMP production in neutrophils

The most well understood signaling function of $G\alpha_i$ -GTP is to inhibit adenylyl cyclase (AC), thus decreasing the production of the second messenger cAMP. In contrast to most cell types, activation of G_i -coupled receptors in neutrophils stimulates cAMP production through a noncanonical pathway involving type 9 AC (AC9). In this pathway, $G\beta\gamma$ released from G_i activates mammalian target of rapamycin (mTOR), which in turn activates protein kinase C β II (PKC β II), leading to the phosphorylation and activation of AC9 (24–26). We hypothesized that concomitant activation of $G\alpha_i$ might counteract the $G\beta\gamma$ -stimulated accumulation of cAMP. To test this, we measured the ability of *f*MLP, Mas7, or 12155 to regulate cAMP abundance in either mouse or human neutrophils. Consistent with previous studies, *f*MLP stimulated an increase in cAMP in primary human neutrophils that peaked at 1 min and decayed over the next 4 min (Fig. 6A). Stimulation with Mas7 also led to a moderate increase in the abundance of cAMP that was comparable to that stimulated by

*f*MLP. In contrast, 12155 led to enhanced stimulation of cAMP accumulation compared to that stimulated by either *f*MLP or Mas7 (Fig. 6A). The 12155-induced increase in cAMP was sustained compared to that by either Mas7 or *f*MLP, for which cAMP abundance returned to almost basal amounts over 5 min. The concentration dependence of 12155-dependent cAMP accumulation was validated in primary mouse neutrophils (Fig. 6B). These data suggest that activation of $G\alpha_i$ plays a role in regulating the amount of cAMP produced in neutrophils in response to $G\beta\gamma$ activation.

Inhibition of PKA restores the polarity of 12155-treated mouse neutrophils

Given the large amounts of cAMP generated in response to 12155, we hypothesized that the increased cAMP abundance inhibited migration. The second messenger cAMP is a dynamic regulator of neutrophil chemotaxis. At low abundance, cAMP is promigratory; however, increased concentrations of cAMP cause decreased migration because of reduced pseudopod protrusion, stronger tail adhesion, and reduced retraction (25, 27, 28). cAMP regulates chemotaxis through the activation of cAMP-dependent protein kinase (PKA), which is required for polarity, but excess activation can inhibit polarity (29). During chemotaxis of *D. discoideum*, the formation of pseudopods is dependent on cAMP-mediated activation of PKA (30). If the ability of 12155 to inhibit migration was due to excess cAMP production, then the inhibition of cAMP signaling might be able to restore migration in 12155-treated cells. We inhibited cAMP-mediated PKA activation with Rp-cAMPs, an analog of cAMP, or with myristoylated protein kinase inhibitor (14–22) amide (myr-PKI). In contrast to cells treated with 12155 alone, cells pretreated with Rp-cAMPs or myr-PKI and then stimulated with 12155 showed strong polarization, with pseudopod formation at the front of the cell and uropod formation at the tail (Fig. 7, A to D, fig. S7, and movie S8). When neutrophils polarize, actin localizes primarily to the front edge of the cells with some actin being found in the tail. Cells treated with DMSO, Mas7, or *f*MLP had actin staining at the front edge of the cells and in the tail depicting a polarized morphology (Fig. 7D). Cells in which only $G\beta\gamma$ was activated (with 12155) displayed a uniform distribution of actin with no visible polarity (Fig. 7D). In neutrophils treated with myr-PKI and then stimulated with 12155, actin showed a polarized localization similar to that seen in cells treated with *f*MLP or Mas7 (Fig. 7D). These data suggest that the enhanced production of cAMP when $G\beta\gamma$ activity is not opposed by $G\alpha_i$ -GTP inhibits the polarization of neutrophils, and that this polarity can be restored by inhibiting cAMP-PKA signaling.

Inhibition of PKA enhances basal migration but does not restore migration or reduce adhesion in 12155-treated neutrophils

Because inhibition of PKA restored polarity in cells treated with 12155 ($G\beta\gamma$ -stimulated cells), we investigated whether it could also restore migration. The inhibition of PKA led to an increase in the speed of migrating cells and the distance of migration compared to those of basal and 12155-stimulated cells (Fig. 8, A and B, and movie S9); however, inhibition of PKA was unable to restore migration in 12155-treated cells, despite restoring cell polarity (Fig. 8, A and B, and movie S10). These cells were unable to migrate possibly because of a tail retraction defect or the inability of the cells to detach from the substrate (movies S8 and S10). We next investigated whether the inhibition of PKA affected the adhesion of these cells. Cells pretreated with or without the PKA inhibitor and then stimulated with 12155

showed increased adhesion to the substrate compared to that of untreated cells or cells treated with PKA inhibitor alone. Epac (exchange protein activated by cAMP), another target of cAMP, is present in very low amounts in neutrophils and does not mediate adhesion in these cells (31, 32), which suggests that cAMP is not involved in the adhesion induced by 12155. $G\alpha_i$ -GTP rescued the adhesion and migration of 12155-treated cells (Fig. 5), indicating that $G\alpha_i$ -GTP regulates adhesion through a cAMP-independent mechanism.

DISCUSSION

The role of $G\alpha_i$ -GTP in neutrophil migration

The primary phenotype of neutrophils stimulated uniformly with the $G\beta\gamma$ activator 12155 on ICAM-1-coated surfaces was a strong radial activation of pseudopodia formation without polarization, strong adhesion to the substrate, and basal suppression of migration. These data suggest that $G\beta\gamma$ subunit signaling alone regulates multiple aspects of neutrophil biology, but that additional signals are required to achieve a polarized morphology and cell motility. Here, we showed that a key additional signal is the activation of $G\alpha_i$. We used multiple approaches to implicate $G\alpha_i$ -GTP signaling in cell migration. The marked phenotypic alteration (increased polarization, migration, and de-adhesion) of 12155-treated cells by overexpression of the constitutively active mutant $G\alpha_{i1}Q204L$ supports a direct role for $G\alpha_i$ -GTP in driving downstream signaling processes. Under basal or *f*MLP-stimulated conditions, neither $G\alpha_{i1}Q204L$ nor wild-type $G\alpha_{i1}$ appreciably altered neutrophil function, which is likely because $G\alpha_i$ signaling is only required in the context of a $G\beta\gamma$ stimulus, and this is supplied by endogenous $G\alpha_i$ under receptor-stimulated or Mas7-treated conditions. Only under conditions in which free $G\beta\gamma$ was generated in response to 12155 was a role for $G\alpha_i$ -GTP uncovered. This effect was independent of the role of $G\alpha_i$ in regulating the release of $G\beta\gamma$ subunits because $G\alpha_{i1}Q204L$ would not be expected to regulate $G\beta\gamma$ because of its constitutive activation, whereas wild-type $G\alpha_i$ and $G\alpha_{i1}Q204L$ did not appreciably modify *f*MLP-dependent responses.

One of the downstream effects of $G\alpha_i$ -GTP, which we demonstrated here, is to counterbalance the increases in cAMP generated by the $G\beta\gamma$ -dependent activation of AC (Fig. 8D). Liu et al. (25) showed that receptor stimulated cAMP is low in abundance at the leading edge and is increased at the trailing edge, although the mechanisms that lead to this polarized distribution have not been described. $G\alpha_i$ -GTP may be involved in locally suppressing cAMP production at the leading edge to a range in which the polarity network can be activated, whereas, in turn, cAMP diffuses to the back of the cell to suppress these signals and activates processes involved in the regulation of uropod dynamics.

At a molecular level, several studies support various aspects of this model. First, the apparently surprising result that the activation of G_i -coupled receptors or $G\beta\gamma$ alone causes increases in cAMP abundance is potentially explained by a noncanonical mechanism involving regulation of AC9, which is abundant in immune cells. In this pathway, $G\beta\gamma$ released from G_i heterotrimers stimulates mTOR activity, which in turn activates the PKC β -dependent phosphorylation of AC9, leading to increased cAMP production (24–26). Our data suggest that under conditions of sole $G\beta\gamma$ activation with 12155, cAMP reaches concentrations that suppress all polarity signaling and likely loses spatial regulation (Fig.

8D). $G\alpha_i$ -GTP inhibits AC9 and thus is poised to reduce the local concentration of cAMP through a membrane-delimited process (33).

PKA activation regulates cell polarity and migration through multiple mechanisms (29), and it is well established that PKA plays a role in regulating cytoskeletal assembly, adhesion, and the directed migration of neutrophils (34, 35). PKA localizes at the leading edge of chemotaxing neutrophils (36, 37), and it inhibits migration and polarity through phosphorylation and inhibition of PIP₃-dependent Rac exchanger 1 (P-Rex1), which activates Rac at the leading edge (38, 39). In contrast, cAMP-dependent PKA activation is also required for cell motility because blockade of cAMP production prevents neutrophil migration through inhibition of tail retraction (25, 26). Thus, cAMP-PKA signaling can play opposing stimulatory and inhibitory roles in cell migration, and fine-tuning of the cAMP concentration and its spatial distribution is critical for motility.

Our data suggest that the inhibition of PKA activity in 12155-treated cells restores polarity, but not migration, and that the cells remain strongly adhered to the substrate. A possible concern is that pharmacological inhibitors such as PKI completely inhibit PKA activation, and the proper balance and location of PKA activation that can be achieved through $G\alpha_i$ regulation is likely required for proper migration. An alternate target of cAMP, the Rap exchange factor Epac, could be involved in adhesion that would be insensitive to PKA inhibition, but Epac does not play a role in neutrophil adhesion (31, 32), which suggests that the cAMP-dependent regulation of Epac is not involved in neutrophil migration. Because expression of the constitutively active mutant $G\alpha_{i1}Q204L$ reduced adhesion, these data suggest that $G\alpha_i$ -GTP regulates adhesion through a cAMP-independent mechanism (Fig. 8D) that remains to be defined.

cAMP as the global inhibitor in the LEGI model

Neutrophils have an inherent ability to polarize even in response to a uniform concentration of a chemoattractant (40). During neutrophil chemotaxis, the asymmetric distribution of lipids, actin, and actin-binding proteins is observed, but the upstream regulatory molecules, including GPCRs and G proteins, are uniformly distributed at the leading edge of migrating cells (3, 4, 41). The uniform distribution of these upstream molecules calls for the localization and regulation of signaling pathways downstream of receptor and G protein activation. The exact mechanism by which these signals are regulated and localized is still undetermined. Several laboratories have developed a LEGI model to explain the ability of a cell to remain polarized under uniform stimulation or very shallow gradients of chemoattractant (17, 18, 42, 43). The model proposes that the chemoattractant stimulates both a self-potentiating positive signal and a slower accumulating globally diffusible inhibitor. The global inhibitor can be overcome locally by the positive feedback signal at the leading edge, but it suppresses the positive feedback loop at the rear of the cell. It is well established that PIP₃ generation is a key component of the positive feedback loop to establish cell polarity, but the identity of the global inhibitor has not been clearly defined.

cAMP is an ideal global inhibitor candidate because it is highly diffusible, its cellular localization is controllable, and it is stimulated by $G\beta\gamma$, which simultaneously stimulates PI3Kg and other positive signals at the leading edge. The kinetics of increases in cAMP

abundance is slow relative to that of PIP₃ production, and as shown here, the concentration of cAMP can be modulated by the dynamic interplay between Gβγ-dependent stimulation and Gα_i-dependent inhibition immediately downstream of GPCR activation.

In summary, using a distinct set of selective reagents, we have dissected the relative roles of Gα and Gβγ signaling and shown a role for Gα_i-GTP subunit signaling in the regulation of neutrophil polarization, adhesion, and migration. This role is played in part through modulation of the concentration of cAMP, which we propose is a key global inhibitor generated downstream of receptor stimulation, and through regulation of cell adhesion by a mechanism that requires further investigation.

MATERIALS AND METHODS

Materials

12155 was originally identified in a screen of the National Cancer Institute Diversity Set with the original identifier number NSC12155 (44). 12155 is also known as surfen [1,3-bis(4-amino-2-methylquinolin-6-yl)urea]. The molecule was prepared as 50 mM stocks in DMSO. Surfen, fMLP, DMSO, and fibronectin were purchased from Sigma-Aldrich. Rp-cAMPs and Mas7 were purchased from Calbiochem. Sp-cAMPs and myr-PKI were purchased from Enzo. Mouse ICAM-1 was purchased from Sino Biological.

Isolation of mouse neutrophils

Neutrophils were obtained from the bone marrow of adult C57BL/6 mice. All procedures were performed on ice with ice-cold buffers. Bone marrow was flushed with phosphate-buffered saline (PBS) (pH 7.4), and red blood cells (RBCs) were lysed with ACK (ammonium-chloride-potassium) lysis buffer. The white blood cells were separated from the lysed RBCs by centrifugation at 325g for 3 min. The cells were then counted, and 1×10^8 cells were used for further isolation. Neutrophils were isolated by magnetic immunodepletion with the Neutrophil Negative Selection Kit (StemCell) according to the manufacturer's protocol.

Isolation of human neutrophils

Blood was drawn from normal healthy donors according to the protocols followed by the University of Rochester and HIPAA (Health Insurance Portability and Accountability Act). Heparinized blood (20 ml) was added onto a gradient separation kit (1-Step Polymorphs, Accurate Chemical & Scientific Corporation). The various types of cells were separated by centrifugation. The cell layer for polynuclear cells was collected and washed, which was followed by lysis of RBCs with a hypotonic 0.1× PBS solution. The remaining cells collected were mostly neutrophils (>90%) as assessed by flow cytometry.

Nucleofection of HL-60 cells

HL-60 cells differentiated in 1.2% DMSO for 4 days were subjected to nucleofection as follows. Cells (5×10^6) were spun down at 80g for 10 min. After removal of the culture medium, the cells were suspended in 100 μl of Ingenio transfection solution (Mirus Bio LLC). This solution was added to 2 mg of DNA (1 mg of a plasmid expressing enhanced

YFP and 1 µg of plasmid expressing the appropriate construct or of empty vector). The mixture was transferred to a 0.2-cm cuvette, and the cells were transfected by nucleofection with an Amaxa Nucleofector II Device (Lonza) on the recommended setting (T-019). After transfection, the mixture was immediately diluted with 500 µl of recovery medium (RPMI 1640 containing 10% fetal bovine serum and 1.2% DMSO) and incubated in an Eppendorf tube at 37°C for 30 min. The cells were then transferred to a dish containing 1.5 ml of recovery medium and incubated overnight at 37°C in an incubator with 5% CO₂. The next morning, the transfected cells were plated on coverslips coated with 5 µg of fibronectin from bovine plasma (Sigma-Aldrich). Cells were allowed to attach for 30 min and then were washed with 1 ml of RPMI 1640 medium and subjected to treatment and microscopic analysis in Hanks' balanced salt solution (HBSS) containing 10 µM Hepes (pH 7.4).

Cell migration assays

Cell migration assays were performed as described previously (45), with modifications. Briefly, Millicell EZ SLIDE 8-well glass (Millipore) or 35-mm glass-bottom dishes were coated with mouse ICAM-1 (1 or 5 µg per well, respectively; Sino Biological) and incubated for 2 hours at 37°C. Naïve mouse neutrophils were isolated and placed on the slide in L-15 medium containing glucose (2 mg/ml; CellGro). Cells were allowed to adhere to the bottom of the slide, and nonadherent cells were washed off. Cells were preincubated with inhibitors for 15 to 20 min, treated with the compounds of interest, and then imaged every 10 s for 25 min. Image acquisition was conducted on a DIC-enabled microscope (Nikon or Olympus) coupled to a Hamamatsu or CoolSNAP HQ (Roper Scientific) camera. Themagnification used was ×10, ×20, or ×60. The cells were tracked with the manual tracking functionality in ImageJ software, and the tracked cells were analyzed with the Chemotaxis tool (ibidi). All cells that appeared healthy were tracked, and no thresholding criteria were applied. Velocity for each cell was calculated by dividing the total distance moved by the total migration time.

Adhesion assays

Primary mouse neutrophils were allowed to adhere on ICAM-1-coated plates in L-15 medium containing glucose (2 mg/ml). Cells were stimulated with the appropriate compounds for 5 min at 37°C. For inhibitor studies, the cells were pretreated with a given inhibitor for 15 min before being stimulated. The reaction was stopped by transferring the dish to ice, and the dish was tapped to detach loosely adherent cells. The culture medium was collected in a tube, the cells were washed with ice-cold PBS, the wash was combined with the collected culture medium, and the number of cells present was counted. Adhesion was calculated as the inverse of the number of detached cells relative to control.

Cell polarity measurements

Primary mouse neutrophils were allowed to adhere on an ICAM-1-coated plate in L-15 medium containing glucose (2 mg/ml) and then were stimulated with the appropriate compounds at 37°C. For inhibitor studies, the cells were pretreated with a given inhibitor for 15 min before being stimulated. After 5 min, the cells were washed twice with ice-cold PBS and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. The cells were washed with ice-cold PBS and permeabilized with PBS containing 0.1% Triton X-100 for 10 min, which was followed by washing with PBS. Acti-stain 555 phalloidin (150 nM;

Cytoskeleton Inc.) was added to the cells and incubated for 20 min, and any excess reagent was washed off with PBS. The cells were visualized, and 10 random images were captured with an epifluorescence microscope (Olympus Inc.) with a 40 $\times$, 0.75-NA (numerical aperture) air objective. Random images were selected, and the polarized cells were labeled. Data were pooled, and the number of polarized cells was calculated. For Fig. 7D, the coverslips were mounted on a slide with Fluoromount-G (SouthernBiotech) and imaged with the confocal functionality of a FV1000-AOM multiphoton system (Olympus Inc.) equipped with a 60 $\times$, 1.35-NA oil immersion lens (Olympus) using 565-nm excitation.

cAMP assays for mouse neutrophils

Briefly, isolated neutrophils were centrifuged and resuspended at 1×10^6 cells/ml in HBSS with calcium, 10 mM Hepes (pH 7.4), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). The cells were incubated at room temperature for 10 to 15 min, and 60,000 cells were used per condition for the assay. The cells were then treated with 0.3 to 30 μ M 12155 or with DMSO for 30 min at room temperature. The cells were then centrifuged and resuspended in HBSS with calcium, 10 mM Hepes (pH 7.4), and 0.5 mM IBMX, and 10 ml of cells (60,000 cells) was added to a 96-well plate ($\frac{1}{2}$ AreaPlate, PerkinElmer). The cells were then incubated with the LANCE Ultra cAMP detection reagents (PerkinElmer), and measurement of cAMP was performed according to the manufacturer's protocol.

cAMP assays for human neutrophils

Briefly, isolated human neutrophils were resuspended in HBSS with Ca^{2+} , and 75,000 neutrophils were used per well in a 384-well plate. The cells were stimulated for the appropriate times (0.5, 1, 2, 3, or 5 min), and the assay was performed with the cAMP-Glo Max kit (Promega) according to the manufacturer's procedure.

Statistical analysis

All statistical analysis was performed by one-way ANOVA using the Bonferroni test functionality. Statistical significance is indicated in the figures as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. G $\beta\gamma$ activation alone reduces neutrophil motility.

(A) Diagram of canonical G protein regulation by GPCRs, including chemoattractant receptors. P_i , inorganic phosphate. (B) Mechanism of action of 12155, which binds directly to G $\beta\gamma$ subunits and results in the release of free G $\beta\gamma$ subunits from G α -GDP without activating the G α subunit. (C) G $\beta\gamma$ activation reduces the basal motility of neutrophils. Primary mouse neutrophils were treated with vehicle (DMSO), 10 μM 12155, or 1 μM fMLP, and then were tracked for 25 min by microscopy and analyzed by ImageJ software. Tracks of individual neutrophils for each treatment are shown for a single experiment and are representative of three experiments. (D) Data from three experiments as represented in (C) were analyzed with the Chemotaxis and Migration tool from ibidi to determine the velocity (left) and the distance traveled (right) by the indicated cells. Each point represents an individual cell from three separate experiments that were pooled and analyzed as indicated below. Data from 20 cells under each condition were analyzed for statistical

significance by one-way analysis of variance (ANOVA) with Bonferroni posttest. * $P < 0.05$ and *** $P < 0.001$. (E) 12155 causes a concentration-dependent decrease in basal migration. Mouse neutrophils were stimulated with the indicated concentrations of 12155 and then were tracked and analyzed as described in (C) and (D). Each point represents the average $\pm$ SEM for 20 cells from the data shown in fig. S3 (C and D), which were pooled from three independent experiments.

Fig. 2. Uniform Gβγ activation promotes the formation of nonpolarized lamellipodia and increases cell adhesion.

(A) Uniform Gβγ activation stimulates the formation of nonpolarized circular lamellipodia. Mouse neutrophils were treated with vehicle (DMSO), 10 μM 12155, or 1 μM fMLP, and then were fixed and imaged by DIC microscopy. Images are representative of multiple cells from four independent experiments. Scale bars, 5 μm. (B) Gβγ activation reduces basal cell polarity. Mouse neutrophils were treated with the indicated reagents as described in (A), fixed, stained for actin, and imaged by epifluorescence microscopy. Cells were scored (between 20 and 50) for each independent experiment in a blinded manner to determine the number of polarized cells. Data are means ± SEM of the percentages of cells that were polarized in three independent experiments. (C) Mouse neutrophils were stimulated with the indicated concentrations of 12155 and were analyzed as described in (B) to determine the percentage of cells that exhibited nonpolarized lamellipodia formation. Data are means ± SEM of three independent experiments. (D) Gβγ activation increases cell adhesion to an

ICAM-1-coated substrate. Mouse neutrophils were uniformly stimulated as described in (A), and the numbers of cells that became detached from the surface were counted. Adhesion was calculated as inversely proportional to the number of detached cells. Data are means $\pm$ SEM of three independent experiments. All data were analyzed by one-way ANOVA with Bonferroni posttest. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Fig. 3. The 12155-dependent effects on cell migration are blocked by GRK2ct.

(A and B) HL-60 cells were transfected by nucleofection with plasmid encoding YFP alone or in the presence of plasmid encoding GRK2ct. Transfected cells were selected on the basis of the abundance of YFP. The cells were further segregated on the basis of whether the cells showed high or low fluorescence, which was interpreted as evidence of increased or decreased amounts of GRK2ct, respectively. (A) Representative individual YFP-expressing cells are shown before and after treatment with 12155. (B) Quantitation of data from the experiments shown in (A). After treatment with 12155, YFP-expressing cells were identified and scored (in a blinded manner) for the appearance of the characteristic flattened fried egg or “normal” morphologies. Cells were imaged at $\times 60$ magnification. Experiments are from four separate sets of transfections, with multiple cells examined in each experiment. Data are means $\pm$ SEM of pooled data from three independent experiments and analyzed by one-way ANOVA with Bonferroni posttest. **** $P < 0.0001$ compared to control cells transfected with YFP alone. The proportions of cells that showed a characteristic flattened symmetrical morphology after treatment with 12155 are as follows: YFP alone, 35 of 41 cells; YFP + GRK2ct (high), 4 of 41 cells; and YFP + GRK2ct (low), 15 of 17 cells.

Fig. 4. Direct activation of G_i heterotrimers is sufficient to stimulate cell migration, induce polarization, and reduce adhesion.

(A) Diagram of the mechanism of action of Mas7, a mastoparan derivative. Mas7 directly interacts with G_i heterotrimers and catalyzes nucleotide exchange on $G\alpha_i$, which leads to the receptor-independent activation of signaling by both $G\alpha_i$ and $G\beta\gamma$. (B) Activation of G_i heterotrimers stimulates neutrophil migration. Mouse neutrophils were treated with vehicle (DMSO), 10 μM 12155, 2 μM Mas7, or 1 μM fMLP, and then were tracked and analyzed as described in Fig. 1A. Data are from a single experiment and are representative of four experiments. (C) Activation of G_i heterotrimers increases both the speed of neutrophil migration (top) and the distance traveled (bottom). Mouse neutrophils were treated, tracked, and analyzed as described in Fig. 1A. Data are means $\pm$ SEM of 20 cells under each condition from three experiments. (D) Activation of G_i heterotrimers induces cellpolarization. Mouse neutrophils were treated with the indicated compounds, fixed, and imaged by DIC microscopy. Images are representative of multiple cells from four individual

experiments. Scale bars, 5 mm. (E) Mouse neutrophils were treated with the indicated compounds, fixed, stained for actin, and imaged for fluorescence. Images were analyzed in a blinded manner as described in Fig. 2B to determine the number of polarized cells. Data are means $\pm$ SEM of the percentages of polarized cells from three independent experiments. All data were analyzed by one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001.

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Fig. 5. Treatment of G α_{i1} Q204L-expressing HL-60 cells with 12155 results in cell polarization and migration.

(A and B) HL-60 cells were cotransfected with plasmid encoding YFP and with plasmid encoding either wild-type (WT) G α_{i1} or the G α_{i1} (Q204L) mutant. Transfected cells were selected on the basis of the abundance of YFP. (A) Representative individual YFP expressing cells are shown before and after a 30-min treatment with either 12155 or fMLP. (B) Quantitation of data from the experiments shown in (A) for 12155 treatment. After treatment, the YFP-expressing cells were identified and scored (in a blinded manner) for the presence of the characteristic flattened fried egg or normal morphologies. Cells were imaged at $\times 60$ magnification. Data are means $\pm$ SEM of pooled data from three independent experiments and were analyzed by a Student's *t* test. *****P* < 0.0001 compared to control transfected cells expressing YFP alone. The proportions of cells that cells showed a characteristic flattened symmetrical morphology after treatment with 12155 are as follows: YFP + WTG α_{i1} , 22 of 29 cells; YFP + G α_{i1} (Q204L), 2 of 20 cells. Fluor, fluorescent cells.

Fig. 6. Stimulation of $G\beta\gamma$ alone results in enhanced and sustained increases in cAMP abundance compared to activation of G_i protein heterotrimers.

(A) Human neutrophils were stimulated for the indicated times with 10 μ M 12155, 2 μ M Mas7, or 1 μ M fMLP. The amount of cAMP generated under each condition was measured as described in Materials and Methods and is presented as the amount of cAMP (pmol) produced per 75,000 cells. Data are means $\pm$ SEM of three independent experiments and were analyzed by one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) 12155 results in a concentration-dependent increase in cAMP. Mouse neutrophils were stimulated with the indicated concentrations of 12155 for 30 min and were analyzed as described in (A). Data are presented as the amount of cAMP (pmol) produced per 100,000 cells. Data are means $\pm$ SEM of three independent experiments.

Fig. 7. Inhibition of PKA restores polarity to 12155-treated cells.

(A) Mouse neutrophils were preincubated with myr-PKI or DMSO before being treated with the indicated compounds, fixed, and imaged by DIC microscopy. Images are representative of multiple cells from three independent experiments. Scale bars, 5 μ m. (B) Mouse neutrophils were preincubated with the indicated inhibitors, treated with the indicated compounds, fixed, and then imaged by DIC microscopy. The images were analyzed in a blinded manner as described in Fig. 2B to determine the numbers of cells with circular uniform pseudopodia. Data are means $\pm$ SEM of three independent experiments and were analyzed by one-way ANOVA. *** $P < 0.001$ compared to cells treated with 12155 alone. (C) Mouse neutrophils were preincubated with inhibitor, treated with the indicated compounds, fixed, stained for actin, and imaged by epifluorescence microscopy. The images were analyzed in a blinded manner as described in Fig. 2B to determine the numbers of actin-polarized cells. Data are means $\pm$ SEM of the percentages of actin-polarized cells from

three independent experiments and were analyzed by one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (D) PMNs were preincubated with myr-PKI or DMSO, treated with the indicated compounds, fixed, stained for actin with Acti-stain 555 phalloidin, and imaged by confocal fluorescence microscopy or DIC. Images are representative of multiple cells from three independent experiments. Scale bars, 5 μm .

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Fig. 8. Inhibition of PKA enhances basal migration but does not restore migration or reduce adhesion in 12155-treated cells.

(A) Inhibition of PKA enhances basal migration, but not in the presence of 12155. Mouse neutrophils were preincubated with 1 μM myr-PKI (m-PKI) or 300 μM Rp-cAMPs, treated with DMSO or 10 μM 12155, and then tracked for 25 min and analyzed by ImageJ software. Tracks of neutrophils treated with the indicated compounds are depicted. Data are from a single experiment and are representative of three experiments. (B) Pooled data from experiments performed as described in (A). The tracked neutrophils were analyzed with the Chemotaxis and Migration tool from ibidi. Data are means ± SEM of 20 cells for each condition from three independent experiments and were analyzed by one-way ANOVA. * $P < 0.05$, *** $P < 0.001$. (C) Inhibition of PKA is unable to reduce Gβγ-stimulated adhesion. Mouse neutrophils were uniformly stimulated with DMSO, 1 μM myr-PKI, 300 μM Rp-cAMPs, 10 μM 12155, or 2 μM Sp-cAMPs, as indicated, and the numbers of cells that detached from the bottom of ICAM-1-coated wells were counted. Adhesion was calculated

as inversely proportional to the number of detached cells. Data are means $\pm$ SEM of three independent experiments and were analyzed by one-way ANOVA. * $P < 0.05$ and *** $P < 0.001$ compared to DMSO-treated control cells. (D) Model depicting the role of G_i proteins in neutrophil migration. When neutrophils are stimulated with the chemoattractant *f*MLP or with Mas7, both $G\alpha_i$ and $G\beta\gamma$ are activated. $G\beta\gamma$ activation stimulates the production of cAMP. In this model, cAMP inhibits cell polarization by activating PKA, but the concurrent activation of $G\alpha_i$ keeps the concentration of cAMP relatively low, perhaps maintaining a cAMP gradient. In parallel, $G\beta\gamma$ stimulates cell adhesion, which is opposed by $G\alpha_i$ -GTP in a cAMP-independent manner.