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Activation of Gαi3 triggers cell migration via regulation of GIV

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D
uring migration, cells must couple direction sensing to signal transduction and actin remodeling. We previously identified GIV/Girdin as a Gαi3 binding partner. We demonstrate that in mammalian cells Gαi3 controls the functions of GIV during cell migration. We find that Gαi3 preferentially localizes to the leading edge and that cells lacking Gαi3 fail to polarize or migrate. A conformational change induced by association of GIV with Gαi3 promotes Akt-mediated phosphorylation of GIV, resulting in its redistribution to the plasma membrane. Activation of Gαi3 serves as a molecular switch that triggers dissociation of Gβγ and GIV from the Gi3–GIV complex, thereby promoting cell migration by enhancing Akt signaling and actin remodeling. Gαi3–GIV coupling is essential for cell migration during wound healing, macrophage chemotaxis, and tumor cell migration, indicating that the Gαi3–GIV switch serves to link direction sensing from different families of chemotactic receptors to formation of the leading edge during cell migration.

Introduction

Cell migration in response to chemotactic stimuli is a key aspect of many physiological and pathological processes (Van Haastert and Devreotes, 2004). During migration, signals from the exterior are relayed to the cell interior via surface receptors to initiate events necessary for efficient directional motility. The most extensively studied chemotactic receptors in the context of cell migration are the growth factor receptor tyrosine kinases (RTKs) studied in fibroblasts and epithelial cells and G protein–coupled receptors (GPCRs) extensively studied in leukocytes and Dictyostelium discoideum (Van Haastert and Devreotes, 2004). Regardless of which receptor system is involved in sensing chemotactic stimuli, they signal via a conserved pathway that culminates in the activation and enhancement of Akt/PKB (Ridley et al., 2003) oriented toward the direction of the chemotactic stimulus.

GIV (Gα-interacting vesicle-associated protein) was discovered based on its ability to bind Gαi3 (Le-Niculescu et al., 2005). Simultaneous work from other groups proposed distinct roles for GIV in endocytosis (Simpson et al., 2005) and in regulating cell migration via its interaction with Akt, actin, and PI4P (Anai et al., 2005; Enomoto et al., 2005). Of particular interest is the fact that GIV is localized at the crossroads of two major signaling pathways, as it is able to interact with α-subunits of G proteins (Le-Niculescu et al., 2005) and enhance PI3K-Akt activity upon RTK stimulation (Anai et al., 2005). Although RTKs and Gβγ-subunits are known to directly increase PI3K activity and subsequently activate Akt (Stephens et al., 1997), the role of Gα-subunits in this process has remained unclear. We set out to define the role of Gα-subunits and the significance of its interaction with GIV during cell migration and to understand how Gα-subunits and GIV link cell surface receptors to downstream signaling events.

In this paper, we show that Gαi3 regulates GIV’s functions during cell migration. We provide mechanistic insights into how activation of the G protein dictates the formation of...
Figure 1. Gα3 redistributes to the cell periphery during cell migration and is necessary for cell migration. (A) Distribution of Gα3 in quiescent (a–f) versus migrating (g–i) HeLa cells after scratch wounding. In quiescent cells, endogenous Gα3 colocalizes with β1GALT in the Golgi (a–c) but not with actin (d–f). In migrating cells, Gα3 is found in puncta that partially colocalize with actin at the leading edge (g–i). Arrows denote direction of migration. Boxed area in i is enlarged in j. HeLa cells were aldehyde fixed 0 (left) and 8 (right) h after scratch wounding, as shown in the diagram, and stained as indicated. Bars, 10 μm.

Results

Gα3 is necessary for cell migration

Both GIV and Gα3 are localized on Golgi membranes and the plasma membrane (PM; Stow et al., 1991; Denker et al., 1996; Enomoto et al., 2005; Le-Niculescu et al., 2005). We reported previously that GIV is predominantly on vesicles near the Golgi during quiescence (Le-Niculescu et al., 2005), and others reported that during cell migration it accumulates at the leading edge where it interacts with Akt and participates in actin remodeling (Enomoto et al., 2005). Because key participants in cell migration (GIV, PI3K, Akt, and actin; Merlot and Firtel, 2003; Enomoto et al., 2005) are enriched at the leading edge where it interacts with Akt and participates in actin remodeling (Enomoto et al., 2005), we asked if Gα3 behaves similarly. We subjected confluent monolayers of HeLa cells to scratch wounding to induce unidirectional cell migration (Kupfer et al., 1982) and examined the distribution of endogenous Gα3 by immunofluorescence (IF).

In quiescent cells far from the wound, Gα3 showed a predominant Golgi localization based on colocalization with a Golgi marker, β1-4 galactosyltransferase (β1GALT; Fig. 1 A, a–c), and was almost undetectable at the cell periphery marked by actin (Fig. 1 A, d–f). In contrast, migrating cells at the edge of the wound showed peripheral Gα3-stained puncta within lamellipodial extensions. Some of these puncta colocalized with actin at the leading edge (Fig. 1 A, g–j). Thus, Gα3 showed different distributions depending on the migratory state of the cell.

Because GIV is a binding partner of the α-subunit of Gi and Gs and is important for cell migration, we asked if Gα3 or Gαs is necessary for cell migration. We depleted Gα3 in HeLa cells using siRNA oligos (Fig. 1 C) and measured the rates of wound closure. Depleting Gα3 (~95%) delayed wound healing (Fig. 1 B, g and h), whereas controls closed the wound as expected (Fig. 1 B, a and b). This impairment in migration was similar to that observed when GIV was depleted (~85%; Fig. 1 B, e and f). In contrast, depletion of Gαs (~90%) had no effect (Fig. 1 B, c and d). Gα3-depleted cells regained their ability to close the wound when Gα3 levels were replenished by expression of siRNA-resistant rat (rGα3wt; Fig. 1 B, i–l).
Live cell imaging revealed that most (~75%) of the Ga3i-depleted cells displayed random oscillatory movements around a virtually motionless nucleus (Fig. 1, D and E; and Video 1, available at http://www.jcb.org/cgi/content/full/JCB.200712066/DC1), resembling cells expressing a dominant-negative migration-defective GIV mutant (Enomoto et al., 2005). When siRNA-resistant YFP-tagged rGa3i (rGa3i-YFP) was introduced into Ga3i-depleted cells, ~75% of the transfected cells migrated toward the wound (Fig. 1 E).

Ga3i preferentially localizes to pseudopods at the leading edge of migrating cells

To study the dynamics of Ga3i localization after wounding, we observed HeLa cells expressing rGa3i-YFP by live cell imaging as they transitioned from quiescence to migration. Previously, we demonstrated that rGa3i-YFP localizes largely to the Golgi and behaves similarly to endogenous Ga3i (Weiss et al., 2001). During migration, Ga3i-YFP localized preferentially within pseudopods at the leading edge as manifest by the transient “blush” of fluorescent signal within these microdomains (Fig. 2 A and Video 2, available at http://www.jcb.org/cgi/content/full/JCB.200712066/DC1). This blush most likely reflects the transient localization of Ga3i-stained puncta (Fig. 1 A, j) that are not resolved by the optical conditions used for live cell imaging (see Materials and methods). Because Ga3i is membrane anchored, membrane redundancy at the sites of pseudopod formation could lead to nonspecific accumulation of membrane-anchored proteins (Servant et al., 1999). However, myristoyl-palmityl-modified YFP (mp-YFP), which has the same modifications as Ga3i-YFP and was previously characterized as a general membrane marker (Zacharias et al., 2002), showed patchy distribution along the entire PM and other membranous compartments without any preference for the leading edge during polarized migration (Fig. 2 B and Video 3). Accumulation of Ga3i was also observed within pseudopods at the leading edge using 1,1’-Diocadecyl-3,3,3’,3’-tetrachloroindodicarbocyanine perchlorate (DiD) as an alternative membrane marker (Fig. S1, available at http://www.jcb.org/cgi/content/full/JCB.200712066/DC1; Servant et al., 1999). We conclude that Ga3i, like its binding partner GIV, is concentrated at the leading edge of a migrating cell. 

Ga3i is necessary for centrosome/MTOC repositioning during cell migration

Because Ga3i-depleted cells failed to undergo directional migration, we looked for defects in centrosome reorientation at the wound edge using HeLa cells stably expressing the centrosome marker Centrin-1-GFP and quantified as described previously (Fig. 3 B; Kupfer et al., 1982; Etienne-Manneville and Hall, 2001). We observed that although ~64% of control siRNA-treated cells repositioned the centrosome in front of the nucleus looking toward the wound, only ~34% of Ga3i-depleted cells (as was expected in the absence of polarization) achieved the same phenotype (Fig. 3 C). GIV or Gas3-depleted cells were similar to controls. When rGa3iwt was introduced into Ga3i-depleted cells, the defect in centrosome repositioning was reversed. Using deconvolution microscopy to visualize the plane of the centrosome, we found that both endogenous Ga3i (Fig. 3 A, a–f) and endogenous (Fig. 3 A, j–l) or overexpressed GIV (Fig. 3 A, g–i) localized at the centrosome with bona fide centrosomal proteins (pericentrin or γ-tubulin) in HeLa and Cos7 cells. Our finding that both Ga3i and GIV localize to the centrosome region and only Ga3i is required for centrosomal repositioning suggests that the latter is a GIV-independent function of Ga3i.

Ga3i is required for enhancement of Akt signaling

Next, we asked whether Ga3i regulates GIV’s functions in activating Akt and remodeling actin after growth factor stimulation, an approach which mimics scratch wound–induced Akt signaling in a more synchronized fashion (Enomoto et al., 2005). When serum-starved HeLa cells were stimulated with insulin, Akt activity peaked at 5 min and was rapidly down-regulated within 15–30 min in controls (Fig. 4 A). In Ga3i-silenced cells, the peak activation was reduced by ~60% (Fig. 4, A and B), which is similar to the effect observed after GIV depletion in HeLa (Fig. 4 B) or HepG2 cells (Anai et al., 2005). The effect was Ga3i specific because Gas depletion did not significantly affect Akt activation (Fig. 4 A) and was reversed when rGa3iwt was restored in Ga3i siRNA-treated cells (see Fig. 5 B). Therefore, Ga3i links Akt activation and cell migration in a manner
similar to that reported for GIV (Anai et al., 2005). To distinguish whether Gai3 and GIV function in a common pathway or in independent parallel pathways mediating enhancement of Akt signaling, we investigated the effect of silencing both proteins. Silencing of Gai3 or GIV alone reduced Akt activation by ∼60 and 80%, respectively (Fig. 4 B). When both were silenced (Fig. 4 B), no significant difference was observed from GIV-depleted cells, indicating that the effect on Akt was not additive. The fact that depletion of Gai3 promoted weaker inhibition of Akt than GIV suggests that other Gi3-independent pathways might exist in which GIV is a common effector. Akt activation was also impaired when Gai3-depleted HeLa cells were stimulated with EGF (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200712066/DC1), which implicates GIV in a common pathway mediating Akt activation upon RTK stimulation.

Gai3 is necessary for remodeling of the actin cytoskeleton

Because PI3K-Akt signaling is associated with actin remodeling during cell migration, we next examined the organization of the actin cytoskeleton in the migration-deficient Gai3-depleted cells. Phalloidin staining for F-actin (filamentous actin) revealed major differences in actin organization of Gai3-depleted cells versus that of controls (Fig. 4 C, a and b): the Gai3-depleted cells were virtually unable to form long stress fibers and displayed a prominent bed of cortical actin but recovered their ability to form stress fibers when transfected with rGai3wt-YFP (Fig. 4 C, c and d). We also investigated actin morphology in Gai3-depleted cells treated with insulin which induces actin remodeling (Ridley et al., 1992) and found that actin remodeling and generation of stress fibers failed to occur upon insulin stimulation (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200712066/DC1). Similar results were obtained in GIV-depleted cells using EGF as a ligand (Enomoto et al., 2005). We conclude that Gai3 plays a major role in the organization of the actin cytoskeleton, most likely via its interaction with GIV, an established actin binding protein.

The distribution of GIV is altered in the absence of Gai3

We next investigated if silencing Gai3 leads to changes in the distribution of GIV. In quiescent cells treated with scr siRNA, we detected GIV on vesicles in and around the Golgi and scattered throughout the cytoplasm (Fig. 4 D, a). Occasionally, GIV also colocalized with actin stress fibers (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200712066/DC1) as reported by Enomoto et al. (2005) in fibroblasts; however, this was an infrequent and inconsistent finding in epithelial cells. We believe that this discrepancy in staining pattern is likely caused by the fact that epithelial cells express lower levels of GIV than mesenchymal cells. Depletion of Gai3 resulted in a more compact concentration of GIV staining in the Golgi region and reduced peripheral staining (Fig. 4 D, b). This phenotype was reversed when Gai3-depleted cells were replenished with rGai3-YFP (Fig. 4 D, c–e). We conclude that Gai3 is necessary for redistribution of GIV from the Golgi to more peripheral locations.

Activation of Gai3 is required for Akt enhancement and actin remodeling

Because heterotrimeric G protein signaling is regulated by the activation state of the α-subunit, we asked whether activation of Gai3 is required for its functions during cell migration. We transfected rGai3Q204L and rGai3G203A mutants, which behave like the GTP- and GDP-bound forms of Gai3, respectively (Hermouet et al., 1991; Coleman et al., 1994), into Gai3-depleted cells and assessed their ability to reverse the effects of
Gαi3 depletion. Transfection of either Gαi3wt or the active rGαi3Q204L mutant restored cell migration (Fig. 5 A), Akt activation in response to insulin (Fig. 5 B), formation of stress fibers (Fig. 5 C), and distribution of GIV (Fig. 5 D). Transfection of the inactive mutant rGαi3G203A had no effect on these parameters (Fig. 5, A–D).

Next, we asked whether the interaction between Gαi3 and GIV is activity dependent and found that the inactive GST-Gαi3G203A mutant consistently bound ~10–15-fold more GIV than the active Gαi3Q204L mutant in in vitro binding assays using HeLa cell lysates or TnT (in vitro–translated) GIV (Fig. 6 A). We confirmed these results by preloading GST-Gαi3 with GDP alone or in the presence of AlF4−, which mimics the active state (Coleman et al., 1994). In the presence of GDP alone, GST-Gαi3 bound ~10–30% of the total GIV, and addition of AlF4− virtually abolished the binding (Fig. 6 B). In contrast, GST-Gαs bound ~100–300-fold less than GST-Gαi3 (Fig. 6 C), which is consistent with our finding that Gαs-depletion had no influence on cell migration (Fig. 1 B), Akt activation (Fig. 4 A), or actin stress fiber formation (not depicted). These results demonstrate that GIV binding is specific for Gαi3 and is greatly reduced upon activation of the G protein. Because inactive GDP-bound Gαi3 interacts with both Gβγ and GIV, we asked whether the α-subunit can interact with GIV when it is assembled into an intact heterotrimer. That this is the case is suggested by the fact that the Gβ subunit could be coimmunoprecipitated from brain lysates with GIV antibodies and that this interaction was abolished in the presence of GDP and AlF4− (Fig. 6 D).

Because the active, but not the inactive, Gαi3 mutant reversed the migration-defective phenotype of Gαi3-depleted cells, activation of the G protein seems to be the key event leading to Akt enhancement during cell migration. However, in vitro binding studies showed that GIV preferentially binds to the inactive G protein. Collectively, these observations support a working model whereby in vivo, the functional interaction between the G protein and GIV needs to be reversible as the G protein cycles between active and inactive states.

Gαi3 promotes phosphorylation of GIV by Akt at S1416

In addition to being an enhancer of Akt activity, GIV is a substrate for Akt at S1416 (Enomoto et al., 2005). Interestingly, S1416 lies within the G binding domain of GIV (Le-Niculescu et al., 2005), raising the question of whether the Gαi3–GIV interaction modulates ser/thr phosphorylation of GIV. We found that in Gαi3-depleted cells GIV was poorly phosphorylated compared with controls (Fig. 7 A). Repletion of Gαi3 with rGαi3wt restored phosphorylation of GIV to control levels (Fig. 7 A). To determine if the Gαi3–GIV interaction enhanced or interfered with Akt mediated phosphorylation of
GIV on S1416, we performed in vitro phosphorylation reactions with immunopurified GIV, GST-Gai3, and recombinant Akt. Because Akt phosphorylates immunopurified GIV at a single site, i.e., S1416 (Enomoto et al., 2005), p-ser/thr antibody was used to estimate the amount of phosphorylation that occurred. We found that preincubation of GIV with GST-Gai3G203A, the inactive mutant that binds GIV most avidly, increased the phosphorylation of GIV ~2.5 fold (Fig. 7 B, lane 6), whereas GST-Gai3Q204L and GST-Gai3wt were not significantly different (Fig. 7 B, lanes 4 and 5). We conclude that binding of Gai3 to GIV promotes phosphorylation of GIV at S1416 by Akt. We reasoned that this is likely via a change in the conformation of GIV that might facilitate access of Akt to S1416. To investigate whether such a change in conformation takes place, we performed limited proteolysis using increasing amounts of trypsin on immunopurified GIV (Fig. 7 C). GIV preincubated with GST, GST-Gai3wt, or GST-Gai3Q204L was relatively resistant to proteolysis, whereas GIV preincubated with GST-Gai3G203A was more susceptible to proteolysis, indicating a change in the conformation of GIV upon binding the inactive mutant.

We also asked whether phosphorylation of GIV at S1416 affects its binding to Gai3 and found that this is not the case as wild-type, phosphorylation mimic (S1416D), or nonphosphorylatable (S1416A) GIV all bound preferentially to the inactive

Figure 5. Activation status of Gai3 determines its role in cell migration. (A) Cell migratory behavior is restored by wild-type (a and b) and active (Q204L; c and d), but not the inactive (G203A; e and f), Gai3 mutant. (Top) HeLa cells expressing rGai3wt, active, or inactive mutants were subjected to scratch wounding. (Bottom) Equal overexpression of Gai3 constructs was confirmed by immunoblotting. In assays in which siRNA was followed by plasmid overexpression, the efficiency of transfection (~45–55%) was similar for wt, active, or inactive Gai3 constructs by IF (n = 3). (B) Insulin-stimulated Akt activation is restored by wt and active (Q204L) but not the inactive (G203A) Gai3 mutant. (Top) Assays were performed as in Fig. 4 (A and B) and samples were immunoblotted for tAkt and pAkt 5 min after stimulation. (Bottom) Bar graph showing percentage of Akt activation in cells treated as in top. Results are shown as mean ± SEM (n = 3). (C) Transfection of active (Q204L; a–c), but not inactive (G203A; d–f), rGai3-YFP restores normal actin organization (Phalloidin, red) in Gai3-depleted cells. *, cell expressing rGai3-YFP visualized with anti-GFP (green). Bar, 10 μm. (D) Transfection of active (Q204L; a–c), but not inactive (G203A; d–f), rGai3-YFP restores the normal (Fig. 4D, a) scattered peripheral and Golgi-associated (arrowheads) punctate distribution of GIV (red). *, cell expressing rGai3-YFP visualized with anti-GFP (green). Bar, 10 μm.
subunits using anti–pan G

assays (Tetreault et al., 2008). To find out how the G

tant for Akt activation during chemotaxis in macrophages and

Previous studies have established that Gi and GPCRs are impor-
tion, i.e., macrophage chemotaxis and cancer cell migration.

Figure 6. Activation status of Gi3 regulates its interaction with GIV. (A and B) GIV binds preferentially to the inactive [Gal3G203A] form of

G protein with similar strength (Fig. 7 D and Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200712066/DC1). This indicates that the activation state of the G protein, and not phosphorylation of GIV at S1416, regulates the Gi3–GIV interaction.

These results show that the Gi3–GIV interaction facilitates phosphorylation of GIV by Akt. We propose that GIV bound to Gi3 adopts a conformation that brings Akt (bound to its C terminus) closer to S1416 in the G binding domain and thus facilitates its phosphorylation (Fig. 7 E).

Gai3 and GIV are necessary for macrophage chemotaxis and cancer tumor cell migration

We also investigated the expression pattern of Gi3 and GIV in cell lines derived from colon adenocarcinoma with variable in vivo metastatic potential. GIV was expressed exclusively in cells with high metastatic potential (HCT116 and DLD1) and was virtually undetectable in those with poor metastatic potential (HT29p and Ls174T, Fig. 8 D), whereas Gi3 and other regulatory proteins of the G protein pathway, i.e., GaIP (Fig. 8 D), showed no such correlation. As in HeLa cells and macrophages, when highly metastatic DLD1 cells were treated with Gi3 or GIV siRNA, they demonstrated inefficient migration in scratch wounding assays (Fig. 8 E) and impaired Akt activation (Fig. 8 F) compared with scr siRNA–treated cells. Because cell motility in scratch wound assays and Akt activity are validated predictors of the metastatic potential of tumor cells (Raz, 1988; Qiao et al., 2007), DLD1 cells depleted of Gi3 or GIV might be expected to have poor metastatic potential compared with the parent cell line. Collectively, these results suggest a broader role for the Gi3–GIV complex in migratory processes where direction sensing through different chemotactic receptors uses this complex for Akt enhancement, actin remodeling, and cell migration.

Discussion

Summary and working model

This paper describes a novel mechanism by which Gi3 serves as a molecular switch that dictates the formation of the leading edge during cell migration via regulation of the distribution, phosphorylation, and functions of GIV. Without Gi3, Akt amplification downstream of both RTK and GPCR failed to occur, actin remodeling was inhibited, and cells failed to undergo polarized migration after scratch wounding. We also showed that Gi3 localizes preferentially within pseudopods at the leading edge and that activation of Gi3 is essential for migration. Collectively, these results support a working model (Fig. 9) of how the switch operates in few key steps: inactive Gi3 heterotrimer interacts avidly with GIV and induces a change in the conformation of GIV. Upon a chemotactic stimulus (when Akt signaling is initiated) the Gi3-bound conformation of GIV facilitates phosphorylation of GIV at a critical Ser residue that is necessary for its functions at the leading edge. Subsequently, activation of Gi3 triggers dissociation of the Gi3–GIV macromolecular complex releasing Gβγ-subunits and GIV simultaneously. Released Gβγ participates in localized PI3K–Akt activation (Lilly and Devreotes, 1995), and released GIV amplifies and propagates (Collins, 1987) into macrophages. GIV expression increased ~18-fold at 48 h after TPA induction compared with undifferentiated monocytes, whereas levels of Gi3 remained unchanged (Fig. 8 A). We then asked if depletion of GIV would impair macrophage chemotaxis induced by activation the Gi-coupled fMLP receptor (Allen et al., 1988). Macrophages were monitored in real time during the rapid phase (0–25 min) of chemotaxis toward a pipette tip continuously releasing fMLP (Fig. 8 B, top; Chen et al., 2006). We found that depletion of either Gi3 (Fig. 8 B, a–d) or GIV (Fig. 8 B, e and f) inhibited chemotaxis and impaired Akt activation (Fig. 8 C). We conclude that Gi3 and GIV are essential for Akt signaling during GPCR-stimulated macrophage chemotaxis.

We also investigated the expression pattern of Gi3 and GIV in cell lines derived from colon adenocarcinoma with variable in vivo metastatic potential. GIV was expressed exclusively in cells with high metastatic potential (HCT116 and DLD1) and was virtually undetectable in those with poor metastatic potential (HT29p and Ls174T, Fig. 8 D), whereas Gi3 and other regulatory proteins of the G protein pathway, i.e., GaIP (Fig. 8 D), showed no such correlation. As in HeLa cells and macrophages, when highly metastatic DLD1 cells were treated with Gi3 or GIV siRNA, they demonstrated inefficient migration in scratch wounding assays (Fig. 8 E) and impaired Akt activation (Fig. 8 F) compared with scr siRNA–treated cells. Because cell motility in scratch wound assays and Akt activity are validated predictors of the metastatic potential of tumor cells (Raz, 1988; Qiao et al., 2007), DLD1 cells depleted of Gi3 or GIV might be expected to have poor metastatic potential compared with the parent cell line. Collectively, these results suggest a broader role for the Gi3–GIV complex in migratory processes where direction sensing through different chemotactic receptors uses this complex for Akt enhancement, actin remodeling, and cell migration.

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this Akt signaling (Chen et al., 2003; Postma et al., 2004). Amplification of cellular Akt activity via sequential coupling and uncoupling of Gai3 and GIV is likely to set up a positive feedback loop that mediates phosphorylation of further GIV molecules to remodel actin and form the leading edge. In this feedback loop, G proteins are presumably directly activated by GPCRs or indirectly transactivated via RTKs.

Gai3 and GIV directly interact with each other and share dual localizations on the Golgi and the PM (Enomoto et al., 2005; Le-Niculescu et al., 2005). Our results provide clues as to where in the cell this sequential Gai3–GIV molecular coupling might occur. In quiescent cells, Gai3 is predominantly found on the Golgi where it partially colocalizes with GIV (Fig. 1 A), and in migrating cells, Gai3 redistributes to the cell periphery and is concentrated in pseudopods at the leading edge (Figs. 1 A and 2 A) where phosphorylated GIV accumulates during migration (Enomoto et al., 2005). Additionally, we showed that depletion of Gai3 leads to accumulation of GIV on Golgi membranes and impairs phosphorylation of GIV at S1416. Previously, phosphorylation of GIV at S1416 by Akt has been demonstrated to specifically result in abolishing GIV’s affinity toward PI4P (Enomoto et al., 2005), a lipid known to be enriched in Golgi membranes. Based on these findings, we postulate that GIV phosphorylation at S1416, which is facilitated by the Gai3–GIV interaction, mediates the redistribution of GIV from the Golgi to the cell periphery. Mobilization of this internal pool of Gai3-bound GIV to the cell periphery sets the stage for G protein activation and subsequent release of phosphorylated GIV.

Gai3, a state-dependent molecular switch for cell migration

Because the active Q204L, but not the inactive G203A, Gai3 mutant reversed the phenotype of Gai3-depleted cells, activation of the G protein emerges as the key event in its functioning as a molecular switch. In the absence of Gai3 or after expression of inactive Gai3, Akt signaling does not occur (Figs. 4 A and 5 B), indicating that the most significant and direct downstream consequence of Gai3 activation is Akt enhancement. As a consequence of this failure to enhance Akt signaling, phosphorylation of GIV on S1416, redistribution of GIV to the cell periphery, actin remodeling, and cell migration failed to occur (Figs. 4 and 5). Because activation of Gai3 was necessary for migration, it came as a surprise that in vivo binding assays the inactive G protein binds GIV ∼10–15-fold more than the active mutant. Collectively, our results suggest that the weaker interaction between the active Gai3 mutant and GIV is sufficient to facilitate targeting, phosphorylation, and the functions of GIV in vivo. Conversely, our finding that a strong interaction between the inactive G protein and GIV has an overall inhibitory effect on GIV’s functions indicates that reversible coupling is essential...
for normal functioning of the switch. We conclude that the Ga\n3-GIV association constitutes an “on” and “off” switch that is regulated by cyclical G protein activation and is essential for phosphorylation, targeting, and subsequent release of GIV during cell migration.

\textbf{Ga\n3 regulates phosphorylation of GIV}

We show here that the Ga\n3–GIV association is required for phosphorylation of GIV at S1416 by Akt. Phosphorylation of GIV at this site was previously demonstrated to play a critical role in cell migration, but the mechanism was unclear (Enomoto et al., 2005). We provide evidence that GIV undergoes a change in conformation when bound to Ga\n3. It is noteworthy that the Akt binding site on GIV (extreme C terminus) is separated in conformation when bound to Ga\n3 by 250 \textmu m, but not low (HT-29p, LS-174T) in vivo metastatic potential. Ga\n3 and GAIP (used as a control) expression are similar in all cell lines. (E) Depletion of Ga\n3 or GIV impairs wound healing in highly metastatic colon cancer cells. DLD1 cells treated with scr (a and b), Ga\n3 (c and d), or GIV (e and f) siRNA were imaged at the end of a fMLP chemotaxis assay. Arrow denotes direction of chemotaxis. Bars: (a, c, and e) 250 \textmu m; (b, d, and f) 50 \textmu m. (C) Immunoblotting performed on lysates harvested at 0 and 36 h after scratch-wounding. (F) Depletion of Ga\n3 or GIV (right) impairs Akt activation (pAkt) in highly metastatic DLD1 colon cancer cells. Immunoblots were performed on lysates harvested at the end of a wounding assay.

\textbf{Ga\n3 dictates formation of the leading edge by simultaneous release of G\n3\m and phosphorylated GIV}

We found that Ga\n3 through its interaction with GIV is required for Akt enhancement and actin remodeling during scratch wound–induced migration in epithelial cells and for rapid chemotaxis in macrophages. However, in \textit{D. discoideum} the Ga\n3-subunit is dispensable and only G\n3\m-subunits are required for PI3K-Akt activation at the leading edge during GPCR-stimulated migration (Lilly and Devreotes, 1995; Van Haastert and Devreotes, 2004). It is noteworthy that there is only one G\n3\m subunit in \textit{D. discoideum} (Lilly and Devreotes, 1995) and, to the best of our knowledge, there is no homologue of GIV. Thus, the differences between HeLa cells and \textit{D. discoideum} may represent an acquired function of Ga\n3 in mammals, as the number of G proteins and their interacting partners expanded during evolution.

\begin{figure}
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\includegraphics[width=\textwidth]{figure8}
\caption{The Ga\n3-GIV signaling complex is necessary for macrophage chemotaxis and tumor cell migration. (A) GIV, but not Ga\n3, expression is induced ~18-fold upon differentiation of monocytes into macrophages. THP1 monocytes were treated with 5 nM TPA or DMSO carrier, and the expression of Ga\n3 and GIV was followed by immunoblotting over 68 h. (B) Depletion of Ga\n3 or GIV impairs macrophage chemotaxis. (Top) Schematic illustration of the chemotaxis assay in which macrophages migrate toward a pipette tip, continuously releasing fMLP to maintain a gradient. The dashed line denotes the edge of the monolayer just before the application of the chemotactic gradient. (Bottom) THP1-derived macrophages treated with scr (a and b), Ga\n3 (c and d), or GIV (e and f) siRNA were imaged at the end of a fMLP chemotaxis assay. Arrow denotes direction of chemotaxis. Bars: (a, c, and e) 250 \textmu m; (b, d, and f) 50 \textmu m. (C) Immunoblotting performed on lysates harvested at the end of the chemotaxis assay (shown in B) demonstrating that depletion of Ga\n3 or GIV impairs Akt activation (pAkt) in THP1-derived macrophages. (D) GIV is expressed in colon cancer cell lines with high (HCT-116, DLD1) but not low (HT-29p, LS-174T) in vivo metastatic potential. Ga\n3 and GAIP (used as a control) expression are similar in all cell lines. (E) Depletion of Ga\n3 or GIV impairs wound healing in highly metastatic colon cancer cells. DLD1 cells treated with scr (a and b), Ga\n3 (c and d), or GIV (e and f) siRNA were monitored at 0 and 36 h after scratch-wounding. Bars, 1 mm. (F) Depletion of Ga\n3 (left) or GIV (right) impairs Akt activation (pAkt) in highly metastatic DLD1 colon cancer cells. Immunoblots were performed on lysates harvested at the end of a wounding assay.}
\end{figure}
et al., 2005) sets the stage for sequential signal amplification of preferential accumulation of G1997; Janetopoulos et al., 2001; Ueda et al., 2001). Our observation fails to account for this steep signaling gradient (Xiao et al., 1997; Anai et al., 2005; Enomoto et al., 2005). Thus, our work provides mechanistic insights into how activation of Gα3 links direction sensing to GIV-mediated PI3K–Akt enhancement and actin remodeling at the leading edge.

Gα3 is required for centrosome repositioning during migration

We found that both Gα3 and GIV are localized on centrosomes and are necessary for polarized migration, whereas Gα3, but not GIV, is required for repositioning the MTOC/centrosome. In contrast, GIV is required for polarized migration but is not essential for centrosome repositioning. This is in keeping with the fact that asymmetrical extension of lamellipodia and centrosome repositioning are not necessarily coupled during polarized migration of epithelial cells (Euteneuer and Schliwa, 1992). Our results suggest a distinct hierarchy within the Gα3–GIV regulatory complex, in which Gα3 regulates additional steps during cell migration besides those performed by GIV. The role of centrosomal GIV could be to nucleate microtubule tracks to the leading edge because, like other members of the Hook family, GIV can bind to microtubules (Simpson et al., 2005).

Direction sensing via different chemotactic receptors converge upon Gα3 and GIV

GIV has been implicated in RTK-stimulated migration (Enomoto et al., 2005), and Gαi is well known to be important for GPCR-stimulated chemotaxis (Thelen, 2001). Our results now demonstrate that activation of Gα3 is required during scratch wound-induced migration where cross talk between GPCRs and RTKs are known to mediate chemotactic movements (Shan et al., 2006a; Yin et al., 2007). Similarly, we have shown that upon direct stimulation with RTK ligands (insulin and EGF), activation of Gα3 is required to elicit a full response in terms of Akt activation, suggesting that prominent transactivation of G proteins takes place. Additionally, we provide evidence supporting the necessity for GIV during GPCR-stimulated Akt signaling or chemotaxis. Although indirect stimulation of some RTKs by GPCR–G protein–dependent intermediates is well established (Luttrel et al., 1999), there is little mechanistic insight into how RTK stimulation might directly signal via G proteins to activate Akt (Waters et al., 2004; Dhanasekaran, 2006). In this paper, we show that in epithelial cells during wound healing, chemotaxing macrophages, and tumor cells, the state-dependent interaction between Gα3 and GIV is essential, and upon depletion of either of these proteins the critical step of amplification of Akt signaling is abrogated.

We conclude that regardless of how the chemotactic signal is propagated from the cell surface to initiate Akt signaling, the common theme is the requirement of Gα3 activation and GIV to promote cell migration. Although we cannot rule out...
that different biochemical events may occur depending on whether GPCRs or RTKs are activated, it is tempting to speculate that molecular coupling between Gαi3 and GIV could serve as a novel platform for receptor cross talk and that together they could be the long-sought missing link between chemotactic receptors and signal amplification.

Materials and methods

Reagents and antibodies

Unless otherwise indicated, all reagents were of analytical grade and obtained from Sigma-Aldrich, and cell culture media were purchased from Invitrogen. Antibodies against Gαi3 and Gαi3 wild type and mutants (Q204L or G203A) were obtained from EMD, and mAb against human GIV (Enomoto et al., 2005), Gαi3 tagged with YFP at the C terminus (L) and short (S) splice variants were obtained from GALT were gifts from S.J. Doxsey (University of Massachusetts, Worcester, MA) and E. Berger (University of Zurich, Zurich, Switzerland).

Full-length GIV cloned into a pcDNA 3.1 vector was provided by M. Takahashi (Enomoto et al., 2005). For plasmid overexpression, the efficiency of transfection was monitored after 38–40 h. In those assays where siRNA was followed by plasmid overexpression, the efficiency of transfection was analyzed after ~38–40 h. In those assays where siRNA was followed by plasmid overexpression, the efficiency of transfection was ~45–55%.

Live-cell imaging

Hela cells grown in chambered coverglass (Thermo Fisher Scientific) were scratched wounded to induce migration. Media was changed to Liebovitz L-15 supplemented with 2.5 mM Hepes, 4.5 g/liter glucose, and 100 mM Na2 pyruvate as cells were placed on the stage of an inverted microscope (Axiovert 200M; Carl Zeiss, Inc.) equilibrated at 37°C (Incubator XL-3 [Carl Zeiss, Inc.] Heating Unit and TempControl 37–2 Digital). Images of many fields of cells along the wound edge were taken at 40x magnification (Plan Neofluar objective [Carl Zeiss, Inc.], 1.3 NA in DIC and YFP channels every 10 min for 8 h using an XYZ moving stage (MS2000; Applied Scientific Instrumentation) and charge-coupled device camera (MicroMax 512 BF; Princeton Instruments) with SlideBook 4.1 software (Intelligent Imaging Innovations, Inc.). Image processing was done using ImageJ software (National Institutes of Health).

In vitro binding

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Cell migration assays

Monolayer cell cultures (~100% confluent) were wounded with a 1-mm sterile pipette tip creating a 1-mm wound, as previously described (Enomoto et al., 2005), and monitored by phase contrast or time-lapse video microscopy over the succeeding 25 min as they migrated toward a steady chemoattractant gradient generated by releasing 100 pM fMLP (Sigma-Aldrich) from a micropipette tip placed on the opposite side of the plate.

Immunoprecipitation

Cell lysates (~1–2 mg of protein) were incubated overnight at 4°C with 2 μl of preimmune or anti-GIV serum in PBS. Protein A agarose beads (Invitrogen) were added and incubated at 4°C for an additional 90 min. Beads were then washed and boiled in SDS sample buffer or used for in vitro phosphorylation or trypsinization assays.

In vitro phosphorylation and limited proteolysis with trypsin

GIV was immunoprecipitated from Cos7 lysates, subdivided into equal aliquots, and preincubated with equal amounts [5 μg] of purified GST or GST-Gαi3 constructs (wt, Q204L, and G203A) for 4 h at 4°C. Phosphorylation was performed in 20 mM Hepes, pH 7.5, 5 mM MgCl2, 20 mM β-glycerophosphate, 1 mM EDTA, 0.1% β-mercaptoethanol, phosphatase, and protease inhibitor cocktail for 90 min at 30°C. The reaction was terminated by adding 50 μM ATP and 1,000 μM human reconstituted activator Ak1 (EMD) and terminated by boiling in SDS sample buffer. Tryptsin digestion was performed as described previously (Ghosh and Kornfeld, 2003).

IF

All the steps for IF were performed at room temperature as described previously (Zheng et al., 2004). In brief, cells were fixed with 3% PFA for

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30 min, permeabilized with 0.2% Triton X-100 for 45 min, and incubated for 1 h with primary and secondary antibodies. Antibody dilutions as follows: affinity purified anti-GIV, 1:10; anti-Gu3 (EMD), 1:30; anti-V5 (Invitrogen), 1:200; anti-Man1, 1:500; anti-pericentrin, 1:1,000; anti-γ-tubulin (Sigma-Aldrich), 1:500; anti-β-GaIA, 1:20; Phalloidin-Texas Red, 1:1,000; secondary Alexa-conjugated antibodies, 1:500; and DAPI, 1:2,000 (Invitrogen). To visualize endogenous GIV or Gu3, cells were permeabilized with 0.05% (weight/vol) saponin and 0.2 mg/ml BSA in PBS for 1 min before fixation. Images were acquired with a microscope (Axioimager M1; Carl Zeiss, Inc.) using a 100× aperture (Plan Neofluar; 1.3 NA), camera (Orca ER; Hamamatsu Photonics), and Openlab software (Improvision). For the centrosome repositioning assay, HeLa-C1 monolayers were scratch wounded, and 8–10 h after wounding the number of cells with centrosomal [GFP-centrin] and Golgi [ManII] positive staining within the 120° sector facing the wound were counted (Fig. 2; Kuper et al., 1982; Etienne-Manneville and Hall, 2001; Grande-Garcia et al., 2007).

For centrosomal localization studies, wide-field microscopy was performed using the untreated 1.3 x 1.3 NA Neofluor oil immersion objective lenses on an inverted microscope (IX-70 DeltaVision Restoration; Olympus). The microscope was equipped with DAPI (360/40 excitation, 457/50 emission), FITC (490/20 excitation, 528/38 emission), and TRITC (555/28 excitation, 617/73 emission) band pass filter sets, a motorized stage (FCS2; Bioptechs), and a charge-coupled device camera (Photometrics CH350; Hamamatsu Photonics). Optical sections were collected at 0.2 μm intervals along the optical–basal axis. Applied Precision software [DeltaVision] was used to deconvolve z-section series of images. All individual images were processed using Image J software and assembled for presentation using Photoshop and Illustrator software (both Adobe).

**Immunoblotting**

Proteins samples were separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with PBS supplemented with 5% nonfat milk and then incubated sequentially with primary and secondary antibodies. Infrared imaging with two-color detection and Quantification of Western blots was performed according to the manufacturer’s protocols using an Odyssey imaging system (Li-Cor Biosciences). Immunoblotting quantification was performed according to the manufacturer’s protocols using an Odyssey imaging system (Li-Cor Biosciences). Western blot images were acquired using Image J software. Western blot images were acquired and assembled for presentation using Photoshop and Illustrator software (both Adobe).

**Statistical analysis**

Experiments were performed at least three times and results are expressed as mean ± SEM. Statistical significance between various conditions was assessed using Student’s t-test. P < 0.001 was considered significant.

**References**


