Activation of Gαi3 triggers cell migration via regulation of GIV

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During 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 receptor systems 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 through phosphoinositide 3-kinase (PI3K) at the leading edge of migrating cells (Merlot and Firtel, 2003; Van Haastert and Devreotes, 2004). Once the Akt signal is adequately enhanced, cells “polarize” and reposition their Golgi apparatus and microtubule-organizing center (MTOC) in front of the nucleus and form the leading edge (Etienne-Manneville and Hall, 2002; 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. Gαi3 redistributes to the cell periphery during cell migration and is necessary for cell migration. (A) Distribution of Gαi3 in quiescent (a–f) versus migrating (g–i) HeLa cells after scratch wounding. In quiescent cells, endogenous Gαi3 colocalizes with β1GalT in the Golgi (a–c) but not actin (d–f). In migrating cells, Gαi3 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. (B) Depletion of Gαi3 (g and h) or GIV (e and f), but not Gαs (c and d), impairs wound healing (compare with scrambled siRNA [scr siRNA] controls; a and b). Repletion of hGαi3 by rat Gαi3 (i and j), but not vector alone (k and l), restores this defect. HeLa cells treated as indicated were subjected to scratch wounding and examined immediately after wounding (0 h) or 16 h later (n = 5). (C) Cell lysates from cells treated as in B were immunoblotted to assess the efficiency of siRNA depletion of Gαi3 (~90%), GIV (~85%), or Gαs (~95%) and the expression of Gαi3s. Several GIV bands are identified that most likely are different posttranslationally modified forms of GIV because they are specifically depleted using GIV siRNA. (D) Comparative trajectories of Gαi3-depleted cells versus those transfected with Gαi3-YFP were followed by live cell imaging for 8 h after wounding. Representative cells at the advancing edge of the wound (dashed line) were traced using a cell tracker application on videos obtained of transfected (solid squiggles) or untransfected (circles) cells through the YFP channel (b) or a simultaneously recorded DIC channel (a; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200712066/DC1). (E) Bar graphs showing the distance covered by cells in D analyzed by the cell-tracker application (top graph) and the percent of untransfected Gαi3-depleted cells versus Gαi3-depleted cells transfected with rGαi3-YFP that displayed directional migration (bottom graph). Results are shown as mean ± SEM (n = 5).

In quiescent cells far from the wound, Gαi3 showed a predominant Golgi localization based on colocalization with a Golgi marker, β1-4 galactosyltransferase (β1-4GalT; 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 Gαi3-stained puncta within lamellipodial extensions. Some of these puncta colocalized with actin at the leading edge (Fig. 1 A, g–j). Thus, Gαi3 showed different distributions depending on the migratory state of the cell.

Because GIV is a binding partner of the α-subunit of Gi and Gαs and is important for cell migration, we asked if Gαi3 or Gαs is necessary for cell migration. We depleted Gαi3 in HeLa cells using siRNA oligos (Fig. 1 C) and measured the rates of wound closure. Depleting Gαi3 (~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). Gαi3-depleted cells regained their ability to close the wound when Gαi3 levels were replenished by expression of siRNA-resistant rat (rGαi3wt; Fig. 1 B, i–l).

the leading edge in a chemotaxing cell by coupling direction sensing through cell surface receptors to Akt enhancement and actin remodeling via GIV.

Results

Gαi3 is necessary for cell migration

Both GIV and Gαi3 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, we asked if Gαi3 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 Gαi3 by immunofluorescence (IF).
Live cell imaging revealed that most (~75%) of the Gai3-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 rGai3 (rGai3-YFP) was introduced into Gi3-depleted cells, ~75% of the transfected cells migrated toward the wound (Fig. 1 E).

**Gai3 preferentially localizes to pseudopods at the leading edge of migrating cells**

To study the dynamics of Gai3 localization after wounding, we observed HeLa cells expressing Gai3-YFP by live cell imaging as they transitioned from quiescence to migration. Previously, we demonstrated that rGai3-YFP localizes largely to the Golgi and behaves similarly to endogenous Gai3 (Weiss et al., 2001). During migration, Gai3-YFP localized preferentially at 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 Gai3-stained puncta (Fig. 1 A, j) that are not resolved by the optical conditions used for live cell imaging (see Materials and methods). Because Gai3 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-palmitoyl-modified YFP (mp-YFP), which has the same modifications as Gai3-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 Gai3 was also observed within pseudopods at the leading edge using 1,1′-Diocadecyl-3,3,3′,3′-tetramethylrhodocarboxyanine 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 Gai3, like its binding partner GIV, is concentrated at the leading edge of a migrating cell.

**Gai3 is necessary for centrosome/MTOC repositioning during cell migration**

Because Gai3-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 Centrin1-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 Gai3-depleted cells (as was expected in the absence of polarization) achieved the same phenotype (Fig. 3 C). GIV or Gas-depleted cells were similar to controls. When rGai3wt was introduced into Gai3-depleted cells, the defect in centrosome repositioning was reversed. Using deconvolution microscopy to visualize the plane of the centrosome, we found that both endogenous Gai3 (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 Gai3 and GIV localize to the centrosome region and only Gai3 is required for centrosomal repositioning suggests that the latter is a GIV-independent function of Gai3.

**Gai3 is required for enhancement of Akt signaling**

Next, we asked whether Gai3 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 Gai3-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 Gai3 specific because Gas depletion did not significantly affect Akt activation (Fig. 4 A) and was reversed when rGai3wt was restored in Gai3 siRNA-treated cells (see Fig. 5 B). Therefore, Gai3 links Akt activation and cell migration in a manner...
Figure 3. Gαi3 and GIV localize at the centrosome/MTOC and Gαi3 is necessary for centrosome repositioning. (A) Deconvolved images through the centrosomes demonstrating the localization of Gαi3 and GIV on centrosomes. HeLa and Cos7 cells were permeabilized, fixed, and stained with a centrosome marker (α-tubulin or pericentrin) and Gαi3 [α–β3, GIV [α–β], or overexpressed V5-GIV [α–β]]. Bar, 10 μm. (B) Schematic illustration (bottom) of the centrosome repositioning assay performed on HeLa cells expressing GFP-Centrin1. Cells located at the edge of a wound are scored positive in which the centrosome [GFP-Centrin1, green] and Golgi (Man II, red) are positioned in front of the nucleus [DAPI, blue] within the 120° tri- dent facing the direction of the wound (arrow). The white box indicates the area magnified below. Bar, 10 μm. (C) Bar graph showing the percentage of cells at the wound edge that achieved centrosome repositioning by 8 h after wounding. Gαi3-depleted cells failed to reposition their centrosomes and transfection of Gαi3wt restored this defect. GIV or Gαs-depleted cells were similar to controls (64%). Results (200–400 cells per experiment; n = 3) are shown as mean ± SEM. *, P < 0.001; #, P > 0.001 (compared with scr siRNA cells).

similar to that reported for GIV (Anai et al., 2005). To distinguish whether Gαi3 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 Gαi3 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 Gαi3 promoted weaker inhibition of Akt than GIV suggests that other Gα3-independent pathways might exist in which GIV is a common effector. Akt activation was also impaired when Gαi3-depleted HeLa cells were stimulated with EGF (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200712066/DC1), which implicates Gαi3 and GIV in a common pathway mediating Akt activation upon RTK stimulation.

Gαi3 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 Gαi3-depleted cells. Phalloidin staining for F-actin (filamentous actin) revealed major differences in actin organization of Gαi3-depleted cells versus that of controls (Fig. 4 C, a and b); the Gαi3-depleted cells were virtually unable to form long stress fibers and displayed a pronounced bed of cortical actin but recovered their ability to form stress fibers when transfected with rGαi3wt-YFP (Fig. 4 C, c and d). We also investigated actin morphology in Gαi3-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 Gαi3 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 Gαi3

We next investigated if silencing Gαi3 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 Gαi3 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 Gαi3-depleted cells were replenished with rGαi3-YFP (Fig. 4 D, c–e). We conclude that Gαi3 is necessary for redistribution of GIV from the Golgi to more peripheral locations.

Activation of Gαi3 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 Gαi3 is required for its functions during cell migration. We transfected rGαi3Q204L and rGαi3G203A mutants, which behave like the GTP- and GDP-bound forms of Gαi3, respectively (Hermouet et al., 1991; Coleman et al., 1994), into Gαi3-depleted cells and assessed their ability to reverse the effects of
Gai3 depletion. Transfection of either Gai3wt or the active rGai3Q204L 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 rGai3G203A had no effect on these parameters (Fig. 5, A–D).

Next, we asked whether the interaction between Gai3 and GIV is activity dependent and found that the inactive GST-Gai3G203A mutant consistently bound ~10–15-fold more GIV than the active Gai3Q204L 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-Gai3 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-Gai3 bound ~10–30% of the total GIV, and addition of AlF₄⁻ virtually abolished the binding (Fig. 6 B). In contrast, GST-Gai3 bound ~100–300-fold less than GST-Gai3 wt (Fig. 6 C), which is consistent with our finding that Gai3-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 Gai3 and is greatly reduced upon activation of the G protein. Because inactive GDP-bound Gai3 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 coimmuno-precipitated 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, Gai3 mutant reversed the migration-defective phenotype of Gai3-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 active, but not the inactive, Gai3 and GIV does not have an additive effect on Akt signaling. (Top) HeLa cells treated as in A were immunoblotted for pAkt and pAkt 5 min after insulin stimulation. (Bottom) Bar graph showing Akt activity in cells treated as in top expressed as percentage of the response in controls. Results are shown as mean ± SEM (n = 3). *P values compared with scr-siRNA are indicated. (C) Depletion of Gai3 alters actin organization. Cells treated with scr siRNA (a) show stress fibers whereas Gai3-depleted cells (b) are unable to form stress fibers and show a prominent bed of cortical actin. The effects on actin in Gai3-depleted cells are reversed by transfecting rGai3wt-YFP (d) but not vector alone (c). HeLa cells treated as indicated were stained with Phalloidin (red) and DAPI (blue). *, cells expressing rGai3wt-YFP visualized with anti-GFP. Bar, 10 μm. [D] Distribution of GIV is less scattered and more concentrated in the Golgi (arrowheads) upon silencing Gai3 (compare a and b). The scattered distribution is restored upon transfecting rGai3wt-YFP (e–g). Hela cells were treated as indicated and stained for Hela (red), GFP (green), and DAPI (blue). *, cells expressing rGai3wt-YFP visualized with anti-GFP mAb. Bar, 10 μm.
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-Gai3wt, GST-Gai3G203A, or GST-Gai3Q204L was relatively resistant to proteolysis, whereas GIV preincubated with 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
GIV interaction. We propose that GIV regulates phosphorylation of GIV by Akt. This indicates that the activation state of the G protein, and thus facilitates its phosphorylation (Fig. 7E).

Figure 6. Activation status of Ga3 regulates its interaction with GIV. (A and B) GIV binds preferentially to the inactive [Ga3GDP303A] form of Ga3. (A, Top) In vitro binding assays were performed using GST-Ga3 (wt or mutants) and either HeLa cell lysates [GIV (HeLa)] or in vitro–translated GIV [GIV (TnT)]. Bead-bound fractions were separated by SDS-PAGE. Bound GIV was analyzed either by immunoblotting or autoradiography (TnT). (B, Top) In vitro binding assay performed as in A with GST-Ga3wt after GDP loading in the absence or presence of AlF4−. (A and B, Bottom) Ponceau S-staining of PVDF membranes to show equal loading of GST proteins. (C) GIV preferentially binds to Ga3. In vitro binding assays were performed as in B with GST-Ga3wt or Gsα in the presence of GDP ± AlF4− loading to compare the relative binding of Gsα and Ga3 to GIV. Gβi-subunits were used as a positive control. Bound GIV from HeLa cell lysates was visualized by immunoblotting (GIV [low exp], low exposure; GIV [high exp], high exposure) and autoradiography [TnT], respectively. (D) GIV interacts with the inactive Ga3βγ heterotrimer in vivo. GIV was immunoprecipitated from rat brain lysate in the absence (lanes 1–3) or presence (lanes 4–6) of GDP + AlF4−, and immune complexes were probed for GIV and Gβi subunits using anti–pan Gβ.

G protein with similar strength (Fig. 7D 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 Ga3–GIV interaction.

These results show that the Ga3–GIV interaction facilitates phosphorylation of GIV by Akt. We propose that GIV bound to Ga3 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. 7E).

Ga3 and GIV are necessary for macrophage chemotaxis and cancer cell migration We also investigated the roles of Ga3 and GIV in other biological processes that require Akt enhancement during cell migration, i.e., macrophage chemotaxis and cancer cell migration. Previous studies have established that Gi and GPCRs are important for Akt activation during chemotaxis in macrophages and neutrophils (Thelen, 2001) and that cancer cell lines, like HeLa, use RTKs for Akt activation during migration in scratch wound assays (Tetreault et al., 2008). To find out how the Ga3–GIV regulatory complex behaves during cell migration in macrophages, we analyzed THP1 human monocytes before and after tetradecanoyl-phorbol acetate (TPA)–induced differentiation (Collins, 1987) into macrophages. GIV expression increased ~18-fold at 48 h after TPA induction compared with undifferentiated monocytes, whereas levels of Ga3 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 Ga3 (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 Ga3 and GIV are essential for Akt signaling during GPCR-stimulated macrophage chemotaxis.

We also investigated the expression pattern of Ga3 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 Ga3 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 Ga3 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 Ga3 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 Ga3–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 Ga3 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 Ga3, 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 Ga3 localizes preferentially within pseudopods at the leading edge and that activation of Ga3 is essential for migration. Collectively, these results support a working model (Fig. 9) of how the switch operates in few key steps: inactive Ga3 heterotrimer interacts avidly with GIV and induces a change in the conformation of GIV. Upon a chemotactic stimulus (when Akt signaling is initiated) the Ga3-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 Ga3 triggers dissociation of the Ga3–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...
Figure 7. Goi3 is necessary for phosphorylation of GIV by Akt. (A) Phosphorylation of GIV is decreased upon depletion of Goi3 (Goi3 siRNA) and restored upon transfecting rGoi3 (Goi3 siRNA and rGoi3). GIV was immunoprecipitated from HeLa cells treated as indicated and phosphorylation determined by immunoblotting with anti-pSer/Thr IgG. (B, top) Goi3 binding to GIV promotes phosphorylation of GIV at S1416 by Akt. Immunoprecipitated GIV was preincubated in the presence or absence of ~5 µg GST (lane 3), GST-Goi3wt (lane 4), Goi3 Q204L (lane 5), or Goi3 G203A (lane 6) and subsequently phosphorylated in vitro with recombinant Akt1, followed by SDS-PAGE. Phosphorylation was determined by immunoblotting with antipSer IgG. (B, bottom) Equal loading of preimmune or anti-GIV IgG and GST proteins was confirmed by Ponceau S staining. (C) Goi3 binding to GIV increases its susceptibility to trypsin- mediated proteolysis. Immunoprecipitated GIV was incubated in the presence or absence of GST-Goi3 proteins as in B and digested with increasing concentrations of trypsin at 37°C for 8 min. The amount of uncleaved (trypsin resistant) GIV was determined by immunoblotting (inset) and displayed as percentage of starting amount (y axis) plotted against trypsin concentration (x axis). (D) GST-Goi3 interacts similarly with in vitro-translated GIV wild-type and phosphorylation mimic (S1416D) or non-phosphorylatable (S1416A) GIV mutants. Semi-quantitative densitometry revealed that 14 ± 2, 13 ± 1, and 14 ± 3% of wild-type, S1416A, and S1416D, respectively, bound to GST-Goi3 (n = 5). (E) Schematic representation of how binding of Goi3 to GIV may mediate phosphorylation of GIV by bringing the C-terminal Akt binding site closer to the Akt phosphorylation site. 

Goi3 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 Goi3–GIV molecular coupling might occur. In quiescent cells, Goi3 was predominantly found on the Golgi where it partially colocalizes with GIV (Fig. 1 A), and in migrating cells, Goi3 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 Goi3 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 Goi3–GIV interaction, mediates the redistribution of GIV from the Golgi to the cell periphery. Mobilization of this internal pool of Goi3-bond GIV to the cell periphery sets the stage for G protein activation and subsequent release of phosphorylated GIV.

Goi3, a state-dependent molecular switch for cell migration

Because the active Q204L, but not the inactive G203A, Goi3 mutant reversed the phenotype of Goi3-depleted cells, activation of the G protein emerges as the key event in its functioning as a molecular switch. In the absence of Goi3 or after expression of inactive Goi3, Akt signaling does not occur (Figs. 4 A and 5 B), indicating that the most significant and direct downstream consequence of Goi3 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 Goi3 was necessary for migration, it came as a surprise that in an in vitro 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 Goi3 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
Figure 8. The Goi3–GIV signaling complex is necessary for macrophage chemotaxis and tumor cell migration. (A) GIV, but not Goi3, 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 Goi3 and GIV was followed by immunoblotting over 68 h. (B) Depletion of Goi3 or GIV impairs macrophage chemotaxis. (Top) Schematic illustration of the chemotaxis assay in which monocytes migrate toward a pipette tip, continuously releasing fMLP to maintain a gradient. The dashed line denotes the edge of the monolayer. (Bottom) THP1-derived macrophages treated with scr (a and b), Goi3 (c and d), or Goi3 (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 μm; (b, d, and f) 50 μm. (C) Immuno­blotting performed on lysates harvested at the end of the chemotaxis assay (shown in B) demonstrating that depletion of Goi3 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. Goi3 and GAIP (used as a control) expression are similar in all cell lines. (E) Depletion of Goi3 or GIV impairs wound healing in highly metastatic colon cancer cells. DLD1 cells treated with scr (a and b), Goi3 (c and d), or Goi3 (e and f) siRNA were moni­tored at 0 and 36 h after scratch-wounding. Bars, 1 mm. (F) Depletion of Goi3 (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.

Goi3 regulates phosphorylation of GIV

We show here that the Goi3–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 Goi3. It is noteworthy that the Akt binding site on GIV (extreme C terminus) is separated from S1416 (which lies within the G binding domain) by ~300 aa (Anai et al., 2005). We speculate that a major molecular rearrangement occurs when GIV binds Goi3 to bring Akt bound to the carboxyl terminus closer to S1416 in the G binding domain and, thus, facilitates its phosphorylation. We also show that activation of Goi3 rather than phosphorylation of GIV is the key trigger for dissociation of GIV from the Goi3–GIV complex.

In the context of when these events take place, our results provide evidence that the cycling of G protein between active and inactive states ensures that phosphorylation of Goi3-bound GIV precedes its subsequent release from the complex. In a highly polarized chemotaxing cell, this is likely to occur on the PM at the leading edge where there is a concentration of ligand-occupied receptors, activated Akt, and activated Goi3 (Ridley et al., 2003; Van Haastert and Devreotes, 2004). Thus, the G protein molecular switch contributes to the spatial bias in accumulation of phosphorylated GIV that was previously demonstrated to occur selectively at the leading edge (Enomoto et al., 2005).

Goi3 dictates formation of the leading edge by simultaneous release of Gβγ and phosphorylated GIV

We found that Goi3 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 D. discoideum the Gxα-subunit is dispensable and only Gβγ-subunits are required for PI3K-Akt activation at the leading edge during GPCR-stimulated migration (Lilly and Devreotes, 1995; Brzostowski et al., 2004). It is noteworthy that there is only one Gβγ subunit in 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 D. discoideum may represent an acquired function of Goi3 in mammals, as the number of G proteins and their interacting partners expanded during evolution.
We found that during cell migration, Gαi3 preferentially localizes to the leading edge where enhancement of Akt signaling occurs (Merlot and Firtel, 2003). It is well established that to form a leading edge and migrate, cells must display bi- phasic Akt activation in response to a chemotactic stimulus in that a brief early phase of generalized Akt activation is followed by an enhanced and prolonged phase. The second phase of enhancement creates a steep PI3K-Akt signaling gradient that is restricted to the stretch of PM destined to form the leading edge (Chen et al., 2003; Postma et al., 2004). However, the uniformity of distribution of chemotactic receptors (RTKs and GPCRs; Servant et al., 1999; Bailly et al., 2000) and shallow anterior–posterior gradient of Gβ subunits (Jin et al., 2000) fails to account for this steep signaling gradient (Xiao et al., 1997; Janetopoulos et al., 2001; Ueda et al., 2001). Our observation of preferential accumulation of Gαi3 together with the localization of phospho-GIV at the leading edge (Enomoto et al., 2005) sets the stage for sequential signal amplification by placing the Gαi-subunit in the immediate vicinity of the ligand-occupied receptors that can activate G proteins. Activation of Gαi3 accounts for a major part of Akt signaling (~60% of the peak Akt activity upon insulin/EGF stimulation) via regulation of GIV, indicating that although Gβγ-subunits initiate Akt signaling through activation of PI3K (Lilly and Devreotes, 1995), accumulation of simultaneously released phosphorylated GIV is essential for localized enhancement of Akt at the leading edge via the PI3K–Akt pathway (Xiao et al., 1997; Anai et al., 2005; Enomoto et al., 2005). Thus, our work provides mechanistic insights into how activation of Gαi3 links direction sensing to GIV-mediated PI3K–Akt enhancement and actin remodeling at the leading edge.

**Gαi3 is required for centrosome repositioning during migration**

We found that both Gαi3 and GIV are localized on centrosomes and are necessary for polarized migration, whereas Gαi3, 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 Gαi3–GIV regulatory complex, in which Gαi3 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 Gαi3 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 Gαi3 is required during scratch wound-induced migration where cross talk between GPCRs and RTKs are known to mediate chemotactic movements (Shan et al., 2006; Yin et al., 2007). Similarly, we have shown that upon direct stimulation with RTK ligands (insulin and EGF), activation of Gαi3 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 Gαi3 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 Gαi3 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 the coiled-coil region of GIV were raised in rabbits and purified as previously described (Le-Niculescu et al., 2005). Antibodies against Gαi3 and Gαq/11 were obtained from EMD, and pan Gβi (M14) and Gαi3 used for immunoblotting were purchased from Santa Cruz Biotechnology, Inc. Rabbit IgG against phospho-Akt (serine 473) was purchased from Cell Signaling Technology, and mAb against total Akt was purchased from BD Biosciences. Mabs against actin, α-tubulin, and rabbit IgG against phosphoserine were purchased from Sigma-Aldrich. Anti–mouse and anti–rabbit Alexa 594– and Alexa 488–coupled secondary antibodies for IF, alkaliphilic Texas Red, and anti-rat were purchased from Invitrogen. Rabbit antisera against mammotribe II (ManII) was prepared as described previously (Velasco et al., 1993). Goat anti–rabbit and goat anti–mouse Alexa Fluor 680 or IRDye 800 F(ab')2 were obtained from LI-COR Biosciences, and DiD was obtained from Invitrogen. The autologous human serum against pericentrin (5051) and mAb against pGALU were gifts from S.J. Doxsey (University of Massachusetts, Worcester, MA) and E.G. Berger (University of Zurich, Zurich, Switzerland), respectively.

Plasmids, mutants, and protein expression
Full-length GIV cloned into a pcDNA 3.1 vector was provided by M. Takahashi (Enomoto et al., 2006). Mutants GIV S314A or GIVi3D6 were generated according to the manufacturer's instructions (QuickChange II; Stratagene) and confirmed by sequencing. Primer sequences are available upon request. Wild-type rat Gαi3 cDNA, Gαi3 (Q204L), and Gαi3 (G203A) mutants, obtained from A. Spiegel (National Institutes of Health, Bethesda, MD), were subcloned into pcDNA 3.1 (Invitrogen) or pGEX-4T-1 (GE Healthcare) vectors. Cloning of Gαi3 tagged with YFP at the C terminus was described previously (Weiss et al., 2001). The plasmid encoding the mYFP was a gift from R. Tsien (University of California, San Diego, La Jolla, CA). cDNA encoding the human Gαs long (I) and short (S) splicing variants were obtained from Guthrie cDNA Resource Center(Zheng et al., 2004) and were subcloned into pGEX-KG vector.

Plasmids encoding GST-Gαi3 wild type and mutants (Q204L or G203A) or GST-Gαs fusion proteins were used to express these proteins in Escherichia coli strain BL21 (DE3) (Invitrogen), and protein expression was induced overnight at 25°C by IPTG. Pelleted bacteria from 1 liter of culture were resuspended in 10 ml of lysis buffer (Buffer A), sonicated four times for 20 s with 1 min between cycles, and centrifuged at 12,000 g at 4°C for 20 min to remove insoluble material. Solubilized proteins were affinity purified using glutathione Sepharose 4B beads (GE Healthcare). Proteins were eluted, dialyzed overnight against PBS, and stored at −80°C.

Cell culture, transfection, and lysis
Cos7, HeLa, and HeLa-C1 cells expressing Centrin 1-GFP (Piel et al., 2000) were grown at 37°C in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 5% CO2. HT115, HT29p, LS174T, and DLD1 cell lines were maintained as specified by American Type Culture Collection. THP1 human monocytic cells were obtained from C.K. Glass (University of California, San Diego, La Jolla, CA) and cultured in RPMI-1640 supplemented with L-glutamine and 10% heat-inactivated FBS (Collins, 1987). For all assays involving serum starvation, serum concentration was reduced to 0.2% for 6 h.

Plasmid DNA and siRNA transfection of HeLa, DLD1, or THP-1 (Kiprather et al., 2006) cells were performed using FUGENE 6 (Roche) or Oligofectamine (Fisher Scientific) and confirmed by sequencing. Primer sequences are available upon request. Reagents and antibodies
Materials and methods

In vitro binding
15–20 μg of purified GST fusion proteins or 30 μg GST alone were immobilized on glutathione Sepharose beads and resuspended in binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% [vol/vol] NP-40, 10 mM MgCl2, 5 mM EDTA, 2 mM DTT, and protease inhibitor cocktail) containing ∼250 μg cell lysate. In some experiments, 5 μCi [35S]met (GE Healthcare)-labeled GST was prepared using the TnT Quick Coupled Transcription/Translation System (Promega). Binding was performed overnight at 4°C with constant tumbling, and the beads were washed (4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% [vol/vol] Tween 20, 10 mM MgCl2, 5 mM EDTA, and 2 mM DTT) and boiled in sample buffer for SDS-PAGE. In the experiments using nucleotide loading, GIV fusion proteins were preincubated with 30 μM GDP or 30 μM GTP, 30 mM MgCl2, and 10 mM NaF in binding buffer for 90 min at room temperature before binding, and the washing buffer was supplemented with GDP or GDP, MgCl2, and NaF as during binding. Bound proteins were either immunoblotted or exposed for autoradiography.

Cell migration assays
Monolayer cell cultures (∼100% confluent) were wounded with a 1-ml 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 12–16 h. For rapid chemotaxis assays, THP1 cells were differentiated using 5 mM TPA. Upon reaching confluency, half of the plate was scraped, and cells were monitored by phase-contrast microscopy during 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.

Live-cell imaging
HeLa cells grown in chambered coverglass (Thermo Fisher Scientific) were scratched wound to induce migration. Media was changed to Liebestiz L-15 supplemented with 2.5 mM Hepes, 4.5 g/liter glucose, and 100 mM sodium 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 40× 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 BFT; Princeton Instruments) with Slidebook 4.1 software (Intelligent Imaging Innovations, Inc.). Image processing was done using ImageJ software (National Institutes of Health).

Immunoprecipitation
Cell lysates (∼1–2 μg 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 either resuspended 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 recombinant activated Ak1 (EMD) and terminated by boiling in SDS sample buffer. Trypsin 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
30 min, permeabilized with 0.2% Triton X-100 for 45 min, and incubated for 1 h with primary and secondary antibodies. Antibody dilutions were as follows: affinity purified anti-GF, 1:10; anti-Gu3 (EMD), 1:30; anti-V5 (Invitrogen), 1:200; anti-ManII, 1:500; anti-pericentrin, 1:1,000; anti-α-tubulin (Sigma-Aldrich), 1:500; anti-βGAILT, 1:20; Phalloidin-Texas Red, 1:1,000; secondary Alexa-conjugated antibodies, 1:500, 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 100x apertur (Plan Neofluar; 1.3 NA) camera (Orca ER; Hamamatsu Photonics), and Openlab software (Improvement). For the centrosome repositioning assay, HelA-C1 monolayers were scratch wounded, and 8–10 h afterward the wounded region of cells with centrosomal [GFP-centrin] and Golgi [ManII] positioning within the 120° sector facing the wound were counted (Fig. 2; Kupfer et al., 1982; Etienne-Manneville and Hall, 2001; Grande-Garcia et al., 2007).

For centrosomal localization studies, wide-field microscopy was performed using the 100x 1.3 N.A 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 (FC52; Biotech), 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). When anti-phosphoprotein antibodies [anti-pSer1/Thr or anti-pAkt] were used, nonfat milk was replaced by BSA throughout the process. The dilution of the primary antibodies was as follows: anti-GIV, serum, 1:500; anti-Gu3, 1:200; anti-Gu3, 1:250; anti-actin, 1:2,000; anti-Akt total, 1:250; anti-pAkt, 1:250; and anti-βSer, 1:200; anti-Ga, 1:200.

Statistical analysis

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

Online supplemental material

Fig. S1 shows validation that Gu3 is preferentially localized within pseudopods at the leading edge during cell migration using DIB as a PM marker. Fig. S2 shows that depletion of Gu3 impairs EGF-stimulated Akt activation. Fig. S3 shows that insulin-stimulated actin remodeling is inhibited in Gu3-depleted cells. Fig. S4 shows that GIV is occasionally distributed along actin stress fibers in epithelial cells. Fig. S5 shows that activation status of the G protein, and not phosphorylation of Giv at S4116, regulates the Gu3-GIV interaction. Video 1 shows that Hela cells expressing Giv-YFP demonstrate directional migration toward the wound where Gu3-depleted cells show random motility. Video 2 shows that Gu3-YFP preferentially localizes within pseudopods at the leading edge of a migrating Hela cell. Video 3 shows that my-YFP is distributed along the PM during polarized cell migration, with no preference for the leading edge. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200712066/D1.

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