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GIV is a nonreceptor GEF for Gαi with a unique motif that regulates Akt signaling

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Heterotrimeric G proteins are molecular switches that control signal transduction. Ligand-occupied, G protein-coupled receptors serve as the canonical guanine nucleotide exchange factors (GEFs) that activate heterotrimeric G proteins. A few unrelated nonreceptor GEFs have also been described, but little or nothing is known about their structure, mechanism of action, or cellular functions in mammals. We have discovered that GIV/Girdin serves as a nonreceptor GEF for Gαi through an evolutionarily conserved motif that shares sequence homology with the synthetic GEF peptide KB-752. Using the available structure of the KB-752·Gαi1 complex as a template, we modeled the Gαi-GIV interface and identified the key residues that are required to form it. Mutation of these key residues disrupts the interaction and impairs Akt enhancement, actin remodeling, and cell migration in cancer cells. Mechanistically, we demonstrate that the GEF motif is capable of activating as well as sequestering the Gα-subunit, thereby enhancing Akt signaling via the Gβγ-PI3K pathway. Recently, GIV has been implicated in cancer metastasis by virtue of its ability to enhance Akt activity and remodel the actin cytoskeleton during cancer invasion. Thus, the novel regulatory motif described here provides the structural and biochemical basis for the prometastatic features of GIV, making the functional disruption of this unique Gαi-GIV interface a promising target for therapy against cancer metastasis.

cell migration | Heterotrimeric G protein | metastasis | PI3K-Akt | RTK

The duration and extent of heterotrimeric G protein-mediated signaling is determined by the lifetime of the Gα subunit in the GTP-bound state. Guanine nucleotide Exchange Factors (GEFs) trigger G protein activation and signaling by binding to inactive G proteins and accelerating the rate of exchange of GDP for GTP (1, 2). In the standard model of G protein signaling, ligand occupied receptors serve as GEFs and activate heterotrimeric G proteins releasing free Gα-GTP and Gβγ, which in turn transduce signals via their respective effectors (3). More recently, a few nonreceptor GEFs have also been described (1, 4–7). In contrast to G protein-coupled receptors (GPCRs), these nonreceptor GEFs are unrelated to one another, no specific motif or domain responsible for the GEF activity has been identified, and their possible physiological functions in mammalian cells are poorly understood (8, 9), which has limited the exploitation of nonreceptor GEFs as pharmacological targets. We discovered GIV as a Gα-interacting protein (10), and others found that GIV (also called Girdin 12) can enhance Akt activation (11), remodel the actin cytoskeleton, and regulate cell migration (12, 13). More recently we have shown that activation of Gαi3 triggers cell migration via regulation of these cellular functions controlled by GIV (14). Here we describe a novel G protein regulatory motif within GIV that links Gαi activation to enhancement of Akt signaling and migration of tumor cells.

Results

Identification of a Novel Domain in GIV Which Binds GDP·Gαi3.
We recently observed (14) that full-length GIV interacts strongly and preferentially with inactive, GDP-bound Gαi3 (Fig. 1A, Top). We had previously identified a Gα-binding domain in GIV, (GBD, aa1343–1424) (Fig. 1B), that interacts with Gα subunits (10). However, this GBD bound similarly to both GDP (inactive) and GDP·AlF$_4^-$ (active)-loaded (15) Gαi3 (Fig. 1A). To identify the sequence responsible for the state-dependent interaction, we performed domain mapping of GIV using GDP or GDP·AlF$_4^-$-loaded Gαi3. We found that the C-terminal domain (GIV–CT, aa1623–1870) was sufficient to mediate strong and preferential interaction with Gαi3·GDP (Fig. 1A). Thus, GIV interacts with Gα subunits through 2 independent domains and GIV–CT is responsible for the state-dependent interaction.

GIV–CT Contains an Evolutionarily Conserved Motif with Similarity to the GEF Peptide KB-752.
Phylogenetic analysis revealed that GIV–CT is conserved in vertebrates from fish (*Danio rerio*) to mammals (*Homo sapiens*), but no homologues are found in *Drosophila melanogaster*, *Caenorhabditis Elegans*, or *Saccharomyces cerevisiae* (Fig. S1). Since some proteins that modulate G protein activity [GEFs and Guanine nucleotide Dissociation Inhibitors (GDIs)] preferentially interact with the inactive Gα subunit, we looked for sequence similarity between the C-terminal domain of GIV and known GEFs and GDIs by BLAST search or sequence alignment, but no significant homology was found. However, we noted that a conserved stretch of 20 residues (aa1674–1694) in human GIV (Fig. S1) was very similar (37.5% identity, 62.5% similarity) to a 16mer synthetic peptide, KB-752 (Fig. 1B), identified in a random sequence peptide screen based on its ability to interact in a state-dependent manner with Gα-subunits (16). This peptide was found to interact specifically with GDP-bound Gαi subunits and to have GEF activity (16). The KB-752 peptide was crystallized with Gαi1, providing the first structural insight into the mechanism for nucleotide exchange exerted by a GEF, but no similarity to GPCRs or any other known Gα interacting protein was identified (16). Using a degenerate sequence based on the KB-752 peptide and GIV 1674–1694 in a MOTIF search (see *SI Methods*), we were unable to identify any other Gα-interacting protein with a related motif in the Swiss-Prot database. We reasoned that the sequence of GIV homologous to KB-752 might represent a unique GEF motif. The hydrogen bond between T4 (donor) and D7 (acceptor) is required for KB-752 to adopt its helical confirmation (16). The fact that the corresponding residues in GIV (T1681 and Q1684) are capable of forming a hydrogen bond suggests that GIV's GEF motif and the KB-752 peptide might fold similarly. We used several secondary structure prediction programs (see *SI Methods*) to evaluate if the putative GEF motif could fold in a similar way to the KB-752 peptide, and the consensus result indicated that GIV's GEF motif could adopt a partially helical conformation analogous to that observed for the KB-752 in the
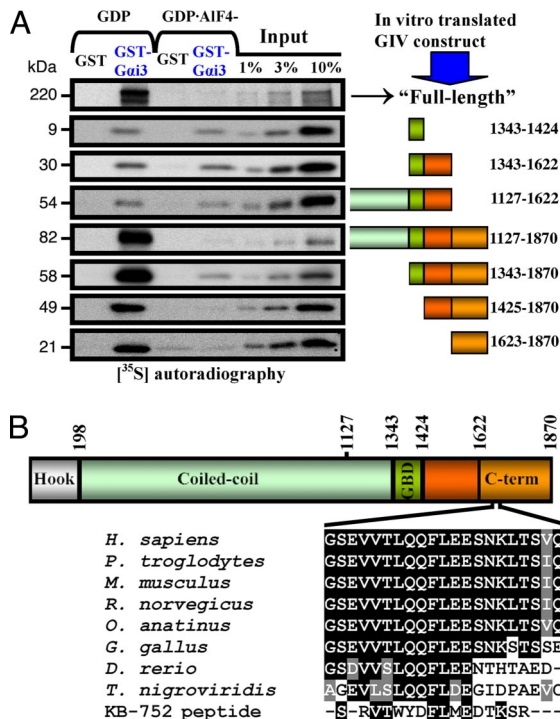
Fig. 1. Identification of a putative novel motif in GIV (amino acid 1623–1870) responsible for the preferential binding to GDP·Gαi3. (*A*) The indicated in vitro translated ^{35}S-Met labeled GIV constructs were incubated with approximately 15 μg GST–Gαi3 or GST preloaded with GDP or GDP·AlF$_4^-$ immobilized on glutathione beads. Bound proteins were analyzed by autoradiography, and equal loading of GST proteins was confirmed by protein staining (data not shown). (*B*) A phylogenetically conserved sequence in the C terminus of GIV (amino acid 1678–1694, ''GEF motif'') shows similarity to the synthetic peptide KB-752. Sequences obtained from the accession numbers (Fig. S1) were aligned using Clustal W. Conserved residues are shaded in black, similar residues in gray.

crystallized complex with Gαi1 (Fig. S2). This result suggests that GIV's GEF motif and the KB-752 peptide might interact with the G protein in a similar manner. To test this, we performed competition assays in which immobilized Gαi3 was incubated with a constant amount of purified GIV–CT, which contains the putative GEF motif but lacks the GBD, and increasing amounts of the KB-752 peptide. The KB-752 peptide reduced the binding of GIV–CT to Gαi3 with an IC$_{50}$ of approximately 2 μM (Fig. S3), a value consistent with the reported K$_D$ for the KB-752/Gαi interaction (approximately 4 μM) (16), indicating that they probably dock onto the Gα-subunit at the same or overlapping binding sites. Taken together, these results indicate that GIV–CT features an evolutionarily conserved motif that shares sequence homology and a Gαi binding site with the KB-752 peptide.

Identification of the Critical Residues Implicated in Forming the Interface Between Gαi3 and the Novel Interacting Motif in GIV. Based on the available structural information on the Gαi1·KB-752 complex (16), we used the Swiss-Model server to model the interface between Gαi3·GDP and the putative GEF motif found in GIV (Figs. 2*A* and S2). The Gαi3·GIV model was found to be reliable based on evaluations by the Verify3D and WHATCHECK programs (Fig. S2), supporting that this interface is analogous to that formed between Gαi1 and the KB-752 peptide. This model predicts that Phe[1685] of GIV would form a hydrophobic interaction with Trp[211] and Phe[215] of Gαi3 (within the "switch II" region), and that Glu[1688] would form an electrostatic contact with Arg[208] of Gαi3
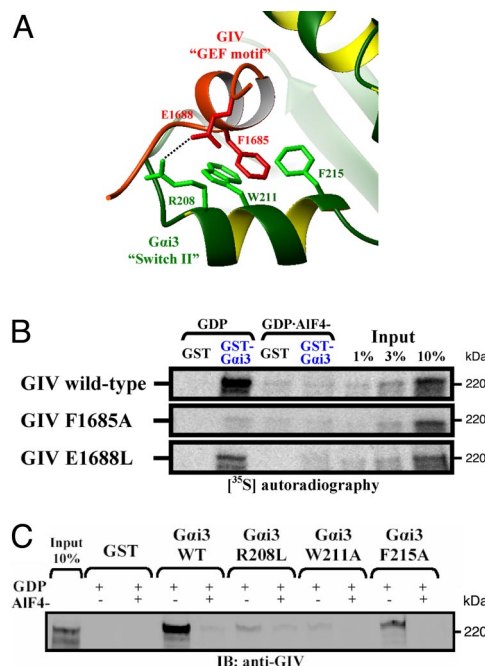

Fig. 2. Identification of the critical residues implicated in forming the interface between Gαi3 and the novel interacting motif in GIV. (*A*) Homology model showing the critical residues forming the predicted interface between Gαi3 and the conserved motif found in GIV. Model was generated by the Swiss-Model server using the KB-752·Gαi1 structure [PDB: 1Y3A] as template. (*B*) F1685A and E1688L mutations in GIV abolish its binding to Gαi3. In vitro translated ^{35}S-Met labeled GIV or the indicated GIV mutants were incubated with approximately 15 μg GST-Gαi3 or GST preloaded with GDP or GDP·AlF$_4^-$ immobilized on glutathione beads. Bound proteins were analyzed by autoradiography. (*C*) R208L, W211A, and F215A mutations in Gαi3's switch II region impair binding to GIV. COS-7 cell lysates were incubated with GST–Gαi3 or the indicated mutants as in (*B*). Bound proteins were analyzed by immunoblotting for GIV. Equal loading of GST proteins in (*B*) and (*C*) was confirmed by protein staining (data not shown).

(also within the "switch II" region). When we mutated either Phe[1685] to Ala (F1685A) or Glu[1688] to Leu (E1688L) in GIV, binding to Gαi3 was virtually abolished (Fig. 2*B*). Similarly, mutation of either of the corresponding residues of Gαi3 that participate in formation of the putative binding interface (Arg[208], Trp[211], Phe[215]) dramatically reduced its binding to GIV (Fig. 2*C*). Taken together these results validate that the predicted model of the GIV·Gαi3 interaction interface based on the KB-752·Gαi1 crystal structure is accurate and identify the critical residues that form the GIV·Gαi3 interface.

GIV Is a Guanine Nucleotide Exchange Factor Specific for Gαi Subunits. We next tested if the newly identified motif in GIV behaves as a GEF by measuring the steady-state GTPase activity of Gαi3 [which directly depends on the rate of nucleotide exchange (1, 6, 7)] in the presence or absence of purified GIV–CT, which contains the putative GEF motif but lacks the GBD. GIV–CT resembled full-length GIV in its binding properties in that it bound strongly and specifically to GDP-loaded Gαi1, Gαi2, and Gαi3 subunits (Fig. S4). The rate of GTP hydrolysis by Gαi3 was increased approximately 2.5-fold over the basal rate (from 0.012 to 0.029 mol GTP·mol^{-1} Gαi3·min^{-1}) in the presence of 1 μM GIV–CT but was not significantly increased by the Gαi3 binding-deficient GIV–CT F1685A (FA) mutant (Fig. 3*A*). We also measured the rate of GTP hydrolysis by Gαi3 in the presence of increasing amounts of GIV–CT. The maximal effect was an approximately 3-fold increase over the basal rate (Fig. 3*B*). This relative increase in the rate of GTP hydrolysis elicited by
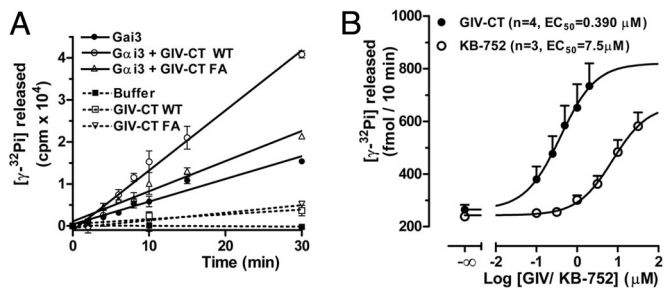
Fig. 3. GIV is a guanine nucleotide exchange factor for Gαi subunits; (*A*) His–GIV–CT (1 μM, open circles) but not His–GIV–CT F1685A (1 μM, open triangles) increases the steady-state GTPase activity of His–Gαi3 over His–Gαi3 alone (50 nM, closed circles). Parallel experiments were run in the absence of His–Gαi3 (dotted lines). (*B*) His–GIV–CT and the KB-752 peptide increase the steady-state GTPase activity of His–Gαi3 in a dose-dependent manner. The amount of GTP hydrolyzed in 10 min by His–Gαi3 was determined in the presence of the indicated amounts of His–GIV–CT or KB-752 peptide and the results were fitted to a sigmoidal curve.

GIV–CT is within the lower range of that observed for other nonreceptor GEFs [which range from approximately 2- to 10-fold, (1, 5–7)] or Gi-coupled GPCRs reconstituted in membranes [which range from approximately 1.5- to 8-fold (17–19)]. We also determined that the EC$_{50}$ was approximately 400 nM, a potency consistent with that described for other nonreceptor GEFs [EC$_{50}$ = 100–500 nM (1, 5–7)]. The KB-752 peptide was used as a positive control (EC$_{50}$ approximately 2 orders of magnitude smaller), and the results obtained were fully consistent with those previously reported (16). These results demonstrate that GIV–CT is a more potent and slightly stronger GEF for Gαi3 than the synthetic peptide. Our previous data indicate that in addition to Gα-subunits of the Gi family, GIV can interact with Gαs (10), although the binding is many fold less than to Gαi-subunits (14). We measured steady-state GTPase activity of Gαs in the presence or absence of GIV–CT and no significant difference was observed, suggesting that GIV is not

a GEF for Gαs (M.G.-M., P.G., and M.G.F., unpublished data). Taken together, these results demonstrate that GIV is a Gαi-specific GEF that functions via a defined motif in vitro.

GIV Enhances Akt Signaling via Gβγ-dependent Activation of PI3K. When we transiently overexpressed GIV in COS-7 cells, Akt phosphorylation was enhanced at steady-state in the presence of 10% serum (Fig. 4*A*), whereas transfection of the GEF-defective GIV F1685A mutant had no significant effect (Fig. 4*A*). Similar results were obtained in HeLa cells under the same conditions (M.G.-M., P.G., and M.G.F., unpublished data). Thus, a functional GEF motif is required for GIV to enhance Akt signaling. We also confirmed (11) that enhancement of Akt phosphorylation upon GIV overexpression is abolished by inhibition of PI3K with LY294002 (Fig. 4*A*). The best characterized link between G protein activation and PI3K-Akt signaling is through release of free Gβγ subunits, which in turn can directly activate PI3K to generate the PIP$_3$ levels required to promote Akt phosphorylation (20, 21). To investigate whether Gβγ release is required for GIV to activate PI3K-Akt, we used gallein, a small molecular inhibitor that selectively targets the "hotspot" in Gβγ responsible for activation of PI3K (22, 23). Treatment of cells with gallein abolished GIV-induced Akt phosphorylation, whereas treatment with the corresponding inactive analogue, fluorescein, had no effect (Fig. 4*B*). Gallein inhibited GIV-dependent activation of Akt with an IC$_{50}$ in the low μM range (Fig. S5), which is consistent with its binding affinity to Gβγ in vitro and with its potency in in vivo experiments (22, 23). In addition, we noted that several residues in the Gα-subunit required for contact with the GEF motif of GIV (Fig. 2 *A* and *C*) are identical to those involved in interacting with Gβγ subunits to form the heterotrimer (24) and that the predicted conformation of the "switch II" in the GIV-bound Gαi-subunit would be incompatible with Gβγ binding (Fig. 4*C*), suggesting that binding of GIV and Gβγ to Gαi3 might be mutually exclusive. Displacement assays confirmed that this is indeed the case, as increasing amounts of purified GIV–CT effectively decreased the amount of Gβ subunits bound to GST–Gαi3·GDP (Fig.
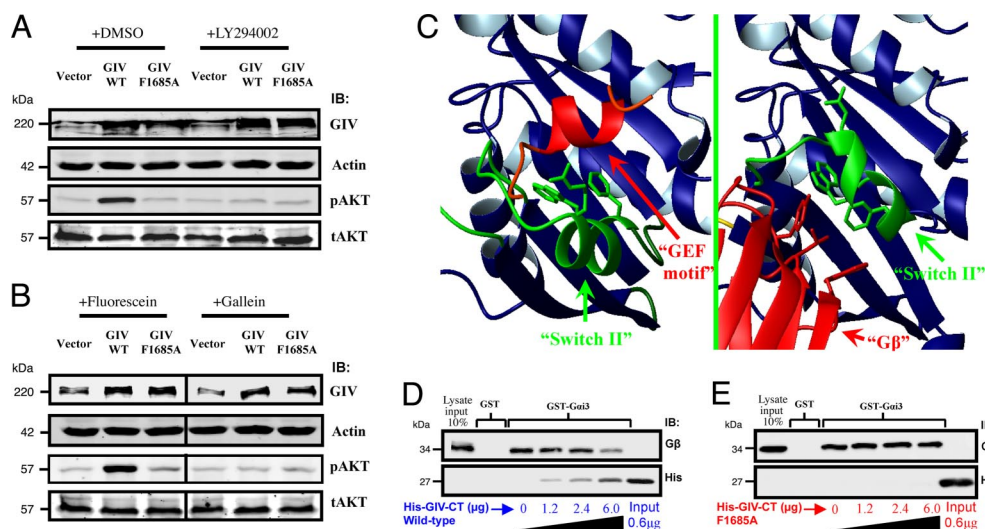

Fig. 4. GIV enhances Akt signaling via Gβγ-dependent activation of PI3K. (*A* and *B*) Inhibition of PI3K and Gβγ signaling blocks GIV-induced Akt phosphorylation. COS-7 cells were transfected with the indicated plasmid constructs and 48 h later incubated with DMSO or 10 μM LY294002 (PI3K inhibitor) for 2 h (*A*), 10 μM gallein (Gβγ "hotspot" inhibitor), or 10 μM fluorescein (negative control for gallein) for 30 min (*B*). Cells were lysed and analyzed by immunoblotting (IB). These experiments were performed in the presence of 10% FBS. (*C*) Structural model of (*Left*) the GEF motif of GIV bound to Gαi3 described in Figs. 2*A* and S2 compared to (*Right*) Gβ-subunit bound to Gαi1 (24). Blue: Gα subunit, Green: Switch II region, Red: "GEF motif" of GIV or Gβ. His–GIV–CT (*D*) but not His–GIV–CT F1685A (*E*) displaces Gβγ from GST–Gαi3 (approximately 7 μg). Equal amounts of preformed GST–Gαi3·Gβγ complexes immobilized on glutathione beads were incubated with increasing amounts of His–GIV–CT, and bound proteins were analyzed by immunoblotting (IB) with the indicated antibodies. Equal loading of GST proteins was confirmed by protein staining (data not shown).
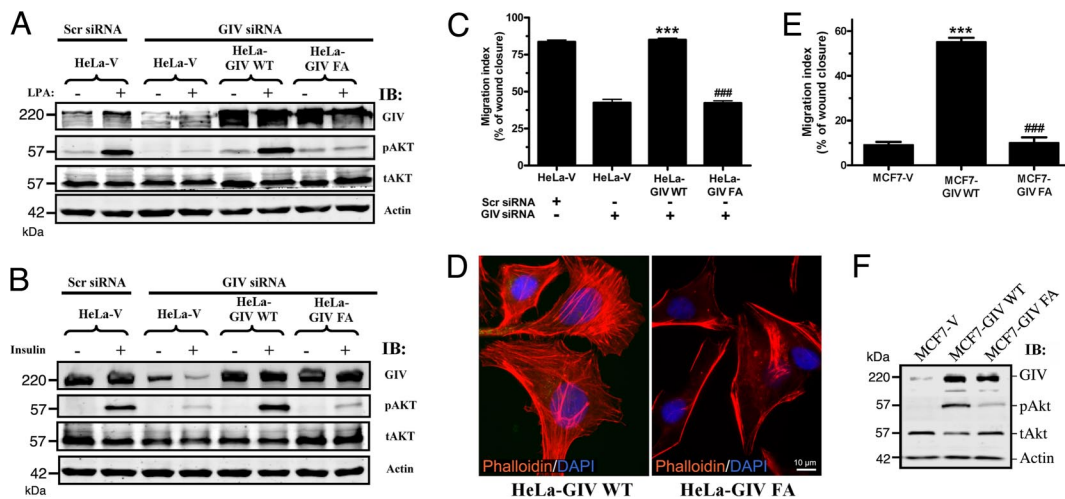
Fig. 5. Role of GIV's GEF motif on Akt activation and tumor cell migration. Depletion of endogenous GIV by siRNA impairs Akt activation (pAkt) upon LPA (*A*) or insulin (*B*) stimulation in HeLa cells stably expressing siRNA-resistant GIV F1685A (HeLa–GIV FA) but not in HeLa cells expressing siRNA resistant wt GIV (HeLa–GIV WT). The different HeLa cell lines were treated with GIV siRNA or control (Scr) siRNA oligos, serum starved for 6 h, and then stimulated with 10 μM LPA for 20 min (*A*) or 100 nM insulin for 5 min (*B*). Cell lysates were analyzed by immunoblotting (IB). (*C*) Only HeLa–GIV WT cells migrated efficiently in scratch-wound assays upon depletion of endogenous GIV. Cell migration was determined as described in *Materials and Methods* after treatment with GIV or control (Scr) siRNA oligos. Endogenous GIV expression was reduced approximately 75% upon siRNA treatment. (*D*) HeLa–GIV FA cells show absence of actin stress fibers compared to HeLa–GIV WT cells. HeLa–GIV WT and HeLa–GIV FA cells were co-stained with phalliodin-Texas red (F-actin, red) and DAPI (DNA, blue) and visualized by fluorescence. (*E* and *F*) MCF7 cells stably expressing GIV (MCF7-GIV WT) but not those expressing F1685A (MCF7-GIV FA) showed enhanced migration in scratch-wound assays (*E*) and enhanced Akt phosphorylation (*F*) compared to MCF7 cells expressing the vector control (MCF7-V). *** = $P < 0.001$ compared to vector control; ### = $P < 0.001$ compared to cells transfected with GIV WT.

4*D*) whereas the same assay performed with GIV–CT F1685A, which cannot bind to Gαi3 efficiently (Fig. 2*B*), did not interfere with Gβ binding (Fig. 4*E*). Taken together, these results delineate a novel signaling pathway in which GIV triggers the release of G$\beta\gamma$-subunits which in turn enhance activation of PI3K-Akt.

Role of GIV's GEF Motif in Akt Activation, Cell Migration, and Organization of the Actin Cytoskeleton after Receptor Stimulation. We have previously shown that GIV and activation of Gαi3 are required for Akt enhancement upon stimulation of GPCRs as well as receptor tyrosine kinases (RTKs) (14). It has also been established that GIV coupling of Akt activation to remodeling of the actin cytoskeleton is essential for cell migration (12, 14). Based on the ability of GIV to activate Gαi in vitro, we hypothesized that the newly discovered GEF motif in GIV might be required for these functions. To test this hypothesis, we generated HeLa cell lines stably expressing the empty vector (HeLa-V) or siRNA-resistant versions of GIV (HeLa–GIV WT) or GIV F1685A (HeLa–GIV FA). Only HeLa–GIV WT cells migrated efficiently (Fig. 5*C*) and showed robust Akt activation in response to activation of either the lysophosphatidic acid (LPA) receptor, a GPCR (Fig. 5*A*), or the insulin receptor, an RTK, (Fig. 5*B*). In addition, the formation of actin stress fibers was impaired in HeLa–GIV FA cells but not in HeLa–GIV WT (Fig. 5*D*) or HeLa-V cells (data not shown). These results indicate that GIV's GEF motif is required for regulating Akt signaling and cell migration upon receptor activation.

The GEF Motif of GIV Endows Epithelial Tumor Cells with Prometastatic Features. Elevation of PIP$_3$ activates multiple pathways that promote enhanced proliferation, cytoskeletal rearrangements, and cell migration. Akt is a kinase target that is activated by PIP$_3$ and its phosphorylation reflects accumulation of PIP$_3$. It has been reported that coupling of Akt activity to cell migration is required for cancer cells to invade and metastasize and that sustained enhancement of Akt is a cardinal feature of metastatic progression of epithelial cancers (25–27). GIV has been reported to be required for cancer metastasis in murine models (13). We

have recently demonstrated that expression of full-length GIV (GIV-fl), an Akt signaling enhancer, is induced several fold in cancer cell lines that are highly metastatic (14) and tumors that carry poor prognosis (M.G.-M., P.G., and M.G.F., unpublished observations). In poorly metastatic cells, GIV-fl is downregulated (vs. normal), resulting in decreased Akt activity and cell migration. Since exogenous expression of GIV-fl in poorly metastatic cells restores the prometastatic properties of Akt enhancement and cell migration (M.G.-M., P.G., and M.G.F., unpublished observations), we asked if the GEF motif is responsible for this change in behavior. To test this, we used the poorly metastatic breast cancer cell line, MCF7, stably expressing GIV (MCF7-GIV WT), the GEF-deficient F1685A mutant of GIV (MCF7-GIV FA) or control vector (MCF7-V). As expected (14), MCF7-GIV WT displayed enhanced Akt signaling and cell migration in scratch wound assays; however, these phenotypes were not seen in MCF7-GIV FA cells (Fig. 5 *E* and *F*). Thus the GEF motif within the C-terminal domain of GIV is the critical determinant for triggering these prometastatic properties. These results provide the molecular mechanism by which GIV achieves sustained enhancement of Akt during tumor cell migration and promotes cancer invasion (13).

Discussion

The classical paradigm of GPCR-mediated activation of heterotrimeric G-proteins (2, 3) has been modified by the discovery of nonreceptor activators of G proteins that are believed to operate in lieu of or in conjunction with GPCRs to enhance signaling (1, 4, 7). Here, we have demonstrated that GIV controls Gi-coupled signaling in vivo through a unique motif in its C terminus. We identified the GEF motif in GIV by virtue of its similarity to the KB-752 synthetic peptide. Most of the critical residues responsible for the GEF·Gα interaction are conserved (GIV's F1685 and E1688 correspond to F8 and E11 in KB-752). In addition, GIV's residues T1681 and Q1684 are capable of forming a hydrogen bond, and it has been shown that the hydrogen bond between the corresponding residues in KB-752 (T4 and D7) is required for the peptide to adopt its helical conformation (16).

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Taken together, our data indicate that the molecular interface formed by GIV's GEF motif and Gαi is structurally and functionally homologous to that formed between the KB-752 peptide and Gαi1 (16).

Our results demonstrate that GIV's GEF motif is required to activate the Gβγ-PI3K pathway, which in turn enhances Akt activity and cell migration. The molecular mechanism by which GIV increases PI3K activity is unknown because binding of GIV's GEF motif to Gαi3 can not only enhance GIV–CT's GEF activity in vitro, but also can displace Gβγ from its association with Gαi3. Either mechanism might contribute to increasing the concentration of free Gβγ, which apparently stimulates PI3K activity. The GEF activity of both GIV and the KB-752 peptide in vitro is relatively low compared to GPCRs (17–19) and therefore might not be sufficient to significantly affect G protein activity in vivo. However, we have shown (Fig. 5A) that an intact GEF motif is required to enhance Gi-coupled activation of Akt in response to LPA in vivo, suggesting that GIV could act as a stronger GEF in the cell. Interestingly, it has been shown that the GIV surrogate peptide KB-752 can synergistically enhance Gαi1 activation mediated by GPCRs (28). Since our data indicate that the KB-752 peptide and GIV bind and activate Gαi-subunits similarly, it seems likely that this mechanism of synergistic activation with GPCRs also applies to GIV and could facilitate GIV's GEF function in vivo. Regardless of whether there is in vivo synergy with other binding partners, it is apparent that GIV's relatively "weak" GEF activity in vitro cannot entirely account for the strong Akt signal enhancement observed in vivo which might depend on GIV-mediated Gα sequestration. Sequestration of Gα would also be expected to result in sustained high levels of free Gβγ that activate the PI3K/Akt pathway. However, since GIV's functions of enhancing Akt phosphorylation and cell migration are also controlled by activation of Gαi3 (14), the GEF function may serve to establish a positive feedback loop by enhancing Gαi3 activation. Further studies are needed to understand the relative contributions of GIV-dependent Gα activation and/or Gα sequestration during Akt signal enhancement.

We have shown that the GEF motif of GIV is required to enhance Akt phosphorylation not only in response to LPA but also in response to insulin (Fig. 5 A and B). Since LPA receptors increase Akt signaling by activating Gi proteins (29), the effect of GIV is likely a consequence of its ability to potentiate activation of Gαi-subunits. The mechanism by which insulin utilizes GIV's GEF motif to enhance Akt signaling is not clear because it does not activate GPCRs directly; however, we have previously found that active Gαi3 is required to achieve full activation of Akt upon stimulation of HeLa cells with insulin (14). Therefore, GIV may enhance Akt activation upon insulin stimulation via its ability to regulate Gi signaling, regardless of the precise biochemical steps involved in the process—i.e., by a GPCR-independent mechanism or by GPCRs via transactivation (30).

The major finding of this work is our discovery of a unique interaction between Gαi3 and GIV and its associated physiological consequences linked to PI3K-Akt activation and cell migration. Here we have shown that targeted disruption of the interface between the GEF motif of GIV and Gαi in vivo abolishes the prometastatic phenotypes that are associated with expression of GIV in tumor cells, i.e., enhanced Akt activation and cell migration (Fig. 5 E and F). Because GIV is a metastasis-related gene (13) (M.G.-M., P.G., and M.G.F., unpublished observations), and we have structurally characterized the GIV·Gαi interface, our results serve as a conceptual basis for identifying small molecular inhibitors of this interface for use as antimetastatic agents. However, greater insight into the molecular mechanisms is needed and will dictate the precise strategy regarding whether targeting Gα or GIV is most appropriate. Inhibitors that will selectively disrupt the GIV·Gαi interface can be further developed for targeted molecular therapy to be used in our armamentarium against cancer metastasis.

Materials and Methods

Reagents and Antibodies. The KB-752 peptide (SRVTWYDFLMEDTKRS, >90% purity) was custom made by GenWay Biotech; gallein was from Fisher Scientific. The sources of the remainder of the reagents and antibodies used were described previously (14).

Plasmids Constructs, Mutagenesis, and Protein Expression. Cloning of Gαi3 and Gαs (short) into pGEX-4T-1 or pET28b were described previously (14). The truncation constructs used for domain mapping and for purification of His–GIV–CT (1623–1870) were cloned from pcDNA 3.1-GIV (12, 14) and inserted between the NdeI and EcoRI restriction sites of the pET28b vector. GIV and Gαi3 mutants were generated using specific primers (sequences available upon request) following the manufacturer's instructions (QickChange II, Stratagene). RNAi-resistant GIV was generated by the silent mutations as described (12). All constructs were checked by DNA sequencing.

Plasmids encoding GST–Gαi3, GST–Gαs, His–Gαi3 or His–GIV–CT fusion constructs were used to express these proteins in *Escherichia coli* as described (14). For the His-tagged Gαi3 or GIV–CT, a similar procedure was followed using His-lysis buffer [50 mM NaH₂PO₄ pH 7.4, 300 mM NaCl, 10 mM imidazole, 1% (v:v) Triton X-100, 2X protease inhibitor mixture (Complete EDTA-free, Roche Diagnostics)], and cobalt resin for purification (HisPur Cobalt Resin, Pierce). His–Gαi3 used for the GTPase assays were buffer exchanged into G protein storage buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM MgCl₂, 1 mM DTT, 10 μM GDP, 5% (v:v) glycerol) before storage at −80 °C. The purified protein (95%) was properly folded as assessed by a protease protection assay (31) (Fig. S6).

Cell Culture, Transfections, Lysates, and Stable Cell Line Preparation. COS-7, MCF7, and HeLa cells were cultured according to ATCC guidelines. Plasmid and siRNA transfections were carried out exactly as described (14). HeLa or MCF7 cell lines stably expressing vector control (HeLa-V, MCF7-V), GIV (HeLa–GIV WT, MCF7-GIV WT), or GIV F1685A mutant (HeLa–GIV FA, MCF7-GIV FA) were selected after transfection in the presence of G418 (500 μg/ml) for 6 weeks, followed by clonal selection. Clones were chosen for each construct that had low expression levels of GIV (2–3 times higher than the endogenous levels). Clones expressing equal amounts of each construct were used for the assays. For assays involving serum starvation, serum concentration was reduced to 0.2% for 6 h. Lysates used for in vitro protein binding assays were prepared exactly as described previously (14).

In Vitro Protein Binding Assays. These assays were performed exactly as described previously (14). For the experiments depicted in Fig. 4 D and E, GST or GST–Gαi3 proteins immobilized on glutathione beads were incubated overnight at 4 °C with rat brain lysate in binding buffer [50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% (v:v) Nonidet P-40, 10 mM MgCl₂, 5 mM EDTA, 2 mM DTT, 1% (w:v) BSA, protease inhibitor mixture supplemented with 30 μM GDP]. Unbound Gβγ-subunits were washed [4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v:v) Tween 20, 10 mM MgCl₂, 5 mM EDTA, 2 mM DTT, 30 μM GDP] and proteins bound to the glutathione beads were divided into equal aliquots containing approximately 7 μg GST-fusion proteins. Aliquots were incubated with increasing amounts of purified His–GIV–CT (1623–1870) in binding buffer supplemented with GDP (approximately 350 μl) for 5–6 h at 4 °C, after which beads were washed and boiled in sample buffer for SDS/PAGE.

Steady-State GTPase Assay. His–Gαi3 (100 nM) was preincubated with His–GIV–CT, His–GIV–CT F1685A, or KB-752 peptide for 15 min at 30 °C in assay buffer [20 mM Na-Hepes, pH 8, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.05% (w:v) C12E10]. GTPase reactions at 30 °C were initiated by addition of an equal volume of assay buffer containing 1 μM [γ-³²P]GTP (approximately 100 c.p.m/fmol). Duplicate aliquots (50 μl) were removed at 0, 2, 4, 6, 8, 10, 15, and 30 min and reactions stopped with 950 μl ice cold 5% (wt/vol) activated charcoal in 20 mM H₃PO₄, pH 3. Samples were then centrifuged for 10 min at 10,000 × g, and 500 μl of the resultant supernatant were scintillation counted to quantify released [³²P]Pᵢ. Parallel reactions were run in the absence of G proteins, in the presence or absence GIV proteins, and the background [³²P]Pᵢ detected at time 0 subtracted from each reaction to determine the specific Pi produced at different time points. For GST–Gαs the same procedure was followed, but the assay was performed at 22 °C.

Cell Migration Assays. Scratch wound assays were carried out as described previously (14). To quantify cell migration (expressed as percentage of wound

area covered), images were analyzed using Image J (National Institutes of Health) software to calculate the difference between the wound area at 0 min and at the end of the migration assay divided by the area at 0 min × 100.

Data Analysis. Each experiment presented in the figures is representative of at least 3 independent experiments. Data shown in Figs. 3*B*, 5*C*, and *E* are expressed as mean ± SEM. Statistical significance between various conditions was assessed with the Student's *t* test. Data shown in Fig. 3*A* is expressed as mean ± SD of one experiment performed in duplicate. Dose dependence curves and parameters were determined by nonlinear regression fitted to a sigmoidal curve using Prism software (GraphPad Software, Inc.).

Other Methods. Immunoblotting and fluorescence were carried out exactly as described previously (14). A detailed description of the bioinformatic techniques used is available in *SI Text*.

1. Tall GG, Krumins AM, Gilman AG (2003) Mammalian Ric-8A (synembryn) is a heterotrimeric Galpha protein guanine nucleotide exchange factor. *J Biol Chem* 278:8356–8362.
2. Oldham WM, Hamm HE (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 9:60–71.
3. Gilman AG (1987) G proteins: Transducers of receptor-generated signals. *Annu Rev Biochem* 56:615–649.
4. Cismowski MJ, et al. (1999) Genetic screens in yeast to identify mammalian nonreceptor modulators of G-protein signaling. *Nat Biotechnol* 17:878–883.
5. Afshar K, et al. (2004) RIC-8 is required for GPR-1/2-dependent Galpha function during asymmetric division of C. elegans embryos. *Cell* 119:219–230.
6. Natochin M, et al. (2005) Characterization of the G alpha(s) regulator cysteine string protein. *J Biol Chem* 280:30236–30241.
7. Lee MJ, Dohlman HG (2008) Coactivation of G protein signaling by cell-surface receptors and an intracellular exchange factor. *Curr Biol* 18:211–215.
8. Malik S, Ghosh M, Bonacci TM, Tall GG, Smrcka AV (2005) Ric-8 enhances G protein betagamma-dependent signaling in response to betagamma-binding peptides in intact cells. *Mol Pharmacol* 68:129–136.
9. Nishimura A et al. (2006) Ric-8A potentiates Gq-mediated signal transduction by acting downstream of G protein-coupled receptor in intact cells. *Genes Cells* 11:487–498.
10. Le-Niculescu H, Niesman I, Fischer T, DeVries L, Farquhar MG (2005) Identification and characterization of GIV, a novel Galpha i/s-interacting protein found on COPI, endoplasmic reticulum-Golgi transport vesicles. *J Biol Chem* 280:22012–22020.
11. Anai M, et al. (2005) A novel protein kinase B (PKB)/AKT-binding protein enhances PKB kinase activity and regulates DNA synthesis. *J Biol Chem* 280:18525–18535.
12. Enomoto A, et al. (2005) Akt/PKB regulates actin organization and cell motility via Girdin/APE. *Dev Cell* 9:389–402.
13. Jiang P, et al. (2008) An actin-binding protein Girdin regulates the motility of breast cancer cells. *Cancer Res* 68:1310–1318.
14. Ghosh P, Garcia-Marcos M, Bornheimer SJ, Farquhar MG (2008) Activation of Gαi3 triggers cell migration via regulation of GIV. *J Cell Biol* 182:381–393.
15. Coleman DE, et al. (1994) Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. *Science* 265:1405–1412.
16. Johnston CA, et al. (2005) Structure of Galpha(i1) bound to a GDP-selective peptide provides insight into guanine nucleotide exchange. *Structure (London)*13:1069–1080.
17. Cerione RA, et al. (1986) Functional reconstitution of the alpha 2-adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J Biol Chem* 261:3901–3909.
18. Cerione RA, et al. (1985) Specificity of the functional interactions of the beta-adrenergic receptor and rhodopsin with guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J Biol Chem* 260:1493–1500.
19. Kurose H, Regan JW, Caron MG, Lefkowitz RJ (1991) Functional interactions of recombinant alpha 2 adrenergic receptor subtypes and G proteins in reconstituted phospholipid vesicles. *Biochemistry* 30:3335–3341.
20. Stephens L, et al. (1994) A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. *Cell* 77:83–93.
21. Maier U, Babich A, Nurnberg B (1999) Roles of non-catalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma. *J Biol Chem* 274:29311–29317.
22. Bonacci TM, et al. (2006) Differential targeting of Gbetagamma-subunit signaling with small molecules. *Science* 312:443–446.
23. Lehmann DM, Seneviratne AM, Smrcka AV (2008) Small molecule disruption of G protein beta gamma subunit signaling inhibits neutrophil chemotaxis and inflammation. *Mol Pharmacol* 73:410–418.
24. Wall MA, et al. (1995) The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2. *Cell* 83:1047–1058.
25. Altomare DA, Testa JR (2005) Perturbations of the AKT signaling pathway in human cancer. *Oncogene* 24:7455–7464.
26. Qiao M, Iglehart JD, Pardee AB (2007) Metastatic potential of 21T human breast cancer cells depends on Akt/protein kinase B activation. *Cancer Res* 67:5293–5299.
27. Ju X, et al. (2007) Akt1 governs breast cancer progression in vivo. *Proc Natl Acad Sci USA* 104:7438–7443.
28. Johnston CA, Siderovski DP (2007) Structural basis for nucleotide exchange on G alpha i subunits and receptor coupling specificity. *Proc Natl Acad Sci USA* 104:2001–2006.
29. Moolenaar WH, van Meeteren LA, Giepmans BN (2004) The ins and outs of lysophosphatidic acid signaling. *BioEssays* 26:870–881.
30. Waters C, Pyne S, Pyne NJ (2004) The role of G-protein coupled receptors and associated proteins in receptor tyrosine kinase signal transduction. *Semin Cell Dev Biol* 15:309–323.
31. Kleuss C, Raw AS, Lee E, Sprang SR, Gilman AG (1994) Mechanism of GTP hydrolysis by G-protein alpha subunits. *Proc Natl Acad Sci USA* 91:9828–9831.

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