A Structural Determinant That Renders Ga4 Sensitive to Activation by GIV/Girdin Is Required to Promote Cell Migration*

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Although several non-receptor activators of heterotrimeric G proteins have been identified, the structural features of G proteins that determine their interaction with such activators and the subsequent biological effects are poorly understood. Here we investigated the structural determinants in Ga13 necessary for its regulation by GIV/girdin, a guanine-nucleotide exchange factor (GEF) that activates Ga subunits. Using G protein activity and in vitro pulldown assays we demonstrate that Ga13 is a better substrate for GIV than the highly homologous Ga4. We identified Trp-258 in the Ga13 subunit as a novel structural determinant for GIV binding by comparing GIV binding to Ga13/Ga subunit chimeras. Mutation of Trp-258 to the corresponding Phe in Ga4 decreased GIV binding in vitro and in cultured cells but did not perturb interaction with other Ga-binding partners, i.e. Gaβγ, AGS3 (a guanine nucleotide dissociation inhibitor), GAIP/RGS19 (a GTPase-activating protein), and LPAR1 (a G protein-coupled receptor). Activation of Ga13 by GIV was also dramatically reduced when Trp-258 was replaced with Tyr, Leu, Ser, His, Asp, or Ala, highlighting that Trp is required for maximal activation. Moreover, when mutant Ga13 W258F was expressed in HeLa cells they failed to undergo cell migration and to enhance Akt signaling after growth factor or G protein-coupled receptor stimulation. Thus activation of Ga13 by GIV is essential for biological functions associated with Ga13 activation. In conclusion, we have discovered a novel structural determinant on Ga4 that plays a key role in defining the selectivity and efficiency of the GEF activity of GIV on Ga4 and that represents an attractive target site for designing small molecules to disrupt the Ga4-GIV interface for therapeutic purposes.

Heterotrimeric G proteins are molecular switches that control signal transduction. G protein cycling between active and inactive states is controlled via interaction with regulatory proteins. Activation is triggered by guanine nucleotide exchange factors (GEFs), and deactivation is greatly enhanced by GTPase-activating proteins (GAPs) (1–3). Because the duration and extent of G protein-mediated signaling is determined by the lifetime of Ga in the GTP-bound state, it is crucial to define the molecular machinery that triggers G protein activation to understand how this signal transduction pathway functions. Ligand-occupied G protein-coupled receptors (GPCRs) are the canonical GEFs of which >800 genes have been identified in the human genome (4). They regulate a myriad of physiological functions and are the most common target for marketed drugs (~30%) (5). Recently, a few non-receptor GEFs have been described, i.e. AGS1 (6), Ric-8 (7, 8), CSPα (9), and Arr4 (10). In contrast to GPCRs, these non-receptor GEFs are structurally unrelated, and their physiological roles are just beginning to be elucidated (8, 11–13). The lack of information on non-receptor GEFs has limited their exploitation as pharmacological targets.

We recently demonstrated that GIV is a non-receptor GEF for Ga4 subunits (11). Originally GIV was identified by its ability to interact with Ga13 in a yeast two-hybrid screen (14). Work from other groups indicated that GIV (also known as girdin) activates Akt signaling (15) and plays a critical role in cell migration via its interaction with Akt and the actin cytoskeleton (16). GIV was shown to be required for cancer metastasis in murine models by virtue of its ability to control cell migration and actin remodeling (17). We subsequently found that active Ga13, like GIV, promotes Akt signaling, remodeling of the actin cytoskeleton, and tumor cell migration (18).

Moreover, we recently reported that GIV activates Ga13 subunits via an evolutionarily conserved GEF motif and that this novel regulatory motif provides the structural and biochemical basis for the pro-metastatic features of GIV (11). We identified the GEF motif of GIV based on its sequence homology with the synthetic GEF peptide KB-752 (19) and showed that mutational disruption of the ability of GIV to activate Ga subunits via this motif abolished the enhanced Akt activation (15), actin cytoskeleton remodeling (16, 17, 20), and cell migration (16, 17) seen in metastatic tumor cells (11).

GIV is the first non-receptor GEF whose function has been shown to be governed by a defined motif. Because the GEF

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The abbreviations used are: GEF, guanine nucleotide exchange factor; GIV, Ga-interacting, vesicle-associated protein; GPCR, G protein-coupled receptor; GAP, GTPase-activating protein; RGS, regulator of G protein signaling; GAIP/Gi-interacting protein; AGS3, activator of G protein signaling 3; GST, glutathione S-transferase; IP, immunoprecipitation; IB, immunoblot; siRNA, small interfering RNA; LPA, lysophosphatidic acid; LPA1, LPA receptor 1; DTT, dithiothreitol; wt, wild type; aa, amino acid(s); GTPγS, guanosine 5′-3-O-(thio)triphosphate; CMV, cytomegalovirus.

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function of GIV appears critical for cancer metastasis, disruption of the interface formed between the GEF motif of GIV and $G_{\alpha_i}$ is potentially of therapeutic significance, and defining the molecular basis and properties of this interface is crucial for the future development of pharmacological agents that target this interface. Here we investigated in depth the structural determinants in the $G_{\alpha_i}$ subunit required for it to interact with GIV and be activated. Using the $G_{\alpha}$ selectivity of GIV to identify such determinants, we found that residues outside of the previously described $G_{\alpha_i}$-GIV interface (11) define the selectivity and efficiency of the GEF activity of GIV on $G_{\alpha_i}$ in living cells and in vitro. These data provide valuable insights that can be used in the design of pharmacological agents that selectively disrupt the $G_{\alpha_i}$-GIV interface for therapeutic purposes.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Unless otherwise indicated all reagents were of analytical grade and obtained from Sigma-Aldrich. Cell culture media were purchased from Invitrogen. All restriction endonucleases and *Escherichia coli* strain DH5a were purchased from New England Biolabs (Cambridge, MA). *E. coli* strain BL21(DE3) was purchased from Invitrogen. Pfu ultra DNA polymerase was purchased from Stratagene (La Jolla, CA). [$\gamma^{32}P$]GTP and [$^{35}$S]GTPyS were from PerkinElmer Life Sciences. Rabbit antisera against AGS3 (21) and the coiled-coil region of GIV (14) were raised as described. Goat anti-rabbit and goat anti-mouse Alexa Fluor 680 or IRDye 800 F(ab′)₂ were from Li-Cor Biosciences (Lincoln, NE). Mouse monoclonal antibodies against alpha-haemastidine (His), FLAG (M2), and a-tubulin were obtained from Sigma-Aldrich. Rabbit anti-pan-β (M14) IgGs was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-Akt and phospho-Akt (S473) IgGs were from Cell Signaling (Beverly, MA).

**Plasmid Constructs and Mutagenesis**—Cloning of rat $G_{\alpha_i}$ into pGEX-4T-1 or pET28b and GIV-CT-(1623–1870) into pET28b was described previously (11, 18). Rat $G_{\alpha_i}$ (isoform 1, $G_{\alpha_{i3}}$, hereafter referred to as $G_{\alpha_i}$) was cloned from pGCT9-$G_{\alpha_i}$ (22) and inserted between the EcoRI and NotI restriction sites of the pGEX-4T-1 vector to generate GST-$G_{\alpha_i}$ or between the NdeI and EcoRI restriction sites of the pET28b vector to generate His-$G_{\alpha_i}$. GIV-CT-(1623–1870) was cloned from pcDNA 3.1-GIV (16) and inserted between the EcoRI and NotI restriction sites of the pGEX-4T-1 vector to generate GST-GIV, or between the NdeI and EcoRI restriction sites of the pET28b vector to generate His-GIV-C. GST-GIV-CT-(1623–1870) was a gift from Dr. Jerold Chun (Scripps Research Institute) and was described previously (23). $G_{\alpha_{i3}}$ and $G_{\alpha_o}$ mutants were generated using specific primers (sequences available upon request) following the manufacturer’s instructions (QuikChange II, Stratagene). All constructs were checked by DNA sequencing (University of California at San Diego Moores Cancer Center Sequencing Facility).

GST-$G_{\alpha_{i/o}}$ chimeras were generated by using an overlapping PCR strategy (24). pGEX-4T-1-$G_{\alpha_{i3}}$ and pGEX-4T-1-$G_{\alpha_o}$ were used as templates to amplify the following sequences in the first PCR: $G_{\alpha_{i3}}$-(1–59), $G_{\alpha_{i3}}$-(1–177), $G_{\alpha_{i3}}$-(61–270), $G_{\alpha_{i3}}$-(178–354), $G_{\alpha_{i3}}$-(271–354), $G_{\alpha_o}$-(60–178), $G_{\alpha_o}$-(60–178), $G_{\alpha_o}$-(179–354), $G_{\alpha_o}$-(179–271), and $G_{\alpha_o}$-(272–354). These cDNA fragments, which contained overlapping sequences in the internal boundaries of the chimeras, were used as templates in successive PCR reactions to generate the full-length chimeras. The full-length chimeras were digested and inserted between the EcoRI and NotI restriction sites of pGEX-4T-1.

**Protein Purification**—GST, GST-$G_{\alpha_{i3}}$, GST-$G_{\alpha_o}$, GST-$G_{\alpha_{i/o}}$ chimeras, His-$G_{\alpha_{i3}}$, His-$G_{\alpha_o}$, His-GIV-CT, or His-GIV-CTs fusion constructs were expressed in *E. coli* strain BL21(DE3) (Invitrogen) as described previously (11) and induced overnight at 25 °C with 1 mM 1-isopropyl-β-D-thiogalactopyranoside. Pelleted bacteria from 1 liter of culture were resuspended in 10 ml of GST-lysis buffer (25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 1% (v/v) Triton X-100, 2× protease inhibitor mixture (Complete EDTA-free, Roche Diagnostics)) or His-lysis buffer (50 mM NaH₂PO₄, pH 7.4, 300 mM NaCl, 10 mM imidazole, 1% (v/v) Triton X-100, 2× protease inhibitor mixture (Complete EDTA-free, Roche Diagnostics)) for GST- or His-fused proteins, respectively. After sonication (4 × 20 s, 1 min between cycles), lysates were centrifuged at 12,000 × g at 4 °C for 20 min. Solubilized proteins were affinity-purified on glutathione-Sepharose 4B beads (Amersham Biosciences) or HisPur Cobalt Resin (Pierce). Proteins were eluted, dialyzed overnight against phosphate-buffered saline, and stored at −80 °C. His-$G_{\alpha_{i3}}$ and His-$G_{\alpha_o}$ 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) prior to storage at −80 °C.

**Cell Culture, Transfection, and Lysis**—COS7 and HeLa cells were grown at 37 °C in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% l-glutamine, and 5% CO₂. siRNA transfection of HeLa cells was carried out using Oligofectamine (Invitrogen) following the manufacturer’s protocol. Oligonucleotides against human $G_{\alpha_{i3}}$ were from Santa Cruz Biotechnology. When reversal of phenotype was attempted, pcDNA3.1-$G_{\alpha_{i3}}$ (untagged) transfection was carried out 8–10 h post-siRNA transfection using GeneJuice (Novagen) following the manufacturer’s protocol, and cells were analyzed after 38–40 h. Transfection of COS7 cells with $G_{\alpha_{i3}}$-FLAG was also carried out using GeneJuice. Lysates used as a source for GIV for *in vitro* protein binding assays or for immunoprecipitation were prepared by resuspending the cells in lysis buffer (20 mM HEPES, pH 7.2, 5 mM Mg(CH₃COO)₂, 125 mM K(CH₃COO), 0.4% Triton X-100, 1 mM EDTA) supplemented with phosphatase (Sigma) and protease (Roche Applied Science) inhibitor mixtures, passed through
a 28-gauge needle at 4 °C, and cleared (10,000 × g for 10 min) before use in subsequent experiments.

In Vitro Protein Binding Assays—Purified GST fusion proteins or GST alone (5–10 μg) were immobilized on glutathione-Sepharose beads and incubated 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, protease inhibitor mixture) containing either 30 μM GDP or 30 μM GDP, 30 μM AlCl₃, 10 mM NaF for 90 min at room temperature. ~250 μg of COS7 cell lysate or 3 μg of purified His-GIV-CT or His-Gαᵢ₃ was added to each tube, and binding reactions were carried out overnight at 4 °C with constant tumbling. Beads were washed (×4) with 1 ml of wash buffer (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, supplemented with GDP or GDP, AlCl₃, and NaF as during binding) and boiled in sample buffer for SDS-PAGE.

Immunoprecipitation—COS7 cell lysates (~1–2 mg of protein) were incubated 4 h at 4 °C with 2 μg of anti-FLAG monoclonal antibody (Sigma) followed by incubation with protein G-agarose beads (Invitrogen) at 4 °C for an additional 60 min. Beads were washed (×4) with 1 ml of wash buffer (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), and the bound immune complexes were eluted by boiling in SDS sample buffer. For immunoprecipitation of FLAG-LPR1 the boiling step was omitted.

Steady-state GTPase Assay—This assay was performed as described previously (11). Briefly, His-Gαᵢ₃ or His-Gαₒ (100 nM) was preincubated with different concentrations of His-GIV-CTs-(1660–1870), for 15 min at 30 °C in assay buffer (20 mM sodium HEPES, pH 8, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.05% (v/v) C₁₂E₁₀). Reactions were initiated at 30 °C by adding an equal volume of assay buffer containing 1 μM [³⁵S]GTPγS (~50 cpm/fmol). Duplicate aliquots (50 μl) were removed at 15 min, and binding of radioactive nucleotide was stopped by addition of 3 ml of ice-cold wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM MgCl₂). The quenched reactions were rapidly passed through BA-85 nitrocellulose filters (Amersham Biosciences) and washed with 4 ml wash buffer. Filters were dried and subjected to liquid scintillation counting. To determine the specific nucleotide binding, the background [³⁵S]GTPγS detected in the absence of G protein was subtracted from each reaction.

Trypsinization of Ga subunits—His-Gαᵢ₃, His-Gαₒ, or the indicated His-Gαᵢ₃ mutants (0.5 mg/ml) were incubated for 120 min at 30 °C in the presence of GDP (30 μM) or GDP-AlF₃ (30 μM GDP, 30 μM AlCl₃, 10 mM NaF). After incubation, trypsin was added to the tubes (final concentration, 12.5 μg/ml), and samples were incubated for an additional 10 min at 30 °C. Reactions were stopped by adding SDS-PAGE sample buffer and boiling. Proteins were resolved by SDS-PAGE and stained with Coomassie Blue.

Immunoblotting—Proteins samples were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with phosphate-buffered saline supplemented with 5% nonfat milk (or 5% bovine serum albumin when probing for Akt) before sequential incubation with primary and secondary antibodies. Infrared imaging with two-color detection was performed using an Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE). Primary antibodies were diluted as follows: anti-GIV (coiled-coil), 1:500; anti-AGS3, 1:1000; anti-His, 1:2000; anti-panGβ, 1:200; anti-pAkt (S473), 1:200; anti-Akt, 1:400; and anti-α-tubulin, 1:2500.

Cell Migration Assays—Scratch wound assays were done as described previously (18). Briefly, 1-mm wounds were created in monolayer cell cultures (~100% confluent) with a 1-ml sterile pipette tip, and the cells were subsequently monitored by phase-contrast microscopy over the succeeding 24 h. To quantify cell migration (expressed as % wound area covered), images were analyzed using ImageJ (National Institutes of Health) software to calculate the difference between the wound area at 0 h and at the end of the migration assay divided by the area at 0 h × 100.
A Novel Structural Determinant of Gα13-GIV Interaction

Statistical Analysis—Experiments were repeated at least three times, and results were presented either as one representative experiment or as mean ± S.E. when data from multiple independent experiments were pooled. Statistical significance between various conditions was assessed with the Student’s t-test. p < 0.05 was considered significant.

RESULTS
Validation of GIV as a Bona Fide GEF for Gα subunits—In our previous work (11) we demonstrated that GIV was capable of increasing the steady-state GTPase activity of Gα13. The steady-state GTP hydrolysis by Gα subunits is a reaction with two major steps, nucleotide exchange (i.e. release of GDP and loading of GTP) and GTP hydrolysis. The GTP hydrolysis step is 10- to 100-fold faster than the nucleotide exchange step. For this reason nucleotide exchange is rate-limiting for the steady-state GTPase reaction (27). Because GIV increases the steady-state GTPase activity of Gα13 and this activity directly depends on the rate of nucleotide exchange, we proposed that GIV is a GEF for Gα subunits.

To rule out an effect of GIV on GTP hydrolysis we performed single-turnover GTPase assays. Under these experimental conditions the nucleotide exchange step is bypassed and the GTPase activity depends solely on the rate of GTP hydrolysis (28). We measured the single-turnover GTPase activity of purified His-Gα13 in the absence or presence of GIV-CTs (1660–1870) (“s” stands for “short”), which contains the GEF motif of GIV. His-GIV-CTs was used, because it behaves the same as His-GIV-CT (1623–1870) (13) in terms of its binding to Gα subunits (data not shown) and modulation of steady-state GTPase activity (Fig. 1A) but gives greater protein yields in E. coli. As a positive control we used GST-GAIP, a well characterized GAP (1) that accelerates the rate of GTP hydrolysis by Gα subunits. GST-GAIP dramatically increased the single-turnover GTPase activity of Gα13, whereas GIV-CTs had no

*FIGURE 1. GIV is a bona fide GEF for Gα13. A, His-GIV-CT (aa 1623–1870) and His-GIV-CTs (aa 1660–1870) are equally efficient in increasing the steady-state GTPase activity of Gα13. The steady-state GTPase activity of purified His-Gα13 (50 nM) was determined in the presence of the indicated amounts (0, 0.1, 0.25, 0.5, 1, and 2 μM) of purified His-GIV-CT (aa 1623–1870, closed circles) or His-GIV-CTs (aa 1660–1870, open circles) by quantification of the amount of [γ-35S]GTP (0.5 μM, ∼50 cpm/fmol) hydrolyzed in 10 min. Data are expressed as % of GTP hydrolyzed by the G protein alone (0 μM His-GIV-CT or His-GIV-CTs). Results are shown as mean ± S.E. of n = 3 independent experiments. B, His-GIV-CTs does not affect the rate of GTP hydrolysis by Gα13. Single-turnover GTPase assays for Gα13 (50 nM) were performed as described under “Experimental Procedures” in the presence of His-GIV-CTs (2 μM, open circles), GST-GAIP (1 μM, “x”), or buffer (closed circles). GST-GAIP increases the rate of GTP hydrolysis by Gα13, whereas His-GIV-CTs has no effect. One representative experiment of four is shown (C) His-GIV-CTs increases GTPγS binding to Gα13. Nucleotide binding activity of purified His-Gα13 (50 nM) was determined in the presence of the indicated amounts (0, 0.1, 0.5, 1, and 2 μM) of purified wild-type His-GIV-CTs (closed circles) or His-GIV-CTs F1685A mutant (open circles) by quantification of the amount of [35S]GTPγS (0.5 μM, ∼50 cpm/fmol) bound in 15 min. Data are expressed as % of GTPγS bound by the G protein alone in the absence of His-GIV-CTs. His-GIV-CTs increases GTPγS binding up to 2.2-fold over basal binding, whereas His-GIV-CTs F1685A has no significant effect. Results are shown as mean ± S.E. of n = 3 independent experiments.*
effect (Fig. 1B) even at concentrations (2 μm) that provoke a maximal increase of the steady-state GTPase activity (Fig. 1A). This result demonstrates that GIV does not affect GTP hydrolysis in the steady-state GTPase reaction.

To further validate the role of GIV as a GEF, we performed GTPγS binding experiments, which directly measure nucleotide exchange activity (8, 26). Purified His-Go13 was incubated in the presence of increasing amounts of wild-type His-GIV-CTs or the Go13 binding-deficient His-GIV-CTs F1685A mutant (11). GTPγS binding to Go13 was increased by His-GIV-CTs in a dose-dependent manner but was not significantly affected by His-GIV-CTs F1685A (Fig. 1C). At the maximal concentration of His-GIV-CTs tested (2 μM) GTPγS binding was increased up to ~2.2-fold over basal binding. This result indicates that GIV increases nucleotide exchange by Go13 via its previously described GEF motif (11). Taken together these results demonstrate that GIV is a bona fide GEF for Go13 and validates the steady-state GTPase activity as a direct measure of its GEF activity.

Comparative Binding of GIV to Go13 and Goαo. We previously reported that GIV interacts with members of the Gı (includes Go11, Go12, Go13, and Goαo) and Gı subfamilies of G proteins in two-hybrid assays (14); however, in vitro pull-down assays GIV preferentially binds to Go13 versus Goαo and binds as efficiently to Go11 and Goαo (11, 18) as to Go13. To gain insights into the structural features responsible for the preferential binding of GIV to Goα subunits, we compared GIV binding to Go13 and Goαo (hereafter referred as Goαo), which also belongs to the Gı family and is the closest to Goαo subunits in sequence and structure (29). We found that both GST-Go13 and GST-Goαo interact with endogenous GIV in pulldown assays on COS7 lysates when the G protein is preloaded with GDP (inactive state (Fig. 2A)); however, GST-Go13 bound ~15- to 20-fold more GIV than GST-Goαo. Neither Go13 nor Goαo bound GIV when preloaded with GDP and AlF4− (mimicking the active state) (Fig. 2A).

Because the state-dependent interaction of GIV with inactive Goα is mediated by the GEF motif located in the C terminus, we next investigated the ability of both GST-Go13 and GST-Goαo, immobilized on beads to bind purified His-GIV-CT (1623-1870) in vitro. The findings were similar to those obtained for endogenous GIV from cell lysates: His-GIV-CT bound preferentially to inactive (GDP-bound) Go13 and Goαo, and binding to Go13 was ~10-fold greater than to Goαo (Fig. 2B). These results indicate that, although GIV-CT can bind to both Go13/GDP and Goαo/GDP, binding to Go13/GDP is much less efficient.

Comparative Activation of Go13 and Goαo by GIV. Based on our recent finding (11) that binding of the GEF motif of GIV to the Goα subunit is required for GIV to exert its GEF function, we hypothesized that its decreased binding to Goαo/GDP might affect its GEF activity. To test if this is the case, we measured the steady-state GTPase activity of purified His-Go13 and His-Goαo in the presence of increasing amounts of purified GIV-CTs. The relative increase in the steady-state GTP hydrolysis exerted by GIV-CTs on Go13 was significantly reduced compared to its effect on Go13 at all concentrations tested (Fig. 2C). At the maximal concentration of GIV-CTs tested (2 μM), activation of Go13 was ~75% less than Go13 (Table 1). Taken together, these results demonstrate that GIV is a more efficient GEF for Go13 than for Goαo, and thus is capable of discriminating among Goα subunits of the Gı subfamily.

### Table 1

| Basal GTP hydrolysis in 10 min | G protein activation by GIV-CTs | Reduction in G protein activation by GIV-CTs compared to wt Go13 |
|-----------------------------|-------------------------------|---------------------------------------------------------------|
| mol GTP/mol Go13            | fold increase                 | %                                             |
| Go13                        | 0.11 ± 0.01                  | 3.17 ± 0.18                                                  |
| Goαo                        | 0.54 ± 0.03                  | 1.47 ± 0.14                                                  |

*GIV-CTs concentrations = 2 μM.

### Figure 3

Amino acids 178–270 of Go13 are responsible for the preferential binding of GIV to Go13 versus Goαo. A, schematic showing the Go13/Goαo chimeras (1–4) used in B. In B, Upper panel, GDP-loaded GST-Go13 chimeras 1 (lane 6) and chimera 3 (lane 10) bind as much full-length GIV as GST-Go13 (lane 4), whereas GST-Go13 chimeras 2 (lane 8) and chimera 4 (lane 12) show dramatically reduced GIV binding. Binding of GIV to GDP-AlF4− loaded G proteins is absent in all cases (lanes 3, 5, 7, 9, 11, and 13). COST cell lysates were incubated with purified GST (lanes 2 and 3), GST-Go13 (lanes 4 and 5) or the indicated GST-Go13 chimeras (lanes 6–13) pre-loaded with GDP (lanes 2, 4, 6, 8, 10, and 12) or GDP-AlF4− (lanes 3, 5, 7, 9, 11, and 13) immobilized on glutathione beads and analyzed as in Fig. 2A. A higher exposure of the same immunoblot (middle panel) shows the weak binding observed for GST-Go13 chimeras 2 (lane 8) and 4 (lane 12). Equal loading of GST proteins was confirmed by Ponceau S staining (lower panel).

### Table 2

Comparative activation of Go13 and Goαo by GIV-CTs

Experiments were performed as described for Fig 2C. Reduction in G protein activation by GIV-CTs was calculated as percent decrease in the -fold activation of Go13 compared to -fold activation of Go13 as described under "Experimental Procedures." All parameters are expressed as mean ± S.E. of n = 9 independent experiments.
domain of \( \text{G}_{\alpha_i} \), bound similar amounts of endogenous GIV from COS7 lysates, whereas \( \text{G}_{\alpha_i3/o} \) chimera 2 (contains the Ras-like domain of \( \text{G}_{\alpha_i} \)), showed dramatically reduced GIV binding (Fig. 3B). In addition, chimera 3 (contains the C-terminal half, aa 272–354) but not chimera 4 (contains the N-terminal half, aa 178–271 of the Ras-like domain of \( \text{G}_{\alpha_i} \)) bound as much GIV as wt \( \text{G}_{\alpha_i} \) (Fig. 3B). None of the chimeras bound GIV when they were preloaded with GDP-\( \text{AlF}_4^- \) (Fig. 3B). These results indicate

**FIGURE 4.** Trp-258 of \( \text{G}_{\alpha_i3} \) is responsible for GIV binding and activation of \( \text{G}_{\alpha_i3} \). 

A, sequence alignment of \( \text{G}_{\alpha_i}, \text{G}_{\alpha_i1}, \text{G}_{\alpha_i2}, \) and \( \text{G}_{\alpha_i3} \) indicating the \( \text{G}_{\alpha_i3} \) mutants studied. Rat \( \text{G}_{\alpha_i}, \text{G}_{\alpha_i1}, \text{G}_{\alpha_i2}, \) and \( \text{G}_{\alpha_i3} \) sequences corresponding to \( \text{G}_{\alpha_i3} \) aa 178–270 were obtained from the NCBI data base and aligned using ClustalW. Conserved identical residues are in black; similar residues are shaded in gray. The secondary structure elements (\( \alpha = \alpha \)-helix, \( \beta = \beta \)-sheet) indicated below the alignment are named according to their crystal structures (29, 33). Residues conserved among \( \text{G}_{\alpha_i1}, \text{G}_{\alpha_i2}, \) and \( \text{G}_{\alpha_i3} \) but different in \( \text{G}_{\alpha_i} \) within the 178–270 region were mutated in GST-\( \text{G}_{\alpha_i3} \) to the corresponding residue in \( \text{G}_{\alpha_i} \) (indicated above with arrows).

B, mutations W258F/T260I and W258F, in the \( \alpha_3/\beta_5 \) loop, impair endogenous GIV binding to GDP-loaded GST-\( \text{G}_{\alpha_i3} \), whereas mutations G217D, L232Q, A235H/E239T/M240T, and K248M do not affect binding (upper panels). GIV binding to GDP-\( \text{AlF}_4^- \)-loaded G proteins is virtually absent in all cases (lower panels). COS7 cell lysates were incubated with GST-\( \text{G}_{\alpha_i3} \) to the corresponding residue in \( \text{G}_{\alpha_i} \) (indicated above with arrows). GIV binding to GDP-\( \text{AlF}_4^- \)-loaded G proteins is virtually absent in all cases (lower panels). COS7 cell lysates were incubated with GST-\( \text{G}_{\alpha_i3} \) to the corresponding residue in \( \text{G}_{\alpha_i} \) (indicated above with arrows).

C, binding of GST-GIV-CT to His-\( \text{G}_{\alpha_i3} \) W258F (lane 6) is reduced (180%) compared with wt His-\( \text{G}_{\alpha_i} \) (lane 3). ∼3 μg of purified wt His-\( \text{G}_{\alpha_i} \) (lanes 1–3) or His-\( \text{G}_{\alpha_i3} \), W258F (lanes 4–6) pre-loaded with GDP were incubated with ∼9 μg of GST (lanes 2 and 5) or GST-GIV-CT (aa 1623–1870, lanes 3 and 6) immobilized on glutathione beads. Bound proteins were analyzed by immunoblotting (IB) for His. Equal loading of GST proteins was confirmed by Ponceau S staining. 

D, mutation of Trp-258 to Phe reduces (60–70%) activation of His-\( \text{G}_{\alpha_i3} \) by His-GIV-CTs at all concentrations tested. The steady-state GTPase activity of purified His-\( \text{G}_{\alpha_i3} \) (closed circles, 50 nM) or His-\( \text{G}_{\alpha_i3} \), W258F (open circles, 50 nM) was determined as in Fig. 2C. Results are shown as mean ± S.E. of nine (\( \text{G}_{\alpha_i3} \)) or five (\( \text{G}_{\alpha_i3}, \text{W258F} \)) independent experiments.

E, mutation of Phe-259 to Trp increases (2-fold) activation of His-\( \text{G}_{\alpha_i} \) by His-GIV-CTs at all concentrations tested. The steady-state GTPase activity of purified His-\( \text{G}_{\alpha_i} \) (closed circles, 50 nM) or His-\( \text{G}_{\alpha_i} \), F259W (open circles, 50 nM) was determined as in Fig. 2C. Results are shown as mean ± S.E. of three independent experiments.
TABLE 2
Comparative activation of wt Gαo3 and Gαo3 W258 mutants by GIV-CTs
Experiments were performed as described for Fig 5A. Reduction in G protein activation by GIV-CTs was calculated as percent decrease in the fold activation of each Gαo3 mutant compared to fold activation of wt Gαo3 as described under "Experimental Procedures." All parameters are expressed as mean ± S.E. of n = 3–9 independent experiments.

| Basal GTP hydrolysis in 10 min | G protein activation by GIV-CTs | Reduction in G protein activation by GIV-CTs compared to wt Gαo3 | Base GTP/mmol Gαo3 | Fold increase | % |
|-------------------------------|-------------------------------|-------------------------------------------------|-----------------|-------------|---|
| Gαo3 wt                       | 0.11 ± 0.01                   | 3.17 ± 0.18                                     | -               | 3.17 ± 0.18 |   |
| Gαo3 W258F                    | 0.11 ± 0.01                   | 1.72 ± 0.03                                     | -               | 1.72 ± 0.03 |   |
| Gαo3 W258A                    | 0.14 ± 0.01                   | 1.13 ± 0.03                                     | -               | 1.13 ± 0.03 |   |
| Gαo3 W258L                    | 0.12 ± 0.01                   | 1.58 ± 0.18                                     | -               | 1.58 ± 0.18 |   |
| Gαo3 W258S                    | 0.11 ± 0.01                   | 1.47 ± 0.04                                     | -               | 1.47 ± 0.04 |   |
| Gαo3 W258Y                    | 0.11 ± 0.01                   | 1.84 ± 0.04                                     | -               | 1.84 ± 0.04 |   |
| Gαo3 W258D                    | 0.11 ± 0.01                   | 1.11 ± 0.03                                     | -               | 1.11 ± 0.03 |   |
| Gαo3 W258H                    | 0.14 ± 0.02                   | 1.42 ± 0.06                                     | -               | 1.42 ± 0.06 |   |

*GIV-CTs concentration = 2 μM.

that aa 178–270 of Gαo3 (corresponding to 179–271 of Gαo1) where switches I, II, and III are located (Fig. 3A) contains the determinants that specify the preferential binding of GIV to Gαo3 versus Gαo1.

Identification of a Single Residue That Determines GIV Preferential Binding and Activation of Gαo3—We reasoned that one or several of the residues within aa 178–270 of Gαo3 that are conserved among Gαo3, Gαo1, and Gαo2 but different in Gαo1 must be responsible for the preferential binding of GIV to Gαo3 subunits. To identify such residues we aligned the sequences of Gαo1, Gαo1, Gαo2, and Gαo3 (Fig. 4A). We mutated residues conserved among Gαo subunits but differing from Gαo3 to the corresponding amino acids of Gαo1 (specified in Fig. 4A) and tested their ability to bind endogenous GIV in GST pulldown assays on COS7 cell lysates. The Gαo3 W258F/T260I double mutant and the Gαo3 W258F single mutant showed a dramatic reduction in GIV binding, whereas the remainder of the mutants were the same as wt Gαo3 (Fig. 4B). Importantly, the GST-Gαo3 W258F mutant bound as much AGS3 and Gβ(3) as wt Gαo3 (data not shown), suggesting that the decreased GIV binding is specific and not due to an overall effect on the structure of Gαo3 subunits. In addition to the mutants specified in Fig. 4A, two other mutants (Gαo3 E193N/Y195H/K197R/M198L and Gαo3 D229G/L239Q) were used in similar pulldown assays, but no differences in GIV binding were observed from wt Gαo3 (data not shown).

To further confirm that the W258F mutation directly affects interaction between GIV and the G protein we carried out protein interaction assays with purified recombinant proteins. GST-GIV-CT was immobilized on glutathione-agarose beads and incubated with His-tagged wt Gαo3 or Gαo3 W258F. Binding of His-Gαo3 W258F was dramatically reduced compared to wt Gαo3 (Fig. 4C). Thus, Trp-258, which is located in the α3/β5 loop of Gαo3, is a critical determinant of its interaction with GIV.

We next investigated the GEF activity of GIV on Gαo3 W258F and found that the relative increase in steady-state GTP hydrolysis exerted by GIV-CTs on Gαo3 W258F was significantly reduced compared with wt Gαo3 (Fig. 4D). At the maximal concentration of the GEF tested (2 μM), activation of Gαo3 W258F was reduced >65% (Table 2), which is comparable to that observed for Gαo1 (Fig. 2C and Table 1). In addition, we tested the GEF activity of GIV on a Gαo1 mutant in which Phe-259 was replaced by the corresponding aa (Trp-258) in Gαo3. We found that mutation of Phe-259 to Trp enhanced the relative increase in steady-state GTP hydrolysis exerted by GIV-CTs on Gαo1 ~2-fold (Fig. 4E), indicating that the F259W mutation is sufficient to make Gαo1 a better substrate for the GEF activity of GIV. From these results we conclude that mutation of Trp-258 in the α3/β5 loop of Gαo3 to the corresponding amino acid (Phe) in Gαo1 dramatically reduces GIV binding and accounts for the reduced GEF activity of GIV on Gαo1.

Trp-258 Is Critical for Activation of Gαo3 by GIV—Mutation of Trp-258 to Phe (aromatic to an aromatic side chain) is a conservative mutation; yet it significantly reduces Gαo3 activation by GIV. Based on this finding we reasoned that functional Gαo3-GIV coupling should be very sensitive to alterations in aa 258 of Gαo3. To test if this is the case, we performed further mutational analysis by replacing Trp-258 with amino acids of different nature, i.e. tyrosine (aromatic), leucine (aliphatic), serine (polar), histidine (basic), aspartate (acidic), and alanine (small). The mutants were then tested for their response to GIV in steady-state GTPase assays. All the Gαo3 Trp-258 mutants showed reduced activation by GIV-CTs compared to wt Gαo3, but the relative decrease in GTPase hydrolysis varied (Fig. 5A). At the maximal concentration of GIV-CTs tested (2 μM), G protein activation was as follows: W258A ~ W258D < W258S ~ W258H ~ W258L < W258Y (Table 2). The W258A and W258D mutants showed a ~90–95% reduction and W258Y an ~60% reduction in activation, a value very similar to that observed for W258F (Table 2). These results highlight the specific requirement for Trp in position 258 to achieve maximal activation by GIV.

Results from pulldown assays with purified recombinant proteins were consistent with the results of the GTPase assay: binding of GST-GIV-CT to His-Gαo3 W258A or W258D was virtually abolished (Fig. 5B) and less than to His-Gαo1 W258F (Fig. 5B). Thus the extent of GIV binding parallels the extent of Gαo3 activation (Figs. 4D and 5A and Table 2).

Some mutations that cause reduced activation of Gα subunits by GPCRs also decrease their activation by AlF4− (30–32), which activates G proteins by mimicking the γ-phosphate of GTP in the transition state (33). To test the ability of the Gα mutants to be activated by AlF4− we took advantage of a well established assay based on differential resistance to proteolysis (31, 34). When Gα subunits are in the inactive GTP-bound conformation, they are readily digested by trypsin, whereas upon AlF4− binding and adoption of the active conformation only a short sequence can be cleaved, and the remainder of the protein remains trypsin-resistant (34). All the His-Gαo3 Trp-258 mutants (see Table 2) as well as His-Gαo1 behaved like wt His-Gαo1 in that they were hydrolyzed by trypsin when pre-loaded with GDP but generated a trypsin-resistant form when preloaded with GDP-AlF4− (Fig. 6). Thus all the Gαo3 subunits tested can efficiently adopt the active conformation upon AlF4− binding. Because activation by AlF4− requires the nucleotide binding site to contain GDP and the G protein to be in an appropriate conformation, these results also indicate that the Gα proteins are properly folded. In addition, all the Gαo3 Trp-258 mutants had similar basal rates of steady-state GTPase hydrolysis (see Table 2), suggesting that the spontaneous
exchange of nucleotide is unaffected and that they fold properly and maintain their native properties. Collectively, these results support the conclusion that mutations in position 258 of Gαi3

**FIGURE 5.** Gαi3 activation by GIV is reduced when Trp-258 is replaced by Tyr, Leu, Ser, His, Asp, or Ala. All the Gαi3 Trp-258 mutants investigated show reduced activation by His-GIV-CT ranging from a ~60% (W258Y) to ~95% (W258D) reduction. Trp-258 of His-Gαi3 was mutated to amino acids of different nature, i.e. tyrosine (aromatic), leucine (aliphatic), serine (polar), histidine (basic), aspartate (acidic), and alanine (small). The steady-state GTPase activity of purified His-Gαi3 (closed circles, 50 nM) or the indicated His-Gαi3 mutants (open circles, 50 nM) was determined as described in Fig. 1C. Results are shown as mean ± S.E. of 4–9 independent experiments. B, binding of GST-GIV-CT to His-Gαi3 mutants W258F (lane 3), W258A (lane 9), and W258D (lane 12) is reduced compared to wt His-Gαi3 (lane 6). ~3 μg of purified His-Gαi3 (lanes 4–6) or the indicated His-Gαi3 mutants (W258F: lanes 1–3, W258A: lanes 7–9, W258D: lanes 10–12) pre-loaded with GDP were incubated with ~9 μg of GST (lanes 2, 5, 8, and 11) or GST-GIV-CT (aa 1623–1870, lanes 3, 6, 9, and 12) immobilized on glutathione beads. Bound proteins were analyzed by immunoblotting (IB) for His. Equal loading of GST proteins was confirmed by Ponceau S staining (lower panel).

**FIGURE 6.** Mutations at Trp-258 do not affect the trypsin sensitivity of Gαi3 after activation by AlF4–. Gαi3 wt and all the Gαi3 mutants tested are digested when pre-loaded with GDP, and all adopt the trypsin-resistant conformation after incubation with GDP-AlF4. His-Gαi3 and the indicated His-Gαi3 mutants or His-Gαi3 (0.5 mg/ml) were incubated in the presence of GDP or GDP-AlF4 and treated or not with trypsin as described under “Experimental Procedures.” A Coomassie Blue-stained gel of a representative experiment is shown. The arrowhead denotes the position of the non-trypsinized full-length proteins loaded, and the asterisk arrowhead denotes the trypsin-resistant form of the active, GDP-AlF4-loaded His-Gαi3 subunit.

**FIGURE 7.** Mutation of Trp-258 to Asp, Ala, or Phe impairs the interaction between Gαi3 and endogenous GIV in cultured cells. Upper panel: co-immunoprecipitation of GIV, Gβγ, and AGS3 with FLAG-tagged Gαi3 mutants W258D (lane 2) or W258A (lane 3) is dramatically reduced compared with wt Gαi3 (lane 4). By contrast, with Gαi3 W258F (lane 5) only co-immunoprecipitation of GIV, but not Gβγ and AGS3 is reduced compared to controls. COS7 cells were transfected with empty vector (lane 1) or plasmids encoding FLAG-tagged Gαi3, W258D (lane 2), Gαi3 W258A (lane 3), wt Gαi3 (lane 4), or Gαi3 W258F (lane 5). 48 h after transfection cells were harvested, and lysates were used for immunoprecipitation (IP) with anti-FLAG IgG (~2 μg) as described under “Experimental Procedures.” IP was followed by immunoblotting (IB) for FLAG (Gαi3), GIV, AGS3, and Gβ (pan-Gβ). Equal IgG loading was confirmed by Ponceau S staining. Lower panel: aliquots of the lysates (10%) were analyzed by immunoblotting (IB) to confirm the equal loading of Gαi3, GIV, AGS3, and Gβ and the expression of Gαi3-FLAG constructs in the different transfected samples.
specifically alter GIV-catalyzed activation without causing global structural changes in the Go subunit.

**Mutation of Trp-258 Impairs Goα3 Binding to GIV in Cultured Cells**—Next, we investigated the effect of mutating Trp-258 on the interaction between GIV and the G protein in cultured cells. COS7 cells were transfected with FLAG-tagged wt Goα3 and Goα3 mutants, and immunoprecipitation was carried out using anti-FLAG IgG followed by immunoblotting for GIV. We found that the amount of endogenous GIV that co-immunoprecipitated with the Goα3 mutants W258F, W258A, and W258D was dramatically reduced compared with wt Goα3 (Fig. 7). These results demonstrate that mutation of Trp-258 impairs the interaction of Goα3 with endogenous GIV in cultured cells, corroborating our observations in vitro.

**Mutation of Trp-258 to Phe Does Not Affect Binding of Gβγ, AGS3, GAIP/RGS19, or LPAR1 to Goα3**—We next investigated if mutation of Trp-258 of Goα3 to Phe interferes with its interaction with other binding partners such as Gβγ and AGS3 (a Goα-guanine nucleotide dissociation inhibitor (21, 35)). We also investigated the behavior of Goα3 W258A and W258D, because these two mutants have reduced GIV binding both in vitro and in cultured cells (Figs. 5 and 7) and show the most dramatic reduction in activation by GIV (Fig. 5 and Table 2). We found that Goα3 W258F binds Gβγ and AGS3 as efficiently as wt Goα3 in co-immunoprecipitation assays (Fig. 7), whereas binding of Goα3 W258A or W258D to Gβγ and AGS3 was dramatically reduced (Fig. 7). From these results we conclude that mutation of Trp-258 to Phe specifically impairs Goα3 interaction with GIV without affecting its interaction with Gβγ and AGS3, whereas this is not the case for mutation of Trp-258 to Asp or Ala.

Thus mutation of Trp-258 specifically to Phe can be tolerated for the interaction of the G protein with binding partners other than GIV (e.g. Gβγ and AGS3), whereas mutation of Trp-258 to Ala or Asp most likely affects the structural properties of the G protein such that they impair its interaction with Gβγ and AGS3. We further investigated the ability of Goα3 W258F to interact with GAIP (RGS19) (22), a GAP for Goα subunits (36). Identical results were obtained with wt Goα3 and Goα3 W258F: GST-GAIP bound robustly to Goα3 preloaded with GDP-AlF4 but showed virtually no binding to inactive, GDP-loaded Goα3 (22) (Fig. 8A), demonstrating that this mutation does not compromise the interaction with GAIP.

We also investigated the ability of Goα3 W258F to interact with LPAR1, a GPCR that couples to Goα and Goβ, and Goα3, Goβ, and Goγ subunits (23, 37, 38). COS7 cells were co-transfected with FLAG-tagged LPAR1 and untagged wt Goα3 or Goα3 W258F, and immunoprecipitation was carried out using anti-FLAG IgG followed by immunoblotting for Goα3. We found that the amount of Goα3 that co-immunoprecipitated with the receptor from lysates of COS7 cells transfected with either wt Goα3 or Goα3 W258F was virtually the same (Fig. 8B), indicating that mutation of Trp-258 to Phe does...
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FIGURE 9. G<sub>α<sub>i<sub>3</sub> W258F fails to rescue Akt activation and cell migration defects observed upon depletion of endogenous G<sub>α<sub>i<sub>3</sub> A and B, HeLa cells treated with scrambled (Scr) or hGα<sub>i<sub>3 siRNA oligonucleotides and the indicated DNA plasmids (empty vector, rGα<sub>i<sub>3 WT, or rGα<sub>i<sub>3 W258F) were serum-starved for 6 h and stimulated with 10 μM LPA (A) or 100 nM insulin (B) for 5 min. Cell lysates were analyzed by immunoblotting (IB) for total (tAkt) and S473 phospho-Akt (pAkt), Gα<sub>i<sub>3, and α-tubulin. Depletion of endogenous Gα<sub>i<sub>3 reduces LPA-stimulated (A) and insulin-stimulated (B) Akt activation by ~70%, which is restored upon transfection of wt Gα<sub>i<sub>3, but not rGα<sub>i<sub>3 W258F. C, in controls (Scr siRNA), HeLa cells cover the majority of the experimental wound area after 24 h, whereas in Gα<sub>i<sub>3-depleted cells wound closure is greatly impaired. The ability to migrate and close the wound area at 24 h is restored by transfection of rGα<sub>i<sub>3 wt but not rGα<sub>i<sub>3 W258F. HeLa cell monolayers treated with the indicated siRNA oligonucleotides, and DNA plasmids were scratch-wounded and examined by light microscopy immediately (0 h) or 24 h after wounding. Scale bar = 500 μm. D, bar graph showing quantification of the wound area covered by cells in C. The area covered by cells was determined by calculating the difference between the wound area at 0 and 24 h expressed as percent of the wound area at 0 h. Results are shown as mean ± S.D. of 8–12 randomly chosen fields from three independent experiments. E, cell lysates from cells treated as in C were immunoblotted to assess the efficiency of siRNA depletion of Gα<sub>i<sub>3 (~95%) and the expression of rGα<sub>i<sub>3 WT or rGα<sub>i<sub>3 W258F.

not affect the interaction of Gα<sub>i<sub>3 with LPAR1, a GPCR. Taken together, these results indicate that the W258F, but not the W258A or W258D mutation, specifically impairs activation of Gα<sub>i<sub>3 by GIV without perturbing other known interactions of the G protein.

Gα<sub>i<sub>3 W258F Fails to Enhance LPA- and Insulin-stimulated Akt Activation and to Promote Cell Migration—We have previously shown that activation of Gα<sub>i<sub>3 enhances Akt signaling after stimulation of both GPCRs and receptor tyrosine kinases (18) and that the GEF motif of GIV is required for these functions (11). These effects might be triggered directly by activation of the G protein by GIV, or alternatively, they could be enhanced by GIV-independent activation of Gα<sub>i<sub>3. To distinguish between these two possibilities we took advantage of the GIV-insensitive Gα<sub>i<sub>3 W258F mutant. Gα<sub>i<sub>3 was depleted (>95%) in HeLa cells using siRNA oligonucleotides that specifically target the human sequence of Gα<sub>i<sub>3 (18), and Akt activation was measured in response to stimulation of either the LPA receptor, a GPCR that enhances Akt signaling by activating G<sub>i<sub>3 (proteins (37, 38), or the insulin receptor, a receptor tyrosine kinase. When serum-starved HeLa cells were stimulated with either LPA (Fig. 9A) or insulin (Fig. 9B), activation of Akt was dramatically reduced (~70%) in Gα<sub>i<sub>3-depleted cells compared with controls, and this effect could be reversed by expression of wt rat Gα<sub>i<sub>3 (which is insensitive to human Gα<sub>i<sub>3-specific siRNA oligonucleotides (18)). Gα<sub>i<sub>3 depletion also impaired the ability of HeLa cells to migrate efficiently in scratch-wound assays (Fig. 9, C–E), and this effect was restored by expression of rat Gα<sub>i<sub>3 wt. By contrast, expression of rat Gα<sub>i<sub>3 W258F failed to restore Akt activation in response to either LPA or insulin (Fig. 9, A and B), and to reverse the defect on cell migration (Fig. 9, C–E). From these results we conclude that direct activation of Gα<sub>i<sub>3 by GIV is required to enhance Akt signaling and to promote cell migration after growth factor or GPCR stimulation.

DISCUSSION

GIV is a recently characterized non-receptor GEF that can activate Gα<sub>i<sub>3 (11). The major finding in this work is the identification of a novel structural determinant on Gα<sub>i<sub>3 that renders the G protein sensitive to activation by GIV. This structural determinant is required to promote efficient cell migration and Akt signaling, two cell functions that we have previously shown to be triggered by active Gα<sub>i<sub>3 (18). Using site-directed mutagenesis, we demonstrate here that Trp-258 located in the α<sub>3</sub>β<sub>5</sub> loop of the Ras-like domain of Gα<sub>i</sub> subunits is required to establish an efficient interaction with GIV and to activate the G protein. When Trp-258 is mutated to Phe, Gα<sub>i<sub>3 is less efficiently activated by GIV, but it retains its ability to interact with Gβγ subunits, AGS3, GAIP (RGS19), and...
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FIGURE 10. Sequence comparison and three-dimensional view of the newly identified structural determinant in Goq required for its activation by GIV. A, representative members of different Gα subfamilies were aligned to compare the sequence corresponding to the newly identified structural determinant in Goq. Alignment of rat Gαo, Gαi1, Gαi2, Gαi3, Gαi12, Gαq, and Gαs sequences obtained from the NCBI database was performed using ClustalW. Conserved identical residues are in black; similar residues are shaded in gray. The secondary structure elements (α = α-helix, β = β-sheet) corresponding to these sequences are named according to previously reported crystal structures (29, 33) and are indicated below the alignment. The arrow denotes the position corresponding to the Trp-258 of Gαi3, and the asterisk denotes the previously identified binding site for GIV (11). B, three-dimensional view of the GEF motif of GIV bound to Gαi3 is shown to depict the relative location of the newly identified structural determinant (Trp-258 in the α3/β5 loop), which is positioned C-terminal to the GEF motif. The homology model of GDP-Gαi3 in complex with the GEF motif of GIV generated as described previously (11) using the structure of the synthetic peptide KB-752 bound to Goq (PDB: 1Y3A) as a template (18). The “Ras-like” domain of Gαi3 is shown in blue, the “switch II” region in green, and Trp-258 in yellow. The GEF motif of GIV is shown in red.

LPAR1, to change conformation upon activation and to efficiently hydrolyze GTP (Figs. 6–8 and Table 2). In addition, the GIV-insensitive Goq W258F mutant fails to enhance LPA- and insulin-stimulated activation of Akt and to promote cell migration (Fig. 9). These results are consistent with our previous work in that the GEF-deficient F1685A mutant of GIV also fails to promote LPA- and insulin-stimulated activation of Akt and cell migration (11).

In our previous work (11, 14, 18) we found that GIV interacts with Gα subunits of the Gq subfamily (Gαq1, Gαq2, Gαq3, and Gαq) and, to a lesser extent, the Gα subfamily (Gαq) but not those of the Gq and G12 (Gα12 and Gα13) subfamilies. Here we demonstrate that GIV can also discriminate within the Gq subfamily, because Gαq is a better substrate for the binding and GEF activity of GIV than the highly homologous Gαo (Fig. 2).

Based on structural studies (39–43) the α3/β5 loop has been proposed to be one of the critical elements that determines the binding specificity of Gα subunits of different families to their respective effectors (42). Our data suggest that the α3/β5 loop may be important not only for determining the effector binding specificity of different Gα subunits, but also for its interaction with GIV, a non-receptor GEF. For example, Gα subunits of the G12 subfamily, which do not bind GIV, have aliphatic amino acids (Val or Leu in Gα12 and Gα13) in the position corresponding to Trp-258 of Gα13 (Fig. 10A). Our finding that mutation of Trp-258 to aliphatic amino acids impairs activation by GIV suggests that the inability of Gα12 and Gα13 to bind GIV (14) is, at least in part, a consequence of this difference within the α3/β5 loop. The sequence of Gα12 and Gα13 also differs from Gq in the Switch II region, which is a previously described binding site for GIV (11) and thus could contribute to their inability to bind GIV.

Analysis of the sequence of Gαq and Gα reveals that they have a Trp conserved in the positions corresponding to the Trp-258 of Gαq13 (Fig. 10A), yet they interact poorly with GIV (14, 18). Therefore, in these cases the Gα specificity of GIV cannot be simply attributed to this single residue. However, Gαq and Gαq show significant sequence divergence from Gαq in the residues of the α3/β5 loop that surround the position corresponding to Trp-258 of Gαq (Fig. 10A). We propose that this difference in the sequence of the α3/β5 loop could modify its structural properties and account for the decreased GIV binding observed for Gαq and Gα (14, 18). In the case of Gαq, this idea is supported by the fact that the α3/β5 loop adopts a conformation completely different from that of Gα subunits (40, 41). In addition, subtle differences between Gαq/Gαq and Gαq in some of the residues close to the GIV binding site in the Switch II region (Fig. 10A) may also contribute to the binding specificity of GIV.
Mutants that selectively abolish the ability of Go3 subunits to be regulated by GAPs (44) or guanine nucleotide dissociation inhibitors (45) have been described and used to evaluate the role of these regulators in Gi functions (44–50). Go3 W258F represents a new addition to the growing battery of mutants that can be used to finely dissect how different regulators of G protein activity control cell fate. Although Gi-coupled GPCRs also couple efficiently to Go3 subunits (51–54), here we show that replacement of a single residue (Trp-258) in Go3 for the corresponding residue in Go (Phe) dramatically and specifically reduces G protein coupling to GIV. Using this mutant we provide evidence that cell migration and Akt signaling, cell functions previously described to be promoted by constitutively active Go3 mutants (18), require Go3 to be specifically activated by GIV. The Go3 W258F mutant will also be useful in the future to distinguish other functions of Go(s) subunits controlled by the GEF activity of GIV.

The data presented here suggest that the footprint of the GIV GEF domain on Go3 probably extends from the previously described binding site within switch II (11) to make contact with an additional binding site located in the a3/b5 loop. Based on our homology modeling (11) depicted in Fig. 10B, the GEF motif of GIV docks within the groove formed between the switch II and the a3 helix of the G protein. The location of the novel structural determinant in Go3 required for binding of GIV raises the interesting possibility that GIV residues C-terminal to the previously described GEF motif may be involved in making direct contact with the a3/b5 loop region surrounding Trp-258. However, at this point allosteric effects cannot be ruled out to explain the decreased interaction between Gi and Go3 upon mutation of Trp-258. Nevertheless, our unpublished work6 favors the possibility of a direct contact site, because mutation of GIV residues C-terminal to the previously described GEF motif impairs the Go3-GIV interaction.

Our identification of a novel structural determinant in Go3 required for GIV binding provides insights that may help in the design of selective pharmacological agents that disrupt the Go3-GIV interface for therapeutic purposes. We previously found that expression of full-length GIV is induced severalfold in cancer cell lines that are highly metastatic (18) and that mutational disruption of the Gi binding to switch II of Go abolishes Akt signaling and tumor cell migration (11), which are hallmarks of cancer metastasis (55–58). Here we describe that GIV binding and these functions are similarly abolished by mutational disruption of the a3/b5 loop of Go3. Because alterations in the switch II may also impair the binding of other molecules to the G protein (59–61), the a3/b5 loop represents a more attractive target than the previously described switch II binding site. Moreover, we show here that alterations in aa 258 of Go3 can impair GIV binding and abolish cell functions controlled by GIV without affecting the interaction of Go3 with its other binding partners Gβγ, Gαs, GαIP/αGI519, and LPAR1. Thus we envision that small molecules that target this site might work as anti-metastatic agents by specifically disrupting GIV-Go3 interaction.

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