Supporting Information

Garcia-Marcos et al. 10.1073/pnas.1120538109

SI 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 DH5α were purchased from New England Biolabs. *E. coli* strain BL21(DE3), oligofectamine, and phalloidin-Texas Red were purchased from Invitrogen. Genejuice transfection reagent was from Novagen. PfuUltra DNA polymerase was purchased from Stratagene. [γ-^{32}P]GTP was from Perkin-Elmer. Silencer Negative Control scrambled (Scr) siRNA was purchased from Ambion, and a previously validated Gα-interacting, vesicle-associated protein (GIV) siRNA sequence was custom-ordered from Dharmacon. Rabbit antisera against the coiled-coil region of GIV was raised as described (1). Goat anti-rabbit and goat anti-mouse Alexa Fluor 680 or IRDye 800 F(ab′)$_2$ used for immunoblotting were from Li-Cor Biosciences. Mouse mAbs against hexahistidine (His), FLAG (M2), and α-tubulin were obtained from Sigma-Aldrich. Rabbit anti–pan-Gβ (M-14) IgG was from Santa Cruz Biotechnology and anti-Akt and phospho-Akt (S473) IgGs were from Cell Signaling.

Plasmid Constructs and Mutagenesis. Cloning of rat Gαi3 into pGEX-4T-1 or pET28b and GIV-CT (aa 1,660–1,870) into pET28b to generate N-terminally GST- or His-tagged proteins in bacteria was described previously (2–4). To generate GIV with three FLAG sequences fused to the C terminus of the protein (GIV-3xFLAG), human GIV was amplified by PCR from pcDNA 3.1-GIV with primers designed to add NotI and BamHI restriction sites at the 5′ and 3′ ends, respectively, and to replace the stop codon of the original cDNA with a serine codon. The PCR product was digested and inserted between the NotI and BamHI restriction sites of p3xFLAG-CMV-14. GIV-3xFLAG was rendered resistant to our RNAi sequence by introducing silent mutations as described previously (4). GIV 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.

Protein Purification. GST, GST-Gαi3, His-Gαi3, or His-GIV-CT fusion constructs were expressed in *E. coli* strain BL21(DE3) (Invitrogen) as described previously (3–5). Briefly, after overnight induction at 25 °C with 1 mM isopropyl β-D-1-thiogalactopyranoside, bacteria expressing GST fusion proteins from 1 L of culture were resuspended in 10 mL 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)]. Bacteria expressing His fusion proteins were resuspended in His-lysis buffer [50 mM NaH_2PO_4 (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, and soluble proteins were affinity-purified on glutathione-Sepharose 4B beads (GE Healthcare) or HisPur Cobalt Resin (Pierce). Proteins were eluted, dialyzed overnight against PBS, and stored at −80 °C. For His-Gαi3, Triton X-100 was omitted from all steps, lysis of bacteria was performed using a French press instead of sonication, and a buffer-exchange step into G-protein storage buffer [20 mM Tris·HCl (pH 7.4), 200 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 10 μM GDP, 5% (v:v) glycerol] using Amicon Ultra centrifugal filters (Millipore) was substituted for the final dialysis step.

Cell Culture, Transfection, and Lysis. COS7 and HeLa cells were grown at 37 °C in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% L-glutamine, and 5% CO_2. Transfection was carried out using Genejuice (Novagen) for DNA plasmids or oligofectamine (Invitrogen) for siRNA oligos following the manufacturers' protocols. HeLa cell lines stably expressing GIV-3xFLAG wild-type or mutants were selected after transfection in the presence of G418 (500 μg/mL) for 6 wk as previously described (5). For each mutant, two different clones were investigated, and similar results were obtained. GIV-3xFLAG expression was verified independently using FLAG and GIV rabbit pAb (data not shown) and was ∼2× the endogenous levels. All these cell lines were maintained in the presence of G418 (500 μg/mL). For assays involving serum starvation, serum concentration was reduced to 0.2% overnight. Whole-cell lysates were prepared after washing cells with cold PBS by resuspending cells in sample buffer and boiling immediately. Lysates used as a source for GIV for in vitro protein-binding assays were prepared by resuspending the cells in lysis buffer [20 mM Hepes (pH 7.2), 5 mM $Mg(CH_3COO)_2$, 125 mM $K(CH_3COO)$, 0.4% Triton X-100, 1 mM DTT, 0.5 mM Na_3VO_4] supplemented with phosphatase (Sigma) and protease (Roche) inhibitor mixtures, passed through a 28G needle at 4 °C, and cleared (10,000 × *g* for 10 min) before use in subsequent experiments.

In Vitro Protein-Binding Assays. Purified GST-Gαi3 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 $MgCl_2$, 5 mM EDTA, 30 μM GDP, 2 mM DTT, protease inhibitor mixture] for 90 min at room temperature. COS7 cell lysate (∼250 μg) or purified His-GIV-CT (1.2–12 μg) 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 wash buffer [4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% (v:v) Tween 20, 10 mM $MgCl_2$, 5 mM EDTA, 30 μM GDP, 2 mM DTT] and boiled in sample buffer for SDS/PAGE. In some cases, the binding and wash buffers were supplemented with 30 μM $AlCl_3$ and 10 mM NaF to generate AlF_4^- and activate Gαi3.

Quantitative Immunoblotting. Protein samples were separated on 10% SDS/PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with PBS supplemented with 5% nonfat milk (or 5% BSA when probing for Akt) before sequential incubation with primary and secondary antibodies. Infrared imaging and quantification of Western blots were performed according to the manufacturer's protocols using an Odyssey Infrared Imaging System (Li-Cor Biosciences). Akt activation shown in Fig. 3 and Fig. S3 was determined as the phospho-Akt (pAkt)/total Akt (tAkt) ratio normalized to the value observed in cells expressing GIV-3xFLAG WT. Primary antibodies were diluted as follows: anti-GIV, 1:500; anti-His, 1:2,000; anti-panGβ, 1:200; anti-pAkt (S473), 1:200; anti-Akt, 1:400; and anti–α-tubulin, 1:2,500.

Steady-State GTPase Assay. This assay was performed as described previously (2, 3, 6). Briefly, His-Gαi3 (100 nM) was preincubated with different concentrations of His-GIV-CT wild type or mutants (aa 1,660–1,870) for 15 min at 30 °C in assay buffer [20 mM

Na–Hepes (pH 8), 100 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 0.05% (w:v) C12E10]. GTPase reactions were initiated at 30 °C by adding an equal volume of assay buffer containing 1 μM [γ-^{32}P]GTP (~50 cpm/fmol). Duplicate aliquots (50 μL) were removed at 10 min and reactions stopped with 950 μL ice-cold 5% (wt/vol) activated charcoal in 20 mM H_3PO_4 (pH 3). Samples were then centrifuged for 10 min at 10,000 × *g*, and 500 μL of the resultant supernatant was scintillation-counted to quantify released [^{32}P]P_i. To determine the specific Pi produced, the background [^{32}P]P_i detected at 10 min in the absence of G protein was subtracted from each reaction. G-protein activation by the different constructs was expressed as percent of the steady-state GTPase activity of Gαi3 alone. The relative guanine nucleotide exchange factor (GEF) activity of the different GIV mutants compared with wild type (Table 1) was calculated using the formula $GEF_{mut} - 100/GEF_{WT} - 100$, where GEF_{mut} and GEF_{WT} are the G-protein activities in the presence of different His-GIV-CT mutants or wild type, respectively, expressed as percentage of the steady-state GTPase activity of Gαi3 alone.

Fluorescence Microscopy. Cells were fixed with 3% paraformaldehyde for 30 min, permeabilized (0.2% Triton X-100) for 45 min, and incubated for 1 h with phalloidin-Texas Red and DAPI. Images were acquired with a Zeiss Axioimager M1 microscope using a 60× aperture (Zeiss Plan Neofluar, 1.30 N.A.), Hamamatsu Orca-ER camera, and Openlab software (Improvision). All individual images were processed using Image J software and assembled for presentation using Photoshop and Illustrator software (both Adobe).

Cell Migration Assays. Scratch-wound assays were done as described previously (2–5). 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 12 h. To quantify cell migration (expressed as percent 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 h and that at the end of the migration assay divided by the area at 0 h × 100.

1. 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.
2. Garcia-Marcos M, Ghosh P, Ear J, Farquhar MG (2010) A structural determinant that renders G alpha(i) sensitive to activation by GIV/girdin is required to promote cell migration. *J Biol Chem* 285:12765–12777.
3. Garcia-Marcos M, Ghosh P, Farquhar MG (2009) GIV is a nonreceptor GEF for G alpha i with a unique motif that regulates Akt signaling. *Proc Natl Acad Sci USA* 106:3178–3183.
4. Ghosh P, Garcia-Marcos M, Bornheimer SJ, Farquhar MG (2008) Activation of Galphai3 triggers cell migration via regulation of GIV. *J Cell Biol* 182:381–393.
5. Ghosh P, et al. (2010) A Galphai-GIV molecular complex binds epidermal growth factor receptor and determines whether cells migrate or proliferate. *Mol Biol Cell* 21: 2338–2354.
6. Garcia-Marcos M, Ear J, Farquhar MG, Ghosh P (2011) A GDI (AGS3) and a GEF (GIV) regulate autophagy by balancing G protein activity and growth factor signals. *Mol Biol Cell* 22:673–686.

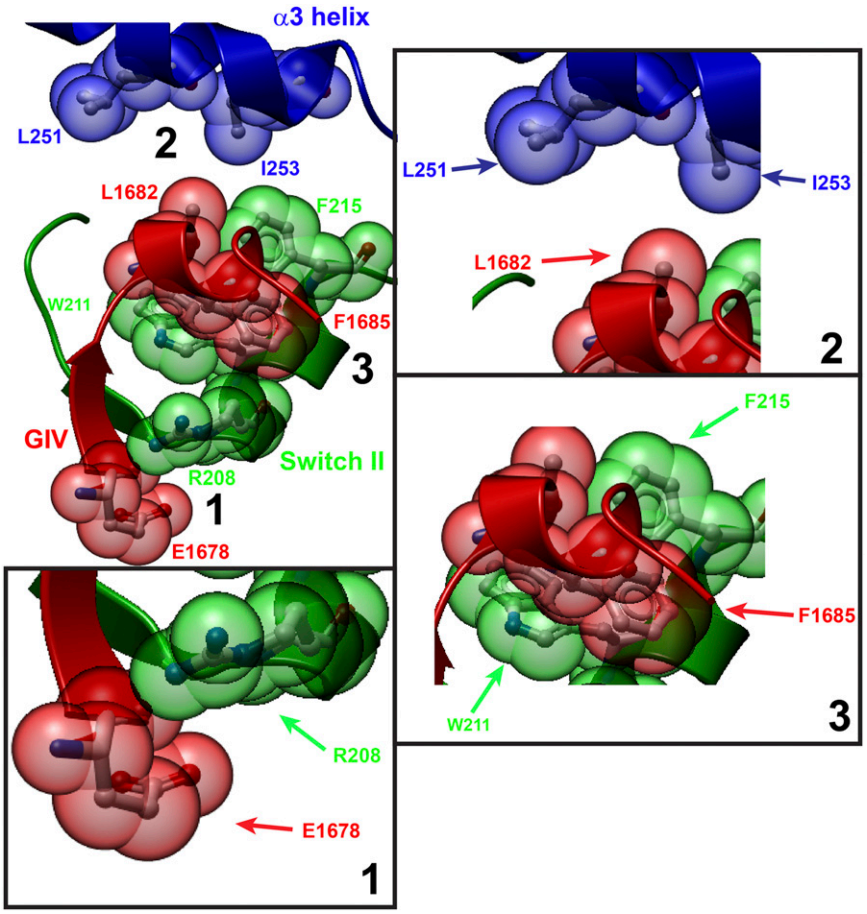

Fig. S1. Detailed view of critical amino acid contacts between GIV and Gαi3 predicted by homology modeling. Enlargement of the homology model shown in Fig. 1*A*. The three relevant structural features of the GIV–Gαi3 interface described in Fig. 1*A* are shown at higher magnification (boxes labeled 1, 2, and 3).

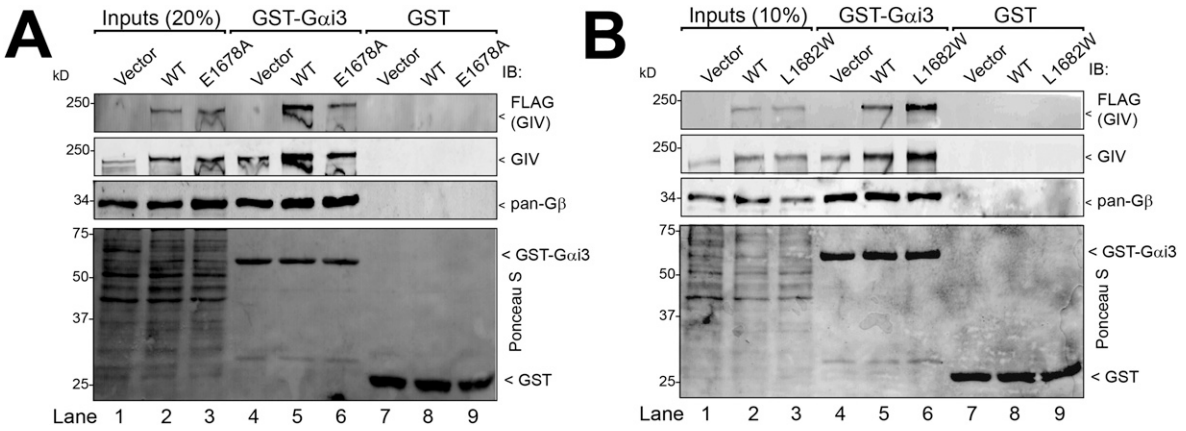

Fig. S2. Effect of E1678A and L1682W mutations on binding of GIV to Gαi3. (*A*) COS-7 cells were transfected with empty vector (lanes 1, 4, and 7) or plasmids encoding GIV-3xFLAG WT (lanes 2, 5, and 8) or GIV-3xFLAG E1678A (lanes 3, 6, and 9). Forty-eight hours after transfection, cells were harvested and lysates were used for pull-down assays with GST-Gαi3 (lanes 4–6) or GST (lanes 7–9) as described in Fig. 1*B*. The full-length GIV-3xFLAG E1678A (lane 6) mutant has impaired binding (~50%) to GST-Gαi3 compared with full-length GIV-3xFLAG WT (lane 5). Inputs: 10% of the lysates from COS-7 cells transfected with vector (lane 1), GIV-3xFLAG WT (lane 2), or GIV-3xFLAG E1678A (lane 3). (*Top two panels*) GIV-3xFLAG WT (lane 2) and GIV-3xFLAG E1678A (lane 3) are expressed at similar levels (approximately two- to threefold) above the endogenous GIV protein level (lane 1, vector-transfected), but GIV-3xFLAG E1678A (lane 6) binds to GST-Gαi3 to a lesser extent than GIV-3xFLAG WT (lane 5). No binding to GST is detected (lanes 7–9). (*Middle*) Gβ binding to GST-Gαi3 is equal for all of the different COS-7 lysates (lane 4–6). No binding to GST is detected (lanes 7–9). (*Bottom*) Ponceau S staining confirming the equal loading of GST proteins. One experiment representative of three is shown. (*B*) Full-length GIV-3xFLAG L1682W (lane 6) mutant has increased binding (approximately twofold) to GST-Gαi3 compared with full-length GIV-3xFLAG WT (lane 5). COS-7 cells were transfected and processed exactly as described in *A*. (*Top two panels*) GIV-3xFLAG WT (lane 2) and GIV-3xFLAG L1682W (lane 3) are expressed at similar levels (approximately two- to threefold) above the endogenous GIV protein level (lane 1, vector-transfected), but GIV-3xFLAG L1682W (lane 6) binds to GST-Gαi3 to a greater extent than GIV-3xFLAG WT (lane 5). No binding to GST is detected (lanes 7–9). (*Middle*) Gβ binding to GST-Gαi3 is equal for all of the COS-7 lysates (lane 4–6). No binding to GST is detected (lanes 7–9). (*Bottom*) Ponceau S staining confirming equal loading of GST proteins. One experiment representative of three is shown.

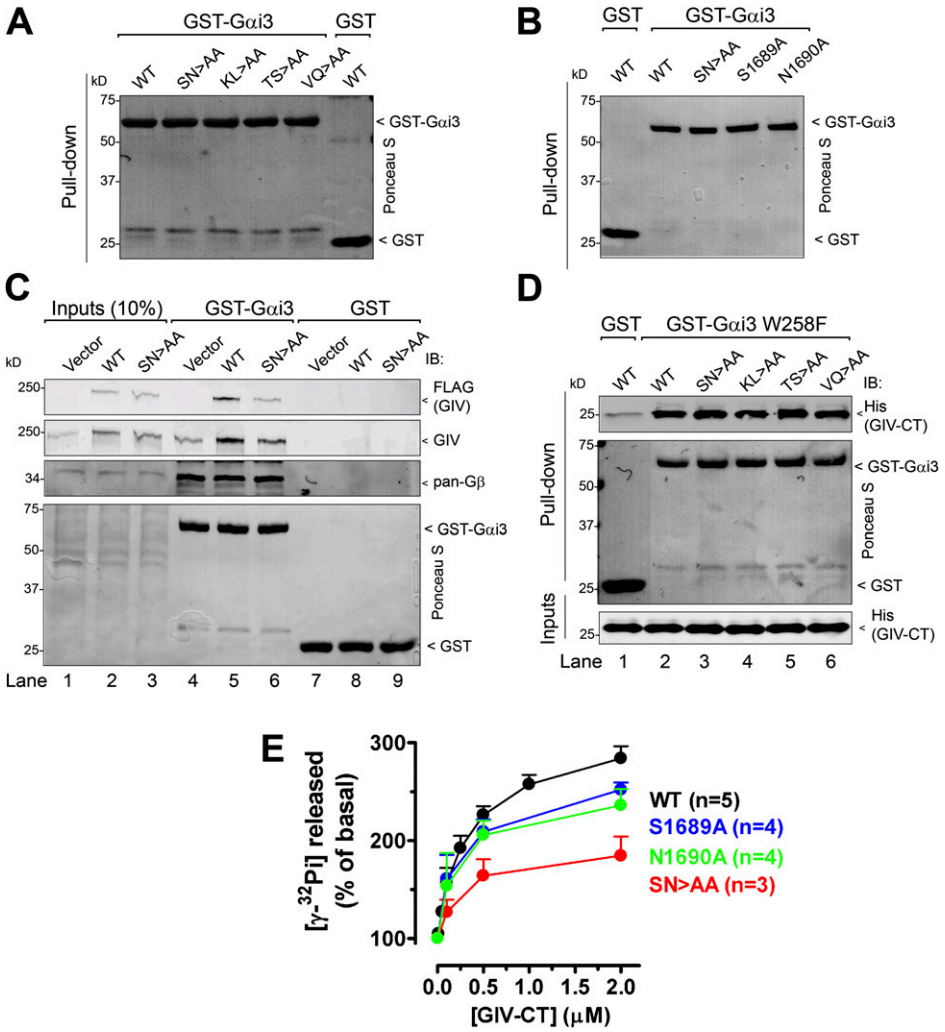

Fig. S3. Effect of S1689 and N1690 GIV mutations on Gαi3 binding and activation. (*A* and *B*) Equal loading of GST proteins for Fig. 2*B* (*A*) and Fig. 2*C* (*B*) was confirmed by Ponceau S staining. (*C*) COS-7 cells were transfected with empty vector (lanes 1, 4, and 7) or plasmids encoding GIV-3xFLAG WT (lanes 2, 5, and 8) or GIV-3xFLAG SN>AA (lanes 3, 6, and 9). Forty-eight hours after transfection, cells were harvested and lysates were used for pull-down assays with GST-Gαi3 (lanes 4–6) or GST (lanes 7–9) as described for Fig. 1*B*. Full-length GIV-3xFLAG S1689A/N1690A (SN>AA, lane 6) has impaired binding to GST-Gαi3 compared with full-length GIV-3xFLAG WT (lane 5). Inputs: 10% of the lysates from COS-7 cells transfected with vector (lane 1), GIV-3xFLAG WT (lane 2), or GIV-3xFLAG SN>AA (lane 3). (*Top two panels*) GIV-3xFLAG WT (lane 2) and GIV-3xFLAG SN-AA (lane 3) are expressed at similar levels above the endogenous GIV protein level (lane 1, vector-transfected), but GIV-3xFLAG SN-AA (lane 6) shows less binding to GST-Gαi3 (~60% reduction) than GIV-3xFLAG WT (lane 5). No binding to GST is detected (lanes 7–9). (*Middle*) Gβ binding to GST-Gαi3 in GIV-transfected cells (lanes 5 and 6) is similar to controls (lane 6). No binding to GST is detected (lanes 7–9). (*Bottom*) Ponceau S staining confirming the equal loading of GST proteins. One experiment representative of three is shown. (*D*) His-GIV-CT WT (lane 2) and the S1689A/N1690A (SN>AA, lane 3), K1691A/L1692A (KL>AA, lane 4), T1693A/S1694A (TS>AA, lane 5), or V1695A/Q1696A (VQ>AA, lane 6) mutants bind equally to GST-Gαi3 W258F. Binding of His-GIV-CT WT to GST is almost negligible (lane 1). His-GIV-CT WT (12 μg) or the indicated mutants were incubated with 10 μg GST or GST-Gαi3 W258F preloaded with GDP immobilized on glutathione beads and analyzed as described in *A*. Note that, to overcome the limitation of the impaired binding of Gαi3 W258F to GIV (2), the concentrations of His-GIV-CT and GST-Gαi3 are increased 10- and 2-fold, respectively, over those used in similar experiments shown in other figures (e.g., Figs. 1*B* and 2 *B* and *C*). One experiment representative of three is shown. (*E*) Activation of His-Gαi3 by His-GIV-CT is only marginally decreased (~10–15%) by the S1689A (blue) or N1690A (green) mutations compared with His-GIV-CT WT (black) whereas the double-mutant S1689A/N1690A (SN>AA, red) shows a more marked decrease (~50%). The steady-state GTPase activity of purified His-Gαi3 (50 nM) was determined in the presence of 0, 0.05, 0.1, 0.25, 0.5, 1, and 2 μM purified His-GIV-CT WT or 0.25, 0.5, and 2 μM of the indicated mutants by quantification of the amount of [γ-^{32}P]GTP (0.5 μM, ~50 cpm/fmol) hydrolyzed in 10 min. Data are expressed as the percentage of GTP hydrolyzed by the G protein alone (0.11 ± 0.02 mol GTP/mol Gαi3 in 10 min). Results are shown as mean ± SEM of the indicated number (*n*) of experiments.

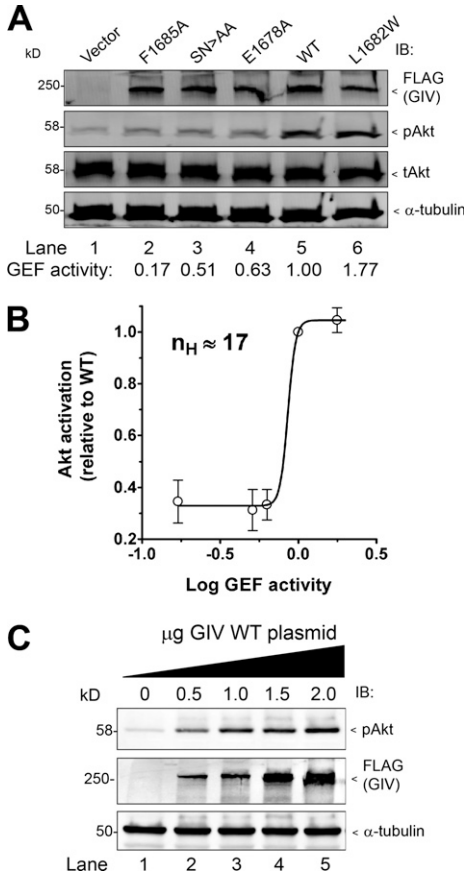

Fig. S4. Activation of Akt by different GIV GEF motif mutants and different amounts of GIV expressed in COS7 cells. (*A*) Expression of GIV-3xFLAG WT (lane 5) or L1682W (lane 6) increased the levels of pAkt ~5× compared with vector-transfected controls (lane 1); GIV-3xFLAG F1685A (lane 2), SN>AA (lane 3), and E1678A (lane 4) promoted only a marginal increase (~1.3×). COS-7 cells were transfected with empty vector (lane 1), GIV-3xFLAG F1685A (lane 2), SN>AA (lane 3), E1678A (lane 4), WT (lane 5), or L1682W (lane 6) and maintained in DMEM media with 10% FBS. Approximately 32 hours after transfection, the cells were switched to DMEM media supplemented with 2% FBS and cultured overnight. Samples were immunoblotted (IB) for FLAG (GIV), S473 phospho-Akt (pAkt), total Akt (tAkt), and α-tubulin. The in vitro GEF activity of each mutant (Table 1) is indicated below the corresponding lanes for reference. One experiment representative of 3 is shown. (*B*) Akt activation is highly sensitive to changes in GIV's GEF activity. Akt activation (*y* axis) for the different mutants investigated in *A* was quantified by infrared immunoblotting as described in *Experimental Procedures* and plotted as a function of GEF activity corresponding to each mutant (*x* axis). The data were fitted to the four-parameter logistic equation using Prism 4.0, resulting in an n_H value of 17. Results are shown as the average ± SEM of three independent experiments. (*C*) Expression of increasing amounts of GIV-3xFLAG WT (from 0.5 to 2 μg DNA transfected/condition) results in a gradual increase of pAkt (from ~4 to ~12×, lanes 2–5) compared with vector-transfected controls (lane 1). COS-7 cells were transfected with the indicated amounts of GIV-3xFLAG plasmid supplemented with empty vector to equalize the total amount of DNA to 2 μg/condition and maintained under the same conditions as in *A*. One experiment representative of three is shown.