GIV/Girdin (Gα-interacting, Vesicle-associated Protein/Girdin) Creates a Positive Feedback Loop That Potentiates Outside-in Integrin Signaling in Cancer Cells*

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Activation of the tyrosine kinase focal adhesion kinase (FAK) upon cell stimulation by the extracellular matrix initiates integrin outside-in signaling. FAK is directly recruited to active integrins, which enhances its kinase activity and triggers downstream signaling like activation of PI3K. We recently described that Gα-interacting, vesicle-associated protein (GIV), a protein up-regulated in metastatic cancers, is also required for outside-in integrin signaling. More specifically, we found that GIV is a non-receptor guanine nucleotide exchange factor that activates trimeric G proteins in response to integrin stimulation to enhance PI3K signaling and tumor cell migration. In contrast, previous reports have established that GIV is involved in phosphotyrosine (Tyr(P))-based signaling in response to growth factor stimulation; i.e. GIV phosphorylation at Tyr-1764 and Tyr-1798 recruits and activates PI3K. Here we show that phosphorylation of GIV at Tyr-1764/Tyr-1798 is also required to enhance PI3K-Akt signaling and tumor cell migration in response to integrin stimulation, indicating that GIV functions in Tyr(P)-dependent integrin signaling. Unexpectedly, we found that activation of FAK, an upstream component of the integrin Tyr(P) signaling cascade, was diminished in GIV-depleted cells, suggesting that GIV is required to establish a positive feedback loop that enhances integrin-FAK signaling. Mechanistically, we demonstrate that this feedback activation of FAK depends on both guanine nucleotide exchange factor and Tyr(P) GIV signaling as well as on their convergence point, PI3K. Taken together, our results provide novel mechanistic insights into how GIV promotes proteovascular cancer cell behavior by working as a signal-amplifying platform at the crossroads of trimeric G protein and Tyr(P) signaling.

Integrins are heterodimeric (αβ) receptors that directly bind extracellular matrix (ECM)2 components and trigger cell responses such as migration, proliferation, survival, and differentiation among others (1–4). Prior to attachment to the ECM, integrins must adopt an active conformation with high binding affinity for substrates. This process is frequently regulated by the so-called “inside-out” integrin signaling, a process by which integrins sense signals from the interior of the cell that enhance their adhesive properties (5, 6). After ECM binding, integrins cluster in focal adhesions and trigger “outside-in” signaling by recruiting intracellular proteins (2, 3, 7, 8). Recruitment of focal adhesion kinase (FAK) is one of the earliest events in this signaling cascade (7, 8). FAK is a tyrosine kinase, and direct binding to the cytoplasmic tail of active integrins induces a conformational change that facilitates its autophosphorylation at Tyr-397. This creates a binding site for Src, which further phosphorylates FAK to promote its maximal catalytic activity. The active FAK-Src module works coordinately to trigger phosphotyrosine-dependent integrin signaling and alter cell behavior. One of the most prominent downstream targets is PI3K-Akt, which is activated by recruitment of the p85 subunit of PI3K to phosphorylated Tyr-397 of FAK (8–10).

In addition to these canonical signaling mechanisms, outside-in integrin signaling also intertwines with other signaling pathways. For example, the cross-talk between integrins and receptor tyrosine kinases (RTKs) has been studied for decades and is now well established (4, 11–13). More recently, it has been shown that trimeric G proteins, which are traditionally activated by G protein-coupled receptors (GPCRs), also participate in integrin outside-in signaling. Du and co-workers (14–16) have shown that active Gα13 directly binds to an EXE motif in the intracellular tail of different integrin β subunits upon ligand binding and is required for activation of downstream signaling. More recently, we have reported that GPCR-independent activation of a G protein of a different class, Gα, also enhances integrin outside-in signaling (17). Activation of Gα in this context is achieved by Gα-interacting, vesicle-associated protein (GIV; also known as Girdin or KIAA1212), a non-receptor guanine nucleotide exchange factor (GEF) that binds to active integrins. GIV subsequently recruits and activates Gα, leading to the enhancement of downstream signaling like PI3K-Akt or small GTPases (17).

GIV is a multifunctional protein involved in cancer, angiogenesis, fibrosis, and neurodevelopment among others (18, 19). The best characterized cellular function of GIV is as an

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2 The abbreviations used are: ECM, extracellular matrix; GIV, Gα-interacting, vesicle-associated protein; FAK, focal adhesion kinase; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; GIVcc, GIV coiled coil domain; GIVct, C terminus of GIV; DMSO, dimethyl sulfoxide; pFAK, phospho-FAK; SH2, Src homology 2; YF, Y1764F/Y1798F; FA, F1685A; IP, immunoprecipitation.
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enhancer of cell migration, which underlies its role as a prometastatic protein in cancer (20–23). Previous work has established that GIV is overexpressed during cancer metastasis (24–31) and promotes PI3K-Akt signaling downstream of different classes of receptors such as GPCRs and RTKs (19, 24, 25, 32). Our recent work on integrin signaling (17) has expanded the repertoire of receptors that utilize GIV as a signaling platform to enhance PI3K signaling and promote tumor cell invasiveness. These findings indicate that GIV enhances prometastatic tumor cell behavior not only in response to soluble factors (i.e. acting on GPCRs and RTKs) but also in response to the ECM. Mechanistically, these prometastatic functions of GIV have been linked to its ability to bind and activate trimeric G proteins (18). GIV belongs to an emerging group of atypical G protein activators called non-receptor GEFs (33–38), which mimic the action of GPCRs but are cytoplasmic factors instead of transmembrane receptors. The GEF activity of GIV is associated with a defined Gα-binding and -activating motif of ~30 amino acids located in its C-terminal region (21, 23) (Fig. 1), and disabling the GEF activity of this motif by site-directed mutagenesis inhibits PI3K activation downstream of GPCRs, RTKs, and integrins (17, 18). The signaling pathway underlying this mechanism appears to be conserved in the context of both soluble factors and ECM stimulation, which involves activation of PI3K by free Gβγ subunits released from Gα proteins upon activation by GIV.

However, it has been recently reported that GIV can also enhance PI3K activation via an alternative mechanism (39). GIV can be directly phosphorylated at two tyrosines (Tyr-1764/Tyr-1798) by both receptor (e.g. EGF receptor) and non-receptor (e.g. Src) tyrosine kinases (Fig. 1). In turn, these phosphorylation sites serve as a docking site for the p85 subunit of PI3K, which results in enhancement of the activity of the p110 catalytic subunit. Importantly, it was shown that GEF- and phosphorytrosine (Tyr(P))-dependent GIV signaling mechanisms worked independently to activate PI3K (39). Moreover, blocking either GIV phosphorylation at Tyr-1764/Tyr-1798 or the GEF activity of GIV separately results in a dramatic reduction of PI3K activation, indicating that both functions are required simultaneously to achieve enhancement of PI3K signaling (39, 40).

Previous work on Tyr(P)-dependent GIV mechanisms was carried out in the context of GPCR and RTK signaling (39, 40) (Fig. 1). Because integrin signaling relies heavily on Tyr(P)-dependent mechanisms and we have recently identified a role for GIV in integrin signaling, we set out to investigate a possible role of GIV in the Tyr(P)-dependent integrin signaling network (Fig. 1). Here we describe how GIV phosphorylation at Tyr-1764/Tyr-1798 works in conjunction with its GEF activity in the context of integrin outside-in signaling to enhance PI3K signaling and tumor cell migration and how, unexpectedly, this sets a positive feedback loop that enhances the activation of FAK.

Experimental Procedures

Reagents and Antibodies—Unless otherwise indicated, all chemical reagents were obtained from Sigma or Fisher Scientific. Escherichia coli DH5α strain was purchased from New England Biolabs. PfuUltra DNA polymerase was from Agilent. Rat tail collagen I (catalog number 354236) was purchased from BD Biosciences. LY294002 (catalog number L9908), poly(r-I)-lysine (P-8920), and PP2 (P-0042) were obtained from Sigma. Gallein and fluorescein were purchased from TCI Chemicals. GTPS (GTPγS) and PP1 (P-0350) were purchased from Sigma-Aldrich. Polyethylene glycol 8000 (P-6545) and Ultrasonicator (64–477) (clone D9E) were purchased from Millipore. Generation of recombinant GIV constructs was performed using the pIRE1 mammalian expression vector purchased from Clontech. Generation of human integrin αvβ3 (clone 4E9) and αvβ5 (clone 4F10) were obtained from Millipore. Generation of human integrin αvβ3 (clone 4E9) and αvβ5 (clone 4F10) were purchased from Santa Cruz Biotechnology.

Mouse monoclonal antibodies raised against α-tubulin (T6074) was obtained from Sigma. Mouse monoclonal antibodies raised against active conformation of β1 integrin (HUTS-4, catalog number MAB20792) or phosphorytrosine (clone 4G10) were purchased from Millipore. Generation of rabbit serum against GIV coiled coil domain (GIVcc) was described previously (41). Rabbit polyclonal antibodies raised against an epitope in the C terminus of GIV (GIVct; T-13) and mouse monoclonal antibodies raised against total Akt (B-1) and total Src (SRC2) were purchased from Santa Cruz Biotechnology. Rabbit antibodies raised against phospho-FAK (Tyr-397) (clone D20B1), total FAK (clone D22R2E), phospho-Akt (Ser-473) (clone D9E), and total β1 integrin (D2E5) were obtained from Cell Signaling Technology. Rabbit antibody raised against phospho-Src (Tyr-418) (catalog number 44660G) was purchased from Cell Signaling Technology. Goat anti-rabbit and goat anti-mouse Alexa Fluor 680 and IRDye 800 F(ab’)2 used as secondary antibodies for immunoblotting were from Invitrogen and LI-COR Biosciences, respectively. Goat anti-mouse Alexa Fluor 488-coupled antibody was from Invitrogen.
Plasmids—All plasmids used in this work have been described previously (17) except the plasmid encoding for GST-C-SH2-p85α (a gift from Dr. Rajala, University of Oklahoma) (42). Briefly, two independent shRNA sequences against GIV (GIV shRNA1, GAAGGAGGGCAACTGGAT; GIV shRNA2, AAGAAGGCTTAGCCAGGAATT) or a control shRNA were cloned into the lentiviral plasmid pLKO.1-puro. GIV was cloned into the lentiviral plasmids pLVX-puro and pLENTI-Blast with a C-terminal 2xMyc tag and bearing silent mutations to make it insensitive to the RNAi sequences described above.

Cell Culture, Transfection, and Generation of Stable Cell Lines—MDA-MB-231, MCF-7, and COLO-357FG cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% l-glutamine (37 °C, 5% CO2). Endogenous and ectopic GIV expression was manipulated using lentiviral transduction followed by selection with the appropriate antibiotics exactly as described previously (17). Briefly, MDA-MB-231 cells stably depleted of GIV were generated by transduction with HEK293FT-packaged lentiviral particles and selection with puromycin. Stable GIV depletion in MDA-MB-231 cell lines by two independent RNAi sequences (GIV shRNA1, ~65% depletion; GIV shRNA2, ~95% depletion) has been validated before (17). For the phenotype rescue experiments, MDA-MB-231 GIV shRNA2 cells were transduced with lentivirus packaged from pLENTI-GIV-2xmyc plasmids, subjected to double puromycin/blasticidin selection for 4–6 days, and used immediately for experiments. MCF-7 cells stably expressing Myc-tagged GIV constructs were generated by transduction with lentivirus packaged from pLENTI-GIV-2xmyc plasmids followed by puromycin selection.

Cell Stimulation by Attachment to Immobilized Collagen I and Other Treatments—Collagen I stimulation was performed exactly as described previously (17). This assay protocol is designed to monitor specifically the response of cells to components of the ECM (in the absence of any other stimuli like soluble growth factors), and we have previously validated (17) that collagen I is the most potent ligand among a battery of purified ECM components for the cells studied here. We have also established that the Akt activation and cell migration responses of these cells to collagen I are β1 integrin-dependent (17). Briefly, tissue culture dishes (p6) and/or glass coverslips were coated with collagen I dissolved in 0.02N acetic acid (1.6 μg/cm2) overnight at 4 °C and washed twice with PBS right before the experiments. In some cases, coverslips were coated with poly-L-lysine by incubation for 5 min at room temperature (5 μg/cm2) followed by PBS washes and drying for 15 min right before use. Cells at 90% confluence were washed with PBS and detached by incubation in PBS supplemented with 20 mM EDTA (pH 7.4) for ~6 min at 37 °C. Cells were washed three times with 10 ml of serum-free DMEM by cycles of centrifugation (450 × g, 2 min) and resuspension. Washed cells at a final concentration of 3 × 105 cells/ml were maintained in suspension in serum-free medium for 1 h at room temperature and subsequently seeded on collagen I-coated plastic dishes and/or glass coverslips (6 × 105 and 1.8 × 106 cells for p6 and p10 dishes, respectively) at 37 °C. Stimulations were stopped at different time points (typically 15, 30, and 60 min) by washing with cold PBS and either freezing the plastic dishes at −20 °C (for subsequent immunoblotting analyses) or fixing the coverslips in 3% paraformaldehyde for 30 min (for subsequent fluorescence microscopy analyses). The time 0 min of the immunoblotting analyses corresponds to an aliquot of the cells in suspension that was pelleted and frozen at −20 °C right before seeding. Experiments for the results shown in Fig. 6C were done the same except that cells were cultured in poly-L-lysine-coated dish (5 μg/cm2) and serum-starved overnight before detachment and collagen I stimulation.

For experiments using the chemicals LY294002, gallein, or fluoroscein, a 10 μM concentration of each compound (or the equivalent volume of DMSO) was added to the medium during the 1-h incubation of the cells in suspension and maintained in the medium during the adhesion to collagen I. For experiments with integrin-blocking antibodies, the β1 integrin-blocking antibody P5D2 (10 μg/ml) was added to cells in suspension for 30 min at 37 °C with constant tumbling before seeding, and the same concentration of antibody was maintained in the medium during the adhesion to collagen I.

Cell Lysis and Quantitative Immunoblotting—Cells frozen at −20 °C on coated tissue culture dishes (as described above) were harvested on ice with lysis buffer (20 mM Hepes (pH 7.2), 5 mM Mg(CH2COO)2, 125 mM K(CH2COO), 0.4% Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate, 0.5 mM Na2VO4 supplemented with a protease inhibitor mixture (Sigma, catalog number S8830)) and cleared (14,000 × g, 10 min) before use. Proteins were quantified by Bradford assay (Bio-Rad), and samples were boiled in Laemmli sample buffer for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with PBS supplemented with 5% nonfat milk or BSA (for antibodies against phosphorylated proteins) before sequential incubation with primary and secondary antibodies. Infrared imaging and quantification of immunoblots were performed according to the manufacturer’s protocols using an Odyssey Infrared Imaging System (LI-COR Biosciences). Data quantified from immunoblots was normalized by a loading control: α-tubulin, 1:2,500; G-actin, 1:250; i3, 1:250; panGIV, 1:1,000; total GIV, 1:2,500; pAkt (Ser-473), 1:1,000; total pAkt (Thr-308), 1:2,500; total pFAK, 1:1,000; total pSrc (Tyr-418), 1:2,500; total Src, 1:1,000; Myc, 1:1,000; total integrin β1, 1:250; and α-tubulin, 1:2,500. All Odyssey images were processed using ImageJ software (National Institutes of Health) and assembled for presentation using Photoshop and Illustrator software (Adobe).

Haptotaxis Assay—This assay was performed using a modified Boyden chamber assay exactly as described previously (17). Briefly, the bottom side of the membrane filters was coated.
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overnight at 4 °C with collagen I, washed twice with PBS, and then blocked with 2% (w/v) BSA for 2 h at 37 °C. Cells were prepared exactly as described under “Cell Stimulation by Attachment to Immobilized Collagen I and Other Treatments.”

The upper chamber was filled with serum-free medium containing 5 × 10^5 MDA-MB-231 cells or 1 × 10^5 MCF-7 cells (300 μl total), whereas the lower chamber was filled with 500 μl of serum-free medium. MDA-MB-231 cells were incubated for 5 h, and MCF-7 cells were incubated for 24 h at 37 °C. Filters were fixed in 3% paraformaldehyde and stained with crystal violet. Cells on the bottom side of the filters were counted for five random fields of two replicates per condition and averaged. The results are presented as the average number of migrating cells in three or more independent experiments. Previously described controls (17) validate that this cell migration is collagen I- and β1 integrin-dependent in the cells utilized in this study.

Immunoprecipitations and Pulldowns—MDA-MB-231 cells were treated essentially as described above under “Cell Stimulation by Attachment to Immobilized Collagen I and Other Treatments.” MDA-MB-231 cells were maintained in suspension or plated for different time points (typically 30 and/or 60 min) on tissue culture dishes coated with collagen I.

Immunoprecipitation of GIV to determine its tyrosine phosphorylation status was carried out following protocols described previously (17).Briefly, cell lysates (~1–2 mg) were prepared in immunoprecipitation (IP) buffer 1 (20 mM Hepes (pH 7.2), 5 mM Mg(CH₃COO), 125 mM K(CH₃COO), 0.4% Triton X-100, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM activated Na₃VO₄ supplemented with a protease inhibitor mixture) and incubated with 3 μg of GIVct rabbit antibody (T-13) or rabbit IgG control (sc-2027, Santa Cruz Biotechnology) for 4 h at 4 °C with tumbling. Protein A-agarose beads (Life Technologies, catalog number 101041) (preblocked with 5% BSA for 2 h at room temperature) were added to the samples and incubated for 1 h at 4 °C. After 3 washes (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 5 mM EDTA, 10 mM MgCl₂, 1 mM DTT, 1 mM activated Na₃VO₄), immunoprecipitated proteins were eluted by addition of Laemmli sample buffer and boiling. For experiments assessing the effect of the Src inhibitor PP2, cells were treated as described above except that PP2 (10 μM or an equivalent volume of DMSO) and 0.1 mM Na₃VO₄ were added to the cells during the 1-h incubation in suspension and after seeding.

GST-p85α-C-SH2 purification and pulldowns were carried out following protocols described previously (17). Cells were stimulated with collagen I for 30 min as described above except 0.1 mM Na₃VO₄ was added to the cells during the 1-h incubation in suspension and after seeding. Cell lysates (~2 mg) were prepared in IP buffer 1 and incubated with 50 μg of GST-p85α-C-SH2 or GST immobilized on glutathione-agarose beads for 1 h at 4 °C with tumbling. After three washes (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween 20, 5 mM EDTA; 10 mM MgCl₂, 1 mM DTT, 1 mM activated Na₃VO₄), immunoprecipitated proteins were eluted by addition of Laemmli sample buffer and boiling.

Immunoprecipitation of active β1 integrins was carried out following protocols described previously (43). Briefly, cell lysates (~1–2 mg) were prepared in IP buffer 2 (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 3 mM MgCl₂, 1% Triton X-100, 10% glycerol, 1 mM Na₃VO₄ supplemented with a protease inhibitor mixture) and incubated with 2 μg of the active β1 integrin mouse antibody (HUTS-4) or mouse IgG control (Santa Cruz Biotechnology) for 180 min at 4 °C with tumbling. Protein G-agarose beads (Thermo Scientific) (preblocked with 5% BSA for 2 h at room temperature) were added to the samples and incubated for 1 h at 4 °C. After three washes with IP buffer 2, immunoprecipitated proteins were eluted by addition of Laemmli sample buffer and boiling.

Statistical Analysis—Each experiment was performed at least three times. Data shown are expressed as mean ± S.E. or as one representative result of each biological replicate. Statistical significance between various conditions was assessed with the Student’s t test. p < 0.05 was considered significant.

Results

GIV Phosphorylation at Tyrosines 1764/1798 Facilitates Integrin-dependent PI3K-Akt Signaling—We have recently reported that GIV is required to facilitate integrin-dependent PI3K-Akt signaling (17). Others have shown that tyrosine phosphorylation of GIV enhances PI3K-Akt signaling in response to non-integrin stimuli (i.e. upon RTK and GPCR activation) (39, 40). It has also been reported that tyrosine phosphorylated GIV concentrates in focal adhesions (44, 45), the site where active integrins cluster and signal. These previous findings prompted us to investigate whether GIV is tyrosine phosphorylated upon integrin stimulation and, if so, whether such phosphorylation enhances downstream activation of PI3K-Akt signaling. For this, we used a previously validated (17) experimental protocol that allows specific assessment of cell responses to ECM stimulation in the absence of any other stimuli like soluble growth factors (Fig. 2A). Briefly, cells are serum-starved in suspension and acutely seeded on collagen I-coated plates in serum-free medium. Metastatic breast cancer MDA-MB-231 cells were stimulated with collagen I as described and lysed at different times, and GIV tyrosine phosphorylation was determined by Tyr(P) immunoblotting after GIV immunoprecipitation (Fig. 2B). We found that GIV Tyr(P) levels were increased ~2-fold in collagen I-stimulated cells compared with unstimulated controls. Next we investigated whether this phosphorylation was Src-dependent. Src (in coordination with FAK) initiates most, if not all, of integrin signaling (7, 8), and it has been reported previously that Src directly phosphorylates GIV in vitro and in cells in response to RTK or GPCR stimulation (39). We found that inhibition of Src with the inhibitor PP2 also reduces GIV tyrosine phosphorylation in response to integrin stimulation by collagen I (Fig. 2C). To further characterize GIV tyrosine phosphorylation in response to collagen I stimulation, we investigated its ability to bind PI3K. It has been shown previously that GIV Tyr(P)-1764 and Tyr(P)-1798, which are phosphorylated in collagen I-stimulated cells compared with unstimulated controls (Fig. 2D). Taken together, these results indicate that GIV is tyrosine phosphory-
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A

Experimental workflow to analyze integrin "outside-in" signaling

Cells are detached with 20mM EDTA

Cells are serum-starved for 1 hour in suspension

Stimulation by cell seeding on collagen I in serum-free media

Phenotypic analysis at different time points

B

FIGURE 2. GIV is tyrosine phosphorylated upon integrin stimulation via Src. A, experimental workflow to monitor ECM-specific integrin outside-in signaling. Cells were lifted, kept in suspension for 1 h in serum-free medium, and seeded on surfaces coated with collagen I in serum-free medium. Cells were harvested at different time points after seeding for subsequent analyses. Under these conditions, the only stimulus for the cells is mediated through attachment to collagen I. B, GIV is tyrosine phosphorylated in response to collagen I in MDA-MB-231 cells. MDA-MB-231 control cells were serum-starved in suspension and stimulated for the indicated times with collagen I as described in A. Cell lysates were subjected to IP with GIVct antibodies or a control IgG followed by Tyr(P) immunoblotting as described under "Experimental Procedures." IPs and lysate aliquots were immunoblotted (IB) with the indicated antibodies. Top and middle panels, immunoblots of one representative experiment. Bottom panel, quantification of GIV tyrosine phosphorylation expressed as average with error bars representing S.E. (n = 3; *, p < 0.05; ***, p < 0.001 compared with t = 0). C, inhibition of Src diminishes GIV tyrosine phosphorylation upon collagen I stimulation in MDA-MB-231 cells. MDA-MB-231 control cells were serum-starved in suspension for 1 h in the presence of the Src inhibitor PP2 (+) or in an equivalent volume of DMSO (−) and stimulated with collagen I for 30 min as described in A. Cell lysates were subjected to IP followed by IB as in (B). Top and middle panels, immunoblots of one representative experiment. Bottom panel, quantification of GIV tyrosine phosphorylation expressed as average with error bars representing S.E. (***, p < 0.001 compared with cells treated with DMSO). D, collagen I-stimulation enhances GIV binding to the C-SH2 domain of p85α. MDA-MB-231 control cells were stimulated with collagen I for 30 min as described in A. Cell lysates were incubated with GST-C-SH2-p85α or GST immobilized on glutathione-agarose as described under "Experimental Procedures." Resin-bound proteins were immunoblotted with the indicated antibodies. Top and middle panels, immunoblots of one representative experiment. Bottom panel, quantification of GIV binding expressed as average with error bars representing S.E. (*, p < 0.05 compared with t = 0). Ctrl, control; Col, collagen.

Next, we investigated whether GIV tyrosine phosphorylation plays a role in modulating PI3K-Akt signaling in response to integrin stimulation by generating a non-phosphorylatable GIV mutant. Although we did not pinpoint the specific tyrosines phosphorylated in GIV after integrin stimulation due to a lack of commercial antibodies for this purpose, we focused on Tyr-1764 and Tyr-1798 for several reasons. (i) These are the two most frequently phosphorylated tyrosines in GIV according to curated phosphoproteomics data (46). (ii) GIV phosphorylated at either Tyr-1764 (45) or Tyr-1798 (44) accumulates in focal adhesions, which are sites of active integrin signaling. (iii) It has been described previously that when these two sites are phosphorylated they bind and activate PI3K (39), and we have found that GIV tyrosine phosphorylated in response to integrin stimulation becomes competent for PI3K binding (Fig. 2D). (iv) Tyr-1764 and Tyr-1798 are direct substrates of Src (39), and we have found that GIV is phosphorylated in a Src-dependent manner upon integrin stimulation (Fig. 2C). (v) We reported previously that GIV-dependent enhancement of PI3K-Akt signaling is blocked by inhibition of Src (17). We generated GIV Y1764F/Y1798F (YF), a non-phosphorylatable mutant that has been validated previously to preclude PI3K binding and subsequent activation (39). We engineered metastatic MDA-MB-231 cells to express exclusively GIV YF mutant and analyzed PI3K-Akt activation after integrin stimulation by immunoblotting for Akt phosphorylation at Ser-473 (pAkt). For this, we depleted endogenous GIV from MDA-MB-231 cells using RNAi (previously validated GIV shRNA2; ∼95% efficiency (17)) followed by ectopic expression of RNAi-resistant GIV WT or GIV YF mutant. For comparison, we also expressed GIV F1685A (FA), a mutant that disrupts the GEF activity of GIV (21, 23) and blunts Akt activation in response to integrin stimulation (17). Importantly, it has been validated previously (39) that the FA and YF mutants affect specifically GEF activity and tyrosine phosphorylation, respectively, and that these two functions of GIV function independently of each other. Consistent with an earlier report (17), we found that GIV depletion impaired Akt activation in response to collagen I stimulation (~70% reduction compared with controls) and that this defect can be partially rescued by expression of GIV WT but not the GEF-deficient GIV FA mutant (Fig. 3A). Moreover, we found that ectopic expression of GIV YF in GIV-depleted cells also failed to rescue the Akt activation defect (Fig. 3A), indicating that GIV tyrosine phosphorylation at Tyr-1764/Tyr-1798 is also required for efficient PI3K signaling in response to integrin stimulation.

Contrary to highly metastatic MDA-MB-231 cells, which naturally overexpress GIV, poorly metastatic breast cancer MCF-7 cells are naturally GIV-deficient (25, 32). We took advantage of the GIV-null background of MCF-7 cells to com-
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FIGURE 3. GIV phosphorylation on tyrosines 1764/1798 facilitates integrin-mediated PI3K-Akt signaling. A, GIV WT but not GIV FA or GIV YF rescues impaired Akt activation in response to collagen I in MDA-MB-231 cells depleted of endogenous GIV. MDA-MB-231 control shRNA and GIV shRNA2 cells were transduced with lentiviral particles carrying the indicated plasmids (empty vector, GIV WT, GIV FA, or GIV YF) and stimulated with collagen I for 30 min as described under "Experimental Procedures." Samples were analyzed by immunoblotting using the indicated antibodies, and Akt activation (as determined by pAkt Ser-473 levels) quantified as described under "Experimental Procedures." Top panel, immunoblots of one representative experiment. Bottom panel, quantification of Akt activation expressed as average with error bars representing S.E. (n = 3; *, p < 0.05 compared with cells expressing exogenous GIV WT). B, GIV WT but not GIV FA or GIV YF enhances Akt activation in response to collagen I in MCF-7 cells. MCF-7 cells stably expressing an empty vector (control), GIV WT, GIV FA, or GIV YF were generated as described under "Experimental Procedures," stimulated with collagen I for 30 min, and immunoblotted (IB) with the indicated antibodies. Top panel, immunoblots of one representative experiment. Bottom panel, quantification of Akt activation expressed as average with error bars representing S.E. (n = 3; *, p < 0.05; **, p < 0.01 compared with cells expressing exogenous GIV WT). Ctrl, control; Col, collagen; tAkt, total Akt.

GIV Phosphorylation at Tyrosines 1764/1798 Facilitates Integrin-dependent Cell Migration—Next, we investigated the impact of GIV Tyr(P)-1764/1798 in tumor cell migration. We reasoned that impaired integrin signaling observed in the absence of GIV tyrosine phosphorylation would also have a deleterious effect on tumor cell processes linked to metastasis like cell migration. To test this, we used a previously validated assay (17) that measures specifically collagen I-driven cell migration (i.e., haptotaxis) in the absence of any other stimuli (Fig. 4A). Expression of endogenous and ectopic GIV constructs (WT or FA/YF mutants) in MDA-MB-231 cells was manipulated as described in the previous section. GIV depletion impairs collagen I-dependent cell migration to a similar extent as PI3K inhibition under the same conditions (~50% decrease compared with controls) (Fig. 4B). As previously reported (17), this defect in cell motility is rescued by expression of GIV WT but not the GEF-deficient GIV FA mutant (Fig. 4B). Similar to the GIV FA mutant, the non-phosphorylatable GIV YF mutant failed to rescue the haptotaxis defect of GIV-depleted cells (Fig. 4B), indicating that GIV Tyr(P)-1764/1798 are required for efficient cell motility in response to integrins. We carried out analogous cell migration experiments in the naturally GIV-deficient MCF-7 cells after ectopic expression of GIV WT or GIV FA/GIV YF mutants. Although GIV WT expression enhanced collagen I-dependent cell migration (~2-fold compared with controls), both GEF-deficient GIV FA and non-phosphorylatable GIV YF failed to do so (Fig. 4C). Taken together, these results show that, much like Akt activation, integrin-dependent cell migration requires both the GEF activity of GIV toward G proteins and tyrosine phosphorylation at residues 1764/1798.
GIV Is Required for Efficient FAK Activation upon Integrin Stimulation—Tyrosine phosphorylation-dependent events play a critical role in integrin outside-in signaling. In fact, binding of the tyrosine kinase FAK to active integrins is the first event in this process (7, 8). FAK engagement to integrins facilitates its activation by autophosphorylation at Tyr-397 and subsequent phosphorylation of substrates, both of which require cooperation with Src (7, 8). Inhibition of Src blocks both GIV tyrosine phosphorylation upon integrin stimulation (Fig. 2C) and GIV-dependent integrin signaling (17), which indicates that GIV functions downstream of the FAK/Src module in the canonical integrin signaling hierarchy. To start understanding the role of GIV in integrin-dependent phosphotyrosine signaling, we measured FAK activation in GIV-depleted cells. First, we validated that FAK activation is integrin-dependent in our experimental system (MDA-MB-231 cells) by confirming that it occurs upon cell attachment to collagen I but not to the non-integrin substrate poly-L-lysine (Fig. 5A) and that it is inhibited by integrin-blocking antibodies (Fig. 5B). We reasoned that GIV depletion would not affect activation of FAK because this is an upstream event in the integrin signaling cascade, but we found that this is not the case. GIV-depleted MDA-MB-231 cells showed a marked decrease in FAK activation (as determined by phosphorylation at Tyr-397) in response to collagen I stimulation compared with controls (~50% decrease) (Fig. 6A). This decrease in FAK activation was reproduced with two independent GIV-targeting shRNA sequences (Fig. 6A), ruling out the possibility that the observed effect is due to an off-target effect of the RNAi. Src activation was also decreased in GIV-depleted MDA-MB-231 cells attaching to collagen I under the same experimental conditions (Fig. 6B). However, Src activity is abnormally high in these cells even when serum-starved in suspension, suggesting that Src activity is dysregulated in these cancer cells and complicating the interpretation of the results. For this reason, we modified the experimental conditions to re-evaluate the effect of GIV depletion on Src activation. Cells were cultured on poly-L-lysine-coated dishes and serum-starved overnight before collagen I stimulation as described in Fig. 2A. Under these conditions, Src activity in MDA-MB-231 cells in suspension was lower than before, and it was induced up to ~3-fold upon collagen I stimulation (Fig. 6C). GIV depletion inhibited collagen I-induced Src activation (Fig. 6C). These results indicate that GIV has a similar effect on FAK and Src activation, supporting the conclusion that GIV affects signaling...
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A

MDA-MB-231

Collagen I
Poly-L-lysine

IB:
pFAK
IFAK
α-tubulin

Time (min)

0 10 20 30 40 50 60

kDa

50

50

50

100

200

FIGURE 5. FAK activation in response to MDA-MB-231 cell attachment to collagen I specifically depends on integrins. A, FAK is activated upon attachment of MDA-MB-231 cells to collagen I but not to the non-integrin substrate poly-L-lysine. Control MDA-MB-231 cells were seeded on collagen I or the non-integrin substrate poly-L-lysine as described under "Experimental Procedures" and analyzed by immunoblotting (IB) with the indicated antibodies at different times after seeding. One experiment representative of three is shown. B, β1 integrin blockade inhibits FAK activation in MDA-MB-231 cells stimulated with collagen I. Control MDA-MB-231 cells were stimulated with collagen I as described under "Experimental Procedures" but in the presence of the β1 integrin-blocking antibody (Ab) P5D2 (or a control IgG). FAK activation was analyzed by immunoblotting with the indicated antibodies. A representative result is shown in the left panel, and the quantification of FAK activation was performed using the indicated antibodies from independent experiments expressed as average with error bars representing S.E. is shown in the right panel (*, p < 0.05). IFAK, total FAK.

events upstream in the integrin signaling cascade. Because activation of FAK and Src are closely intertwined and interdependent in the context of integrin signaling (7, 8), we focused our subsequent efforts on FAK activation to maintain consistent experimental conditions across the studies presented here.

To validate that the observed reduction of FAK activation upon GIV depletion is not cell type-specific, we carried out experiments in COLO-357FG (Fig. 6D), another invasive tumor cell line of different origin (pancreatic) that expresses high levels of GIV (25). We found that GIV depletion impairs FAK activation after collagen I stimulation in this cell line. In conclusion, these results demonstrate that GIV is required for efficient FAK activation in response to integrin activation in different cell types.

GIV Depletion Does Not Impair the Conformational Activation of β1 Integrins upon Collagen I Attachment—The results presented so far suggest a feedback mechanism by which GIV facilitates the activation of FAK. To further pinpoint at what level of the integrin signaling cascade GIV exerts its effect to modulate FAK, we measured the activation of integrins per se, which occurs immediately upstream of FAK. For this, we used an antibody that specifically recognizes the active conformation of β1 integrin, a subunit that is required for collagen I-dependent activation of FAK and Akt in MDA-MB-231 (Fig. 5B and Ref. 17). We compared β1 conformational activation upon collagen I stimulation in control and GIV-depleted MDA-MB-231 cells using two independent assays. First, we seeded cells on collagen I for 30 min and immunostained them for active β1 integrin (Fig. 7A). Control MDA-MD-231 cells seeded on collagen I displayed active β1 integrin staining resembling strip-shaped focal adhesions at the cell periphery (Fig. 7A). This specific pattern of staining was not observed when cells were seeded on the non-integrin substrate poly-L-lysine. The active β1 integrin staining in GIV-depleted cells was similar to that observed in controls (Fig. 7A), indicating that GIV depletion does not affect the conformational activation of integrins. As a complementary and more quantitative approach, we determined integrin activation levels after collagen I stimulation by immunoprecipitation with active β1 integrin antibodies followed by immunoblotting for total β1 integrins (Fig. 7B). We found that activation of β1 integrins was similarly increased after collagen I stimulation in control and GIV-depleted cells (Fig. 7B). Taken together, these results indicate that GIV depletion does not affect β1 integrin activation per se, which suggests that GIV exerts its action on intracellular steps of the integrin signaling cascade without perturbing the ability of integrins to adopt an active conformation and bind the ECM. These findings are in keeping with previous data (17) showing that defective integrin signaling in GIV-depleted MDA-MB-231 cells cannot be rescued by inducing integrin activation with Mn2+ and that GIV-depleted cells adhere normally to different ECM substrates.

GIV Enhances Integrin-dependent FAK Activation via PI3K—To further dissect the molecular mechanisms involved in the positive feedback regulation of FAK by GIV, we exploited the GIV-null background of MCF-7 cells. First, we investigated whether GIV expression is sufficient to enhance FAK activation in response to collagen I stimulation and found that this is the case. FAK phosphorylation at Tyr-397 in response to collagen I stimulation was enhanced 2–2.5-fold in MCF-7 cells stably expressing GIV WT compared with controls (Fig. 8A). As shown in Fig. 3 and in previous work (17), GIV plays a critical role in enhancing PI3K-dependent signaling (e.g. Akt activation). For this reason, we investigated whether the effect of GIV on FAK activation is PI3K-dependent (Fig. 8B). To this end, we treated MCF-7 control and MCF-7 GIV WT cells with the pharmacological PI3K inhibitor LY249002 prior to cell stimulation with collagen I (Fig. 8C). In MCF-7 control cells (i.e. GIV-deficient), activation of FAK was not affected by PI3K inhibition (Fig. 8C). Consistent with the results in Fig. 8A, activation of FAK in MCF-7 GIV WT cells was enhanced ~2–2.5-fold compared with MCF-7 control cells in the absence of LY249002 (Fig. 7C). Treatment of MCF-7 GIV WT cells with LY249002 reduced FAK activation in response to collagen I to levels identical to those in MCF-7 control cells (Fig. 8C). Collectively, these results indicate that GIV promotes integrin-mediated FAK activation via a PI3K-dependent positive feedback loop. It is noteworthy that PI3K inhibition in GIV-expressing cells blocks FAK activation only up to the levels of activation observed in GIV-deficient control cells, which indicates that regulation of FAK is a property specific to GIV-dependent PI3K activity.

Both GIV-mediated G Protein Activation and GIV Tyr(P)-dependent Signaling Are Required for Enhancing FAK Activation in Response to Collagen I Stimulation—Our results indicate that 1) GIV enhances FAK activation via a PI3K-dependent positive feedback loop (Fig. 8) and 2) both the GEF activity of GIV and
GIV phosphorylation at Tyr-1764/1798 are required for PI3K-dependent signaling (Fig. 3). For these reasons, we investigated whether the GEF activity of GIV and/or GIV phosphorylation at Tyr-1764/1798 are responsible for the feedback activation of FAK (Fig. 9A). We used the previously described MCF-7 cells ectopically expressing GIV WT or GIV FA/GIV YF mutants and found that neither cells expressing GEF-deficient GIV FA nor cells expressing non-phosphorylatable GIV YF were able to recapitulate the enhancement of FAK activation in response to collagen I observed in GIV WT cells (Fig. 9B). These results indicate that the GEF activity of GIV and Tyr(P)-1764/1798 are simultaneously required for the feedback regulation of FAK, which is consistent with the same requirement observed for efficient PI3K-dependent signaling (Fig. 3).
FIGURE 7. **GIV depletion does not affect the conformational activation of β1 integrins upon collagen I stimulation.** A, MDA-MB-231 control shRNA and GIV shRNA2 cells were seeded for 30 min on plates coated with collagen I or poly-L-lysine as indicated. Cells were fixed and immunostained with an antibody (HUTS-4) that recognizes the active conformation of β1 integrins. Both MDA-MB-231 control shRNA (middle) and GIV shRNA2 (right) cells show a similar staining for active β1 integrins in focal adhesions when seeded on collagen I. No focal adhesion-specific staining is observed in cells plated on the non-integrin substrate poly-L-lysine (left). B, MDA-MB-231 control shRNA cells and GIV shRNA2 cells were serum-starved in suspension and stimulated with collagen I for the indicated times as described in Fig. 2B. Cell lysates were subjected to IP with active β1 integrin antibodies (HUTS-4) or a control IgG followed by total β1 integrin immunoblotting (IB) as described under “Experimental Procedures.” Left, immunoblots of one representative experiment (IPs, top; lysate aliquots, bottom) Right, quantification of the ratio of active/total β1 integrin (n = 3; average with error bars representing S.E.; *, p < 0.05; **, p < 0.01, compared with t = 0). Ctrl, control; Col, collagen.

FIGURE 8. **GIV-dependent FAK activation occurs via PI3K.** A, GIV WT enhances FAK activation upon collagen I stimulation. MCF-7 cells stably overexpressing GIV WT or an empty plasmid (control) were stimulated with collagen I for 15, 30, and 60 min as described in Fig. 2B. Upper panel, representative immunoblots (IB) for the time course of FAK activation (as measured by levels of pFAK Tyr-397) upon collagen I stimulation. Lower panel, quantification of FAK activation (as described under “Experimental Procedures”) expressed as average with error bars representing S.E. (n = 3; *, p < 0.05; ***, p < 0.001). FAK is not activated when MCF-7 cells are seeded on the non-integrin substrate poly-L-lysine (data not shown), indicating that collagen I specifically activates FAK. B, schematic depicting FAK- and GIV-dependent pathways of PI3K activation in response to integrin stimulation and a putative positive feedback loop from PI3K to FAK (dotted line). LY292002 is a pharmacological inhibitor of PI3K. C, GIV-induced enhancement of FAK signaling requires PI3K. MCF-7 cells stably expressing vector control (blue) or GIV WT (red) were treated with the PI3K inhibitor LY294002 (LY; open circles) or vehicle (DMSO; closed circles) and stimulated with collagen I as described under “Experimental Procedures.” A representative result is shown on top, and the quantification of three independent experiments expressed as average with error bars representing S.E. is shown on the bottom (*, p < 0.05 for LY294002 versus DMSO in GIV WT cells). Col, collagen; tFAK, total FAK.
Although tyrosine phosphorylated GIV directly binds and activates PI3K (39), the GEF activity of GIV has been shown previously to activate PI3K indirectly via Gβγ subunits released from active Gα proteins (17, 21). To test whether this mechanism is also utilized by the GEF activity of GIV to enhance FAK activation in response to integrins, we used the Gβγ inhibitor gallein (Fig. 9C), which blocks its binding to PI3K (47, 48). In MCF-7 control cells, activation of FAK in response to collagen I stimulation was not affected by gallein (Fig. 9C). In MCF-7 GIV WT cells, gallein suppressed the enhancement of FAK activation induced by GIV (Fig. 9C). These results are in good agreement with previous data showing that gallein suppresses GIV-mediated PI3K-Akt activation (17) and that PI3K is required for GIV-mediated enhancement of FAK activation in response to integrins (Fig. 8). Taken together, these findings not only pinpoint the mechanism by which the GEF motif of GIV enhances FAK activation in response to integrin outside-in signaling (Gβγ-dependent) but also support the conclusion that both the GEF activity and Tyr(P) residues of GIV are simultaneously required to create the PI3K-FAK positive feedback loop.

Discussion

In this study, we provide novel mechanistic insights into how GIV, a metastasis-associated protein, works as a signal-amplifying node for different signaling networks. In the context of integrin outside-in signaling, our data support a model (Fig. 10) in which up-regulation of GIV expression promotes the enhancement of PI3K-Akt signaling and tumor cell migration via a two-pronged mechanism; i.e. both GEF-dependent and Tyr(P)-dependent GIV mechanisms are required in these processes. Furthermore, GIV-dependent PI3K activation enhances the activation of FAK, indicating that GIV establishes an activation loop that feeds into the initial steps of integrin outside-in signaling. Taken together, these findings reveal how integrin signaling in cancer cells overexpressing GIV is rewired to amplify responses that favor tumor cell migration. GIV not only transmits signals from different classes of receptors (RTKs/GPCRs (18) and integrins ((17) and this work)) but also serves as a convergence platform that integrates trimeric G protein and Tyr(P) signaling.

Disabling either the tyrosine phosphorylation or GEF activity of GIV independently results in a decrease of signaling activation equivalent to that observed in cells lacking GIV (Figs. 3, 4, and 9). This rules out a possible additive effect of the two functions and indicates that they must work coordinately to achieve PI3K signaling enhancement in response to integrins. Two possible scenarios could explain this. One is that simultaneous activation via two parallel pathways (i.e. GEF-dependent and Tyr(P)-dependent) is required to overcome a threshold of PI3K activation. The other scenario is that both GEF and Tyr(P) GIV activities are interdependent. We favor the former possibility because previous evidence supports that these two functions of GIV work independently of each other. It has been demonstrated that phosphorylation of GIV at Tyr-1764/1798 per se does not affect its GEF activity (39). This indicates that the impaired activation of Akt observed in cells expressing the YF mutant (Fig. 3, A and B) is not due to an indirect effect on its GEF activity. Conversely, GIV FA mutant is efficiently phosphorylated at Tyr-1764/1798 in vitro and in cells (39). This indicates that the impaired activation of Akt observed in cells expressing the YF mutant (Fig. 3, A and B) is not due to an indirect effect on Tyr-1764/1798 phosphorylation. The view of GIV Tyr(P) and GEF signaling as independent pathways that converge on PI3K is in keeping with previous observations for GIV-dependent PI3K activation in the context of signaling in response to soluble factors stimulating RTKs or GPCRs (39, 40). This conserved mechanism of PI3K-Akt signaling enhancement by GIV may arise from a requirement for the synergistic action of tyrosine phosphorylated peptides and
Gβγ subunits (39), which activate specific PI3K isoforms using different molecular mechanisms (49–52).

An unexpected consequence of PI3K activation above the threshold of activity observed in cells not expressing GIV is the feedback regulation of FAK. Only when PI3K activity is enhanced by GIV does FAK become sensitive to PI3K inhibition. This contrasts with the classical view of this pathway in which FAK is an upstream component of the pathway and largely insensitive to PI3K (7, 8, 10). However, recent reports have independently shown that serine/threonine phosphorylation of FAK by Akt, a major effector of PI3K, enhances its activation (53, 54). Our results provide a plausible explanation for this apparent discrepancy in the literature because we showed that FAK becomes sensitized to PI3K-Akt regulation only when GIV enhances this pathway above a critical threshold. Thus, it is possible that the interplay between FAK and PI3K depends on the GIV expression status in different systems.

While this manuscript was under revision, a study by Lopez-Sanchez et al. (45) also described that both the GEF activity and tyrosine phosphorylation of GIV are required for integrin signaling in other experimental systems. We think that their findings are complementary to ours. Lopez-Sanchez et al. (45) generated an antibody specific for GIV Tyr(P)-1764 and showed that it localizes to focal adhesions. These results are similar to those also reported recently by Omori et al. (44) for GIV Tyr(P)-1798 that together support the role of GIV tyrosine phosphorylation at Tyr-1764/Y1798 in integrin signaling described here. Although Lopez-Sanchez et al. (45) also described an effect of GIV depletion on the pFAK status of cells, the conclusion from these observations was unclear. This is because they only analyzed pFAK by immunostaining and observed a decrease in the pFAK signal normally associated with focal adhesions (45).

Because GIV depletion impairs the formation of focal adhesions (17, 45), it is not possible to know from these results whether the decrease of pFAK staining in focal adhesions was due to reduced FAK activation or impaired focal adhesion assembly. Our results presented here provide not only definitive biochemical data on the role of GIV in facilitating FAK activation by integrins but also novel insights into the molecular mechanisms involved (i.e. the positive feedback loop described above). For example, our data in Fig. 7 show a normal pattern of activation of integrins per se in GIV-depleted cells, indicating that GIV exerts its action at the immediate postreceptor level (i.e. FAK) and that the defects observed upon GIV depletion are not simply due to abnormal substrate engagement by integrins in adhesion points.

Although binding of GIV to p85α after integrin stimulation (Fig. 2D) suggests that GIV is phosphorylated at Tyr-1764/1798, our study does not provide direct evidence for the specific phosphorylation of these two sites. However, the fact that mutation of these two residues impairs GIV-dependent signaling and cell migration strongly indicates that this is the case. This is further supported by the recent reports by Lopez-Sanchez et al. (45) and Omori et al. (44) showing direct phosphorylation of Tyr-1764 or Tyr-1798 in the context of integrins. It is still possible that other tyrosines in GIV become phosphorylated upon integrin stimulation, although their biological significance remains unclear.

Another complementary aspect of the study by Lopez-Sanchez et al. (45) and the current study is related to the nature of the kinase(s) involved in phosphorylating GIV upon integrin stimulation. Here we showed that inhibition of Src, which phosphorylates GIV Tyr-1764 and Tyr-1798 in vitro (39), diminishes GIV tyrosine phosphorylation upon collagen I stimulation.

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**FIGURE 10.** Proposed model for the mechanisms used by GIV to potentiate outside-in integrin signaling in cancer cells. **Left,** in the absence of GIV, FAK is activated by its recruitment to active integrins in coordination with Src-mediated phosphorylation. Subsequently, active FAK directly binds and activates PI3K. **Right,** in invasive cancer cells overexpressing GIV, integrin-dependent PI3K activation is enhanced by the simultaneous action of the GEF activity of GIV (via Gβγ) and GIV Tyr(P)-1764/1798 (which directly bind and activate PI3K). GIV-enhanced PI3K activity creates a positive feedback loop (dotted line) by further activating FAK and potentiating integrin outside-in signaling.
This is in agreement with previous observations that inhibition of Src reduces GIV Tyr (p)-1798 staining in focal adhesions (44) and inhibits GIV-dependent integrin signaling (17). Conversely, Lopez-Sanchez et al. (45) found that inhibition of FAK inhibits GIV tyrosine phosphorylation upon integrin stimulation. Much like Src, FAK can also directly phosphorylate Tyr-1764 and Tyr-1798 (45). These results are not contradictory or mutually exclusive because it is well established that FAK and Src are interdependent in the first steps of integrins signaling; i.e. activation of Src requires prior phosphorylation of FAK, and full FAK activation is only achieved upon Src action (7, 8).

In addition to Src and FAK, GIV Tyr-1764 and Tyr-1798 are also substrates for RTKs. Although our results cannot formally rule out that phosphorylation of GIV upon collagen I stimulation is mediated by a “transactivated” RTK, we believe that this is unlikely. The primary stimulus in our experiments was an integrin substrate (collagen I) in the absence of RTK ligands (serum starvation), so the level of activation of RTKs, if any, is expected to be low compared with the level of activation of integrins. Moreover, collagen I stimulation triggers the recruitment of GIV to active integrin complexes (17) where both FAK and Src are located. In fact, tyrosine phosphorylated GIV is found enriched in focal adhesions where integrins, FAK, and Src form active signaling complexes. For these reasons, the simplest explanation for our results is that GIV is phosphorylated by an integrin-associated kinase rather than an RTK. In summary, the findings reported here indicate that when GIV expression is up-regulated in cancer cells integrin signaling is reconfigured to hyperactivate pro-oncogenic signaling.

Author Contributions—M. G.-M. conceived and coordinated the study, designed experiments, and analyzed results. M. G.-M. and A. L. wrote the paper. A. L. performed most of the experiments and study, designed experiments, and analyzed results. M. G.-M. and A. L. performed most of the experiments and participated in the manuscript writing. All authors reviewed the results and approved the final version of the manuscript.

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