Direct Interaction of Focal Adhesion Kinase with p190RhoGEF*

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Rho GTPases are members of the Ras GTPase superfamily that play an important role in a wide range of biological processes, including actin cytoskeleton reorganization, cell motility, gene expression, and cell cycle progression (as reviewed in Refs. 1 and 2). Rho GTPases act as molecular switches through which it can modulate the activity of several intracellular signaling pathways. Rho activity can influence the formation of distinct actin cytoskeletal structures such as lamellipodia and stress fibers in part through effects on small Rho GTPases, although the molecular interconnections of these events are not well defined. Here, we report that FAK interacts with p190RhoGEF, a RhoA-specific GDP/GTP exchange factor, in neuronal cells and in brain tissue extracts by co-immunoprecipitation and co-localization analyses. Using a two-hybrid assay and deletion mutagenesis, the binding site of the FAK C-terminal focal adhesion targeting (FAT) domain was identified within the C-terminal coiled-coil domain of p190RhoGEF. Binding was independent of a LD-like binding motif within p190RhoGEF, yet FAK association was disrupted by a mutation (Leu-1034 to Ser) that weakens the helical bundle structure of the FAK FAT domain. Neuro-2a cell binding to laminin increased endogenous FAK and p190RhoGEF tyrosine phosphorylation, and co-transfection of a dominant-negative inhibitor of FAK activity, termed FRNK, inhibited laminin-stimulated p190RhoGEF tyrosine phosphorylation and p21 RhoA GTP binding. Overexpression of FAK in Neuro-2a cells increased both endogenous p190RhoGEF tyrosine phosphorylation and RhoA activity, whereas these events were inhibited by FRNK co-expression. Because insulin-like growth factor 1 treatment of Neuro-2a cells increased FAK tyrosine phosphorylation and p190RhoGEF-mediated activation of RhoA, our results support the conclusion that FAK association with p190RhoGEF functions as a signaling pathway downstream of integrins and growth factor receptors to stimulate Rho activity.

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‡ The abbreviations used are: GEF, guanine nucleotide exchange factor; IP, immunoprecipitation; FAK, focal adhesion kinase; FAT, focal adhesion targeting; FBS, fetal bovine serum; FRNK, FAK-related non-kinase; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GFP, green fluorescent protein; EGFP, enhanced GFP; HA, hemagglutinin; IGF-1, insulin-like growth factor-1; IP, immunofluorescence; pTyr, phosphorysine; PTK, protein tyrosine kinase; RBD, Rho binding domain; SH3, Src homology 3; GST, glutathione S-transferase; PMSF, phenylmethylsulfonyl fluoride.
In this study, we have identified FAK as an endogenous and direct binding partner for p190RhoGEF, a ubiquitously expressed RhoA-specific GEF (15, 16). In addition to the tandem Dbl-pleckstrin homology domain, p190RhoGEF contains several potential regulatory motifs, including an N-terminal leucine-rich region, a cysteine-rich zinc finger domain, and a large C-terminal domain with a potential coiled-coil region that has been implicated in cell adhesion and cytoskeletal interactions (17), a destabilizing element in the 3′-untranslated region of neurofilament mRNA (17), and to either 14-3-3 proteins (18) or the phosphotyrosine binding domain of JIP-1 (19) in a phosphorylation-independent manner. Despite the identification of these binding partners, the in vivo regulation of p190RhoGEF GDP-GTP exchange activity remains unknown. Here, we show that FAK overexpression or laminin stimulation of Neuro-2a cells promotes p190RhoGEF tyrosine phosphorylation. FAK overexpression with p190RhoGEF is associated with the enhanced activation of p21 RhoA by p190RhoGEF and inhibiting FAK activity via expression of the FAK C-terminal domain termed FRNK blocks this signaling event. These studies support a direct role for p190RhoGEF in linking FAK to Rho activation.

EXPERIMENTAL PROCEDURES

DNA Constructs and Reagents—Expression vectors for p190RhoGEF (full-length and C-terminal domain) were described previously (18). Murine FAK and FRNK expression vectors were used as described previously (20). Coding sequences for RhoA and the Rho-binding domain (RBD) of Rhotekin (amino acids 7–89) (21) were amplified from a mouse spinal cord cDNA library (Stratagene) and cloned into pCMV (Roche Applied Science) and pGEX-6p-1 (Amersham Biosciences), respectively. Mutagenesis was performed using the QuikChange™ sitedirected mutagenesis kit (Stratagene), and all constructs were verified by DNA sequencing. Monoclonal antibodies (clone 12CA5) to hemagglutinin (H) and c-Myc (clone 9E10) were from Roche and monoclonal antibodies to FAK (clone 77) and phosphotyrosine (pTyr, clone RC20) were from BD Transduction Laboratories. Goat anti-mouse or goat anti-rabbit IgG (Molecular Probes) for 24 h at 4 °C followed by incubation with secondary antibodies to FAK or p190RhoGEF (1:1000; J. Weis, University of Michigan) and goat anti-mouse IgG (Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) for 24 h at 4 °C. After mounting, cells were examined by laser-scanning confocal microscopy.

RESULTS

Identification of FAK as a Binding Partner for p190RhoGEF—To explore the interactive properties of p190RhoGEF, residues 1276–1582 in the C-terminal domain of p190RhoGEF (p190RhoGEF-C) were used as bait in a two-hybrid screen of a mouse embryonic spinal cord cDNA library. Two clones were identified with open reading frame sequences matching the C-terminal 140 residues of FAK. To verify the specificity of this interaction, yeast were co-transformed with the respective cDNAs and tested for their abilities to grow on selective media and to activate a β-galactosidase (LacZ) reporter through binding to a bipartite GAL4 transcription factor. As shown in Table 1, only co-transformants containing both p190RhoGEF-C and the C-terminal sequence of FAK (FAK-C) grew on selective media and activated LacZ. Although previous studies using a LexA-FAK C-terminal domain construct promoted “prey”-independent promoter activation (22), no growth was observed in cells transfected with pad-FAD-C alone (data not shown).

Interactions of p190RhoGEF and FAK in Vivo—To determine whether p190RhoGEF interacts with FAK in mammalian cells, co-immunoprecipitation (co-IP) assays were performed with Neuro-2a cells co-transfected with HA-tagged p190RhoGEF or HA-tagged p190RhoGEF-C along with either EGFP-tagged FAK or EGFP-tagged FRNK (13) that encompasses the entire C-terminal domain of FAK. Whereas in the absence of exogenous HA-p190RhoGEF expression there was no association of FAK with anti-HA antibodies (data not shown), both FAK and FRNK co-immunoprecipitated with normalized for the protein content (Pierce) and incubated with antibodies to HA, c-Myc, FAK, or p190RhoGEF, respectively. Immunocomplexes were then precipitated with protein A/G PLUS-agarose, washed twice in lysis buffer, eluted by boiling in SDS-PAGE sample buffer, and subjected to Western blot analysis.

Cell Stimulation with Laminin—Neuro-2a cells were grown to 90% confluence, detached with limited trypsin treatment, and suspended in growth medium for 30 min, then the cells were either lysed or allowed to adhere to laminin-coated plates (BD Biosciences) for 1 h. Cell lysates were divided into three parts: two of which were used to immunoprecipitate FAK and blotted for either p190RhoGEF association or for pTyr. p190RhoGEF was directly immunoprecipitated from the remaining third of the lysate and blotted for pTyr. The FAK or p190RhoGEF IPs were sequentially re-probed with antibodies to FAK or p190RhoGEF, respectively, as described previously (20). To perform analyses of laminin-stimulated Rho activity, cells were serum-starved (0.5% FBS) for 18 h and resuspended in Dulbecco’s modified Eagle’s medium with 0.5% bovine serum albumin prior to replating.

Rho Pull-down Assay—Neuro-2a cells were lysed in Rho-binding lysis buffer (50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 10 mM MgCl₂, 1 mM Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1× protease inhibitor mixture, and 1 mM PMSF). Cell lysates were cleared by centrifugation at 14,000 rpm for 4 °C for 10 min, and equal amount of lysates (500 μg of protein content) were incubated with 20 μg of immobilized GST-RBD at 4 °C for 45 min. The beads were washed four times with wash buffer (7.2, 50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 10 mM MgCl₂, 1 mM Triton X-100, 1× protease inhibitor mixture, and 1 mM PMSF). The bound RhoA was eluted by boiling in sample buffer for SDS-PAGE and subjected to RhoA immunoblot analysis. The relative level of GTP-bound RhoA was determined by densitometry scanning of immunoblots and normalized to the amount of total RhoA expression in the lysates as determined by blotting of whole cell lysates. Data is presented as fold activation compared with the levels of GTP-bound RhoA in cells transfected with p190RhoGEF. Significance between data sets was determined by one-way analysis of variance.

Immunofluorescence and Confocal Microscopy—Neuro-2a cells plated on round coverslips were fixed in 3.7% formaldehyde for 1 h on ice and permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature. The fixed cells were incubated with either affinity-purified rabbit anti-p190RhoGEF (1:1000) or mouse monoclonal anti-FAK (1:2000) for 72 h at 4 °C followed by incubation with secondary antibodies Alexa Fluor® 594 chicken anti-rabbit IgG or Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) for 24 h at 4 °C. After mounting, cells were examined by laser-scanning confocal microscopy.

REFERENCES

1. R. Canete-Soler, unpublished data.
HA antibodies in cells co-transfected with full-length or p190RhoGEF-C (Fig. 1).

To explore the interaction between endogenous p190RhoGEF and FAK, polyclonal antibodies were generated to p190RhoGEF residues 1457–1582. When whole cell lysates of Neuro-2a cells transfected with HA-tagged p190RhoGEF-C or full-length p190RhoGEF (Fig. 2A) were blotted with affinity-purified antibodies to anti-p190RhoGEF (Fig. 2A, lanes 1 and 2) or with anti-HA antibodies (Fig. 2A, lanes 3 and 4), strong immunoreactive p190RhoGEF-C and p190RhoGEF bands were detected. Notably, the anti-p190RhoGEF antibody also recognized a similar sized band as full-length HA-p190RhoGEF in lysates of p190RhoGEF-C-transfected cells (Fig. 2A, lane 1). This band most likely represents endogenous p190RhoGEF in Neuro-2a cells.

Polyclonal antibodies to p190RhoGEF and to FAK were then used to determine whether endogenous FAK and p190RhoGEF were associated in lysates of neuronal cells (Fig. 2B) and in brain extracts (Fig. 2, C and D). By co-IP, p190RhoGEF was associated with FAK in lysates from Neuro-2a cells and DRG sensory neurons (Fig. 2B). Maximal p190RhoGEF association with FAK was detected in lysates from E19 mouse brain extracts (Fig. 2C) compared with the weaker binding of FAK and p190RhoGEF in lysates of adult brain (Fig. 2, C and D). One reason why p190RhoGEF and FAK binding is less in adult mouse brain is that p190RhoGEF may be subject to proteolytic degradation as a faster migrating band is present in p190RhoGEF-C (Fig. 2D). Nevertheless, these results confirm the co-expression of these proteins in neuronal cell lines and mouse brain and show the endogenous interaction of p190RhoGEF with FAK.

Co-localization of p190RhoGEF and FAK—To determine the intracellular distribution of these proteins, Neuro-2a cells were stained with affinity-purified antibodies to p190RhoGEF (Fig. 3A), a monoclonal antibody to FAK (Fig. 3B), and confocal microscopy was used to detect the indirect immunofluorescence (IF) of secondary antibody distribution. p190RhoGEF IP was observed in the cytoplasm, associated with short and long neurites (Fig. 3A, arrows), as well as in distal growth cone projections (Fig. 3A, arrowhead and inset). Merging of the p190RhoGEF IF with the FAK IF revealed a similar but not identical co-distribution (Fig. 3C). Co-localization of p190RhoGEF and FAK was especially evident in the tips of neurites and in distal growth cones. Antibodies to both p190RhoGEF and to FAK also revealed a distinctly reticular and non-overlapping staining pattern in the nucleus (Fig. 3C). Taken together with the co-IP results, these findings support the potential for signaling connections between FAK and p190RhoGEF in vivo.

Identification of FAK Binding Sites on p190RhoGEF—As identified in the two-hybrid screen, the C-terminal region of p190RhoGEF interacts with the C-terminal 140 amino acids of FAK. Previous studies have shown that the adaptor protein paxillin binds to this region of FAK through interactions mediated by an LD motif in paxillin that is formed by the consensus sequence, LDXXLXX (23). Primary sequence analyses revealed that p190RhoGEF-C contains a potential LD-like motif between residues Leu-1376 and Leu-1383, LTRLLYSL. To determine the importance of this putative LD motif in p190RhoGEF for FAK binding, this region was deleted by site-directed mutagenesis in p190RhoGEF-C, and co-IP assays were performed with co-transfected FAK or FRNK in Neuro-2a cells (Fig. 4A). Surprisingly, p190RhoGEF-C lacking this potential LD motif still associated with both FAK and FRNK, indicating that this region of p190RhoGEF is not essential for FAK binding (Fig. 4A).

To identify the FAK binding region in p190RhoGEF-C, a series of truncations surrounding and within the coiled-coil domain (Fig. 4B) were created and tested for interaction with FRNK using a yeast two-hybrid assay (Fig. 4C). Loss of growth in selective media and β-galactosidase activity occurred when N-terminal deletions were extended from residues 1276–1353 within p190RhoGEF-C, whereas C-terminal truncations to residue 1472 did not disrupt the two-hybrid association (Fig. 4C). To verify the importance of this 1276–1353 region in mediating interactions with FAK in mammalian cells, HA-tagged N-terminal truncations or an internal deletion mutant of p190RhoGEF-C were tested for co-IP with Myc-tagged FAK upon co-transfection of Neuro-2a cells (Fig. 4D). Whereas deletion of residues 1276–1292 in p190RhoGEF-C did not affect FAK binding, truncation of p190RhoGEF-C from residues 1276 to 1301 resulted in the expression of a protein that did not associate with FAK (Fig. 4D). Notably, the internal deletion of residues 1292–1301 within p190RhoGEF-C also disrupted binding to FAK (Fig. 4D). These studies support the conclusion...
that residues 1292–1301 (DVSQSSSEESP) are required for the binding of the p190RhoGEF-C to FAK.

*p190RhoGEF Binding to the FAK FAT Domain*—Structural studies have shown that the C-terminal 140 residues of FAK that associate with p190RhoGEF comprise the focal adhesion targeting (FAT) domain of FAK and that this FAT region forms a four-helical bundle structure (24). Previous studies have shown that a point mutation at Leu-1034 to Ser disrupts paxillin binding to the FAK FAT domain but not the association of talin, which also binds to the FAK FAT domain region (25). This Leu-1034 to Ser mutation alters a hydrophobic interaction within the interior of the four-helical bundle structure (24). When the Ser-1034 mutation is introduced into FRNK, it results in the inactivation of FAT-mediated targeting of FRNK to focal contacts (20). To determine whether FAT domain function is required for binding to p190RhoGEF, Neuro-2a cells were co-transfected with GFP-p190RhoGEF-C and either HA-FRNK or HA-FRNK Ser-1034 (Fig. 4E). GFP-p190RhoGEF-C was associated with anti-HA IPs in cells transfected with HA-FRNK but not with HA-FRNK Ser-1034. Because both FRNK and FRNK Ser-1034 were equivalently expressed in Neuro-2a cells (Fig. 4E), our results support the conclusion that FAK FAT domain function is required for p190RhoGEF binding. Taken together, our findings support the conclusion that the p190RhoGEF coiled-coil domain binds directly to the FAK FAT domain and this novel interaction is mediated in an LD motif-independent manner.

Laminin-stimulated p190RhoGEF Tyrosine Phosphorylation and p21 RhoA GTP Binding Are Blocked by FRNK—In neuronal cells, extracellular matrix ligands such as laminin can promote survival and neurite outgrowth (26) and laminin-binding integrins such as $\alpha_1\beta_1$, $\alpha_5\beta_1$, or $\alpha_2\beta_1$ can activate FAK (12). To determine whether cell adhesion regulates FAK binding to p190RhoGEF, lysates of Neuro-2a cells were prepared under normal growth conditions (control), after limited trypsin treatment and holding the cells in suspension for 30 min (suspension), or after replating the cells onto laminin-coated dishes for 1 h (laminin) (Fig. 5). Co-IP assays with antibodies to FAK showed that the endogenous FAK-p190RhoGEF complex remained associated under suspension conditions; however, increased p190RhoGEF binding was detected after cell stimulation by laminin (Fig. 5A).

In many cell types, FAK tyrosine phosphorylation is regulated in an adhesion-dependent manner, and FAK activity is inhibited when cells are held in suspension (12). Accordingly, FAK is tyrosine-phosphorylated in proliferating Neuro-2a cells, it becomes dephosphorylated when Neuro-2a cells are held in suspension, and FAK becomes rapidly re-phosphorylated when cells are plated onto laminin-coated dishes for 1 h (laminin) (Fig. 5). Upon transfection of Neuro-2a cells with FRNK, FAK tyrosine phosphorylation is reduced in proliferating cells and laminin-stimulated FAK tyrosine phosphorylation at 1 h is inhibited by co-expression of FRNK (Fig. 5B). This result is consistent with the inhibition of integrin-stimulated FAK activation by FRNK overexpression, acting as a specific and competitive inhibitor of FAK activity (12).

To determine whether p190RhoGEF may be a component of an integrin-stimulated signaling pathway, p190RhoGEF IPs from proliferating, suspended, or laminin-replated Neuro-2a cells were evaluated by anti-pTyr blotting (Fig. 5C). Low levels of p190RhoGEF tyrosine phosphorylation were detected in control lysates and p190RhoGEF was not detectably tyrosine-phosphorylated in control lysates and p190RhoGEF was not detectably tyrosine-phosphorylated.
phosphorylated in suspended cells. Notably, Neuro-2a cell stimulation by laminin promoted elevated p190RhoGEF phosphotyrosine levels and transfection of Neuro-2a cells with FRNK dramatically inhibited laminin-stimulated p190RhoGEF tyrosine phosphorylation (Fig. 5C).

Because both FAK and p190RhoGEF tyrosine phosphorylations were increased by laminin stimulation of Neuro-2a cells, co-transfection assays with HA-p190RhoGEF and HA-FRNK or HA-FRNK Ser-1034 were performed to determine whether these phosphorylation events were connected to changes in p21 RhoA GTP binding (Fig. 5D). Cells were serum-starved, and lysates were made from either cells held in suspension for 1 h or suspended cells plated onto laminin-coated dishes for an additional hour. Endogenous levels of GTP-bound RhoA were indirectly evaluated by association with a GST fusion protein encompassing the Rho-binding domain (RBD) of Rhotekin in an in vitro pull-down assay (27). Whereas only basal levels of p21 RhoA GTP binding were detected in suspended cells, laminin stimulation promoted a 2-fold increase in endogenous p21 RhoA binding to GTP compared with suspended cells (Fig. 5D, Mock). Notably, co-transfection of FRNK blocked laminin-stimulated Rho GTP binding, whereas expression of FRNK Ser-1034 did not significantly inhibit Rho activation after laminin stimulation (Fig. 5D). Because FRNK expression did not inhibit Neuro-2a cell binding to laminin and FRNK Ser-1034 expression did not block laminin-stimulated FAK or p190RhoGEF tyrosine phosphorylation (data not shown), our results using FRNK as a dominant-negative inhibitor of FAK activity support a role for FAK in the regulation of laminin-stimulated p190RhoGEF tyrosine phosphorylation. Additionally, our results support the hypothesis that FRNK but not FRNK Ser-1034 binding to p190RhoGEF (Fig. 4E) may act to block FAK association with p190RhoGEF and subsequent signaling leading to Rho activation.

FAK Enhances p190RhoGEF Tyrosine Phosphorylation and GEF Activity—To determine whether FAK activity could enhance p190RhoGEF tyrosine phosphorylation in vivo, Neuro-2a cells were transfected with either wild-type FAK alone or in combination with FRNK (Fig. 6A). FAK overexpression enhanced p190RhoGEF tyrosine phosphorylation, and this was inhibited by co-expression of FRNK. Transfection of increasing amounts of FRNK reduced the basal level of p190RhoGEF tyrosine phosphorylation without affecting the level of endogenous p190RhoGEF expression (Fig. 6A). To determine whether FAK or FRNK expression could modulate the level of p190RhoGEF-associated changes in RhoA activity, co-transfection experiments were performed in Neuro-2a cells (Fig. 6B).

Compared with cells transfected with constitutively active V14Rho as a positive control (Fig. 6B, lane 2), no detectable levels of GTP-bound RhoA were visualized in mock-transfected cells (Fig. 6B, lane 1). Overexpression of p190RhoGEF in Neuro-2a cells promoted increased GTP loading of RhoA (Fig. 6B, lane 3) compared with mock-transfected cells, and this value was set to 1 for normalization. Upon co-overexpression of p190RhoGEF with FAK, the level of GTP-bound Rho was significantly increased (p < 0.02) compared with overexpression of p190RhoGEF alone (Fig. 6B, lanes 3 and 4). Notably, increased signaling to RhoA was blocked by transfection of FRNK along with p190RhoGEF and FAK (Fig. 6B, lane 5). Taken together, these findings show that FAK not only increases tyrosine phosphorylation of p190RhoGEF, but that FAK also enhances the GEF activity of p190RhoGEF for p21RhoA.

FAK and p190RhoGEF Act to Coordinate Rho Activation Downstream of IGF-1 Stimulation—In addition to mediating signaling events downstream of integrins, FAK tyrosine phosphorylation and activity can be modulated by growth factor receptor activation (28). In neuronal cells, insulin-like growth factor-1 (IGF-1) can stimulate FAK tyrosine phosphorylation (29) and IGF-1 can promote increased signaling leading to p21Rho and Rho kinase activation (30). In Neuro-2a cells, IGF-1 addition led to increased FAK tyrosine phosphorylation with a maximal change occurring after 30 min (Fig. 7A). To determine whether IGF-1-stimulated FAK tyrosine phosphorylation promoted changes in Rho activation, co-transfection assays with p190RhoGEF, FAK, and FRNK were performed in Neuro-2a cells (Fig. 7B). Compared with no detectable levels of GTP-bound RhoA in serum-starved Neuro-2a cells (data not shown), IGF-1 stimulation promoted a modest increase in RhoA-GTP levels in mock-transfected cells (Fig. 7B, lane 1).

Overexpression of either p190RhoGEF or FAK in combination with IGF-1 stimulation led to increases of 2- and ~3-fold, respectively, in RhoA-GTP levels (Fig. 7B, lanes 2 and 3) compared with mock transfected cells. Combined overexpression of p190RhoGEF with FAK led to ~4.5-fold enhanced RhoA activity compared with mock transfected cells (Fig. 7B, lane 4), and...
this elevation of Rho GTP levels was significantly different compared with p190RhoGEF (p = 0.01) and FAK (p = 0.04) overexpression alone. Notably, the importance of FAK activity in promoting elevated Rho-GTP levels through p190RhoGEF was confirmed by the co-transfection of FRNK that blocked the combined increased in Rho-GTP levels observed upon co-transfection of FAK and p190RhoGEF (Fig. 7B, lane 5). Taken together, our results support the conclusion that FAK association with p190RhoGEF functions as a signaling pathway downstream of integrins and growth factor receptors to stimulate Rho activity.

**DISCUSSION**

Our findings of a functional interaction between FAK and p190RhoGEF provide an additional perspective on the multiple pathways that can regulate Rho activity in cells. As illustrated in the summary model (Fig. 8), FAK can become activated following ligand binding to either growth factor, G-protein linked, or integrin cell surface receptors. Because reports have linked FAK signaling to Rho inhibition as well as Rho activation, our results support the hypothesis that distinct and direct target protein interactions with FAK can shift the output of
FAK signaling to either Rho inhibition or activation. In this context, FAK function could be best described as a regulated and switchable scaffolding protein.

Although many early reports established correlations between increased FAK tyrosine phosphorylation with increased actin stress fiber formation (13), an indirect measure of Rho activity in many cells (12), no clear linkage between FAK signaling and Rho activity changes were defined until the generation of FAK-deficient cells (31). Contrary to expected findings, FAK-null fibroblasts form an abundance of actin stress fibers and focal contact sites in cell culture that result in refractory motility responses. Reconstitution studies have shown that c-Src recruitment to FAK is an initial event promoting focal contact turnover and enhanced motility of FAK-null cells (12, 32). These changes are due in part to the ability of FAK (28) and c-Src (33) to transiently inhibit Rho GTPase activity allowing actin and focal contact remodeling.

**Fig. 5.** FRNK blocks laminin-stimulated FAK and p190RhoGEF tyrosine phosphorylation; inhibition of signaling to p21RhoA. A, proliferating Neuro-2a cells (control) were either held in suspension for 30 min or plated onto laminin-coated dishes for 1 h prior to cell lysis. Co-IP assays were performed with antibodies to endogenous FAK and blotted for p190RhoGEF association (top panel) or FAK expression (bottom panel). B, Neuro-2a cells were either mock-treated or transfected with 1 μg of FRNK expression plasmid prior to preparing cell lysates from control, suspended, or laminin-replated cells as described above. FAK IPs were analyzed by anti-pTyr (top panel) followed by anti-FAK (bottom panel) blotting. C, Neuro-2a cells were mock treated or transfected with FRNK, and cell lysates were prepared from control, suspended, or laminin-replated cells as described above. p190RhoGEF IPs were analyzed by anti-pTyr (top panel) followed by anti-p190RhoGEF (bottom panel) blotting. D, Neuro-2a cells were transfected with p190RhoGEF and either 1 μg of HA-FRNK, HA-FRNK Ser-1034, or control plasmid (Mock). Cells were serum-starved prior to preparing cell lysates from suspended or laminin-replated cells. p21RhoA activation was visualized by in vitro pull-down assays using GST-Rhotekin RBD followed by Rho blotting (top panel). Total RhoA expression is shown in whole cell lysates (middle panel). RhoA activity is expressed as a fold increase with respect to mock transfected cells with p190RhoGEF alone (lane 3). Values are means ± S.D. from three independent experiments.

**Fig. 6.** FAK enhances p190RhoGEF tyrosine phosphorylation and GEF activity. A, Neuro-2a cells were transfected with 1 μg of FAK with or without the indicated amount of FRNK. Endogenous p190RhoGEF was isolated from the transfected Neuro-2a cells and analyzed by anti-pTyr (top panel) followed by anti-p190RhoGEF (bottom panel) blotting. B, Neuro-2a cells were transfected with either empty vector or 1 μg of the indicated constructs, and p21RhoA activity was visualized by in vitro pull-down assays using GST-Rhotekin RBD followed by Rho blotting (top panel). Total RhoA expression is shown in whole cell lysates (middle panel). RhoA activity is expressed as a -fold increase with respect to Neuro-2a cells transfected with p190RhoGEF alone (lane 3). Values are means ± S.D. from three independent experiments.

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for both FAK and c-Src in mediating Rho inhibition include GRAF (GAP for Rho associated with FAK) (34) and p190RhoGAP (35). The importance of FAK signaling leading to Rho inhibition for cell motility is supported by the fact that pharmacological inhibition of Rho effectors such as Rho kinase and myosin light chain kinase can partially reverse the morphological and motility defects of FAK-null cells (36). Because recent studies have shown that FAK-mediated signaling to targets such as p130Cas and Dock180 promote Rac activation (37), and Rac signaling can feedback to inhibit Rho activation (2), it is also possible that FAK can indirectly inhibit Rho through enhanced signaling to Rac.

Alternatively, recent studies identifying PDZ-RhoGEF, LARG (11), and Trio (14) as FAK substrates and/or binding partners have provided possible molecular links leading to Rho activation as a consequence of FAK-mediated signaling in cells. Here, we identify p190RhoGAP as an endogenous binding partner of FAK. Our results show that laminin stimulation promotes p190RhoGAP tyrosine phosphorylation, that changes in p190RhoGAP phosphorylation are coincident with the activation of FAK, and that FAK or FRNK overexpression promote or inhibit p190RhoGAP-mediated RhoA activation, respectively. As both FAK and FRNK bind to p190RhoGAP, it is unlikely that these binding associations act to modulate p190RhoGAP activity. Instead, our results support the hypothesis that FAK-mediated tyrosine phosphorylation of p190RhoGAP is important in promoting GDP-GTP exchange activity. Ongoing studies are aimed at mapping the p190RhoGAP phosphorylation sites and determining whether these modifications trigger changes in p190RhoGAP activity.

Our studies also showed that both FAK and p190RhoGAP are highly expressed in Neuro-2a cells, rat sensory neurons, and in mouse developmental and adult brain lysates. FAK and p190RhoGAP exhibit overlapping distributions within Neuro-2a cells being concentrated in the tips of neurite-like processes and in growth cones. Previous studies have shown that FAK localization is enriched at hippocampal growth cone tips (38), that FAK and RhoA co-distribute in growth cone point-contacts formed on laminin (39), and that FAK is involved in the regulation of neurite outgrowth induced by growth factor and integrins (29). Because neurite outgrowth and guidance are mediated by the local control of actin dynamics, which in turn are regulated by Rho GTPases (40), our studies suggest a potential role for p190RhoGAP downstream of FAK activation in the control of these processes. Notably, recent studies have shown that chromophore-assisted laser inactivation of c-Src signaling complexes leads to neurite outgrowth of DRG neurons on laminin (41) and that RhoGAP-mediated activation of RhoA is associated with growth cone collapse (42). We hypothesize that FAK-specific signaling connections to p190RhoGAP may be involved in the local fine-tuning of Rho GTPase activity, which is essential for growth cone turning and guidance in response to extracellular cues.

Another important finding of our studies is the identification
of p190RhoGEF as the third protein in addition to paxillin and talin that binds to FAK through interactions mediated by the FAK FAT domain. Whereas the molecular interactions between FAK and talin remain uncharacterized (43), structural studies have shown that the peptide LD motifs of paxillin (LDXXLXL) bind in an amphipathic manner to surface grooves on opposite sides of the FAT domain four-helical bundle (24). We determined that it was the coiled-coil region of the p190RhoGEF C-terminal domain that directly bound to FAK. Moreover, p190RhoGEF binding to the FAT domain required the integrity of the four-helical bundle structure, because Leu-1034-Ser mutation, which is within the hydrophobic core of the FAT domain, acted to disrupt p190RhoGEF binding.

Interestingly, p190RhoGEF contains a potential LD motif sequence between residues Leu-1376 and Leu-1383, LTRLLYSL. However, deletion of this region did not disrupt or weaken p190RhoGEF binding to FAK. Instead, truncation or internal deletion of residues Asp-1292 to Pro-1301 (DVSSQSEESP) inhibited p190RhoGEF association with FAK as determined by two-hybrid and co-transfection/co-IP assays performed with Neuro-2a cells. Taken together, our findings support the conclusion that the p190RhoGEF coiled-coil domain binds directly to the FAT domain and this novel interaction is mediated in an LD motif-independent manner. Because the coiled-coil region of the GIT1 ArfGAP protein also has been shown to bind FAK (44), further site-directed mutagenesis studies will be needed to identify the surface residues of the FAT domain required for p190RhoGEF binding and, further, how p190RhoGEF residues 1292–1301 (DVSSQSEESP) function to promote FAK association.

In summary, we have identified an important link between FAK and Rho. FAK forms a complex with p190RhoGEF, FAK promotes p190RhoGEF tyrosine phosphorylation, and these events are associated with the enhanced activation of RhoA by p190RhoGEF. Our findings add to a growing list of RhoGEF proteins that interact with or are phosphorylated as a consequence of FAK activation and provide a molecular basis of how FAK signaling can lead to Rho activation.

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