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Abbreviations used in this paper: Ad, adenoviral; FA, focal adhesion; FN, fibronectin; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GST-RBD, GST–Rhotekin Rho binding domain; MEF, mouse embryonic fibroblast; PTK, protein tyrosine kinase; Pyk2, proline-rich kinase 2; Scr, scrambled; shRNA, short hairpin RNA; TA, tetracycline transactivator; WT, wild type.

The online version of this paper contains supplemental material.

Introduction

Cell migration is a highly regulated process that involves the continuous formation and turnover of cell–substratum contact sites that regulate migration. RhoA GTPases facilitate FA formation, yet FA-associated RhoA-specific guanine nucleotide exchange factors (GEFs) remain unknown. Here, we show that proline-rich kinase-2 (Pyk2) levels increase upon loss of focal adhesion kinase (FAK) in mouse embryonic fibroblasts (MEFs). Additionally, we demonstrate that Pyk2 facilitates deregulated RhoA activation, elevated FA formation, and enhanced cell proliferation by promoting p190RhoGEF expression. In normal MEFs, p190RhoGEF knockdown inhibits FN-associated RhoA activation, FA formation, and cell migration. Knockdown of p190RhoGEF-related GEFH1 does not affect FA formation in FAK−/− or normal MEFs. p190RhoGEF overexpression enhances RhoA activation and FA formation in MEFs dependent on FAK binding and associated with p190RhoGEF FA recruitment and tyrosine phosphorylation. These studies elucidate a compensatory function for Pyk2 upon FAK loss and identify the FAK–p190RhoGEF complex as an important integrin-proximal regulator of FA formation during FN-stimulated cell motility.
Figure 1. Pyk2 knockdown affects FAK−/− MEF morphology, FA formation, and paxillin tyrosine phosphorylation. (A and B) Compensatory increase in Pyk2 levels upon inactivation of FAK expression. Lysates from FAK+/+, FAK−/−, and FAK+/+(loxP/loxP) MEFs were analyzed by anti-FAK, Pyk2, Cre recombinase, and paxillin blotting. Lysates from Ad-Cre–infected FAK+/+(loxP/loxP) MEFs were analyzed after 48 h or 7 d. Paxillin levels were used as a loading control. (B) Human diploid fibroblasts were treated with lentiviral Scr or FAK shRNA and protein lysates were evaluated by anti-FAK, Pyk2, and actin blotting after 7 d. (C) Stable Pyk2 reduction in FAK−/− MEFs by lentiviral anti-Pyk2 shRNA expression. Lysates from the indicated MEFs were analyzed by antiphosphotyrosine (pY), Pyk2, actin, and GFP blotting. (D) Phase-contrast images show that FAK−/− Pyk2 shRNA MEFs exhibit more of a fibroblast phenotype than FAK−/−.
reaction enhanced by GTPase-activating proteins (GAPs). Transient inhibition of RhoA during FN adhesion is mediated in part by the Src family protein tyrosine kinase (PTK) phosphorylation of p190RhoGAP, which leads to elevated RhoGAP activity (Arthur et al., 2000). The GEFs important in facilitating RhoA reactivation and FA formation upon FN adhesion remain unknown.

Many PTKs facilitate Rac- and Cdc42-specific GEF activation, whereas Rho-specific GEF activation by PTKs is less common (Schiller, 2006). FAK activation by integrins facilitates the recruitment of Src family PTKs into a signaling complex localized to FAs (Mitra et al., 2005; Mitra and Schlaepfer, 2006). FAK- or Src-mediated tyrosine phosphorylation of various RhoGEFs is associated with RhoA activation (Chikumi et al., 2002; Medley et al., 2003; Zhai et al., 2003), but it remains unclear whether these events are linked to FA formation or the regulation of cell motility. An unexplained phenomenon is that FAK-null (FAK−/−) mouse embryonic fibroblasts (MEFs) exhibit constitutively high RhoA activity, enhanced FA formation, and refractory cell motility responses (Illic et al., 1995; Owen et al., 1999; Sieg et al., 1999; Ren et al., 2000). Inhibition of RhoA (Ren et al., 2000) or Rho kinase (Chen et al., 2002) in FAK−/− MEFs decreases FA formation. Although FAK can suppress RhoA activity via p190RhoGAP tyrosine phosphorylation (Holinstat et al., 2006), it is unclear whether loss of FAK accounts for constitutive RhoA activation. Alternatively, expression of the FAK-related proline-rich kinase 2 (Pyk2) PTK is elevated in FAK−/− MEFs (Sieg et al., 1998), but the role of Pyk2 in FAK−/− MEFs remains undefined.

In this paper, we show that Pyk2 promotes FAK−/− MEF proliferation and aberrant FA formation through the regulation of p190RhoGEF expression affecting RhoA activation. p190RhoGEF is a ubiquitously expressed RhoA-specific GEF that can bind microtubules and also associates with FAK (Gebbink et al., 1997; van Horck et al., 2001; Zhai et al., 2003). In FAK−/− MEFs, Pyk2 associates with p190RhoGEF, and knockdown of Pyk2 or p190RhoGEF results in FAK−/− MEFs with normal FN-associated RhoA regulation but extreme motility and trailing-edge retraction defects. These findings show that some FAK-null phenotypes are caused by compensatory Pyk2 signaling effects. In normal MEFs with FAK and little Pyk2, p190RhoGEF knockdown limits RhoA activation, FA formation, and cell motility on FN. Knockdown of p190RhoGEF-related GEFH1 (Ren et al., 1998; Rossman et al., 2005) was without effects on FA formation in FAK−/− and normal MEFs. As deletion of the p190RhoGEF FAK-binding region prevents p190RhoGEF localization to FAs, inhibits p190RhoGEF-associated RhoA activation and FA formation, and blocks integrin-stimulated p190RhoGEF tyrosine phosphorylation, our studies have identified the FAK–p190RhoGEF signaling complex as an important integrin-proximal regulator of FA formation during cell migration.

Results

Compensatory Pyk2 expression upon FAK loss in mouse and human fibroblasts

FAK−/− MEFs exhibit elevated Pyk2 expression (Sieg et al., 1998), and it has been proposed that this may reflect genetic instability associated with p53 coactivation (Tilghman et al., 2005). To determine whether Pyk2 levels change upon FAK inactivation in culture, primary FAK(loxpP/loxpP) MEFs were transduced with adenooviral (Ad) Cre-recombinase and FAK-Pyk2 levels were monitored by immunoblotting (Fig. 1 A). High Cre levels were observed within 48 h of transduction with slight reduction of FAK but no changes in Pyk2 expression. Loss of FAK was complete by day 7 after Ad-Cre transduction at which time Pyk2 levels were greatly elevated (Fig. 1 A). To determine whether this compensatory change in Pyk2 occurs in other cells, human diploid fibroblasts were infected with scrambled (Scr) or anti-FAK short hairpin RNA (shRNA) lentivirus (Fig. 1 B).

After 7 d, FAK expression was selectively inhibited by FAK shRNA expression and Pyk2 levels were greatly elevated relative to actin and control Scr shRNA—expressing cells. Although stable FAK shRNA–mediated knockdown in various tumor cell lines did not result in alterations in Pyk2 expression (Mitra et al., 2006a,b), compensatory Pyk2 expression occurs upon loss of FAK in MEFs and human fibroblasts.

Pyk2 knockdown in FAK−/− MEFs reduces FA formation and paxillin tyrosine phosphorylation

To elucidate the role of Pyk2 in FAK−/− MEFs, Pyk2 expression was inhibited by lentiviral-mediated Pyk2-specific shRNA (Fig. 1 C). The shRNA lentivirus coexpresses GFP, and FAK−/− vector-only, Scr, or Pyk2 shRNA MEF pooled cell populations were enriched by FACS. Stable Pyk2 knockdown of ~90% in FAK−/− Pyk2 shRNA MEFs resulted in cells with a normal fibroblast-like phenotype compared with the more rounded FAK−/− parental (not depicted) or control FAK−/− Scr shRNA MEFs (Fig. 1 D).

When plated onto FN for 60 min, Scr shRNA and Pyk2 shRNA FAK−/− MEFs were equally spread (Fig. 1 E), but cells with less Pyk2 formed fewer FAs of larger size (Fig. 1, F and G) and...
Pyk2 promotes FAK−/− MEF proliferation, but not motility, at a level equal to FAK
signaling can promote cell cycle progression (Zhao et al., 2003), but FAK−/− MEFs do not exhibit proliferation defects in culture compared with FAK-reconstituted (clone DP3) and normal FAK+/+ MEFs (Fig. 2 A). Analyses of FAK−/− Scr shRNA MEFs showed no differences in growth compared with control FAK−/− Scr and vector shRNA controls (Fig. 2 B). Importantly, transient Ad-FAK or human Ad-Pyk2 expression in FAK−/− Pyk2 shRNA MEFs enhanced cell proliferation at a level equivalent to FAK−/− controls (Fig. 2 C and D).

Pyk2 shRNA MEFs infected with Ad-FAK or Ad-Pyk2 were resolved by SDS-PAGE and analyzed by HA-tag, Pyk2, and actin blotting. (E) FAK promotes FAK−/− MEF motility better than Pyk2. The indicated MEFs were analyzed for 10 μg/ml-FN–stimulated Boyden chamber motility over 4 h and values are expressed as a percentage of FAK−/− vector control (set to 100). As indicated, FAK−/− vector shRNA or FAK−/− Pyk2 shRNA MEFs were infected with Ad-FAK or Ad-Pyk2 for 48 h and analyzed for motility responses. Values ± SD are from three separate experiments.

Figure 2. Pyk2 facilitates FAK−/− MEFs' proliferation and motility. (A) FAK−/−, FAK+/+, and FAK-reconstituted (DP3) MEF proliferation was evaluated over 5 d and shows that FAK−/− MEFs do not exhibit growth defects. (B) FAK−/− vector, FAK−/− Scr shRNA, and FAK−/− Pyk2 shRNA MEF proliferation was evaluated over 5 d and shows that Pyk2 shRNA expression is associated with reduced growth. (C) Transient Ad-FAK or Ad-Pyk2 expression enhances FAK−/− Pyk2 shRNA MEF proliferation. Cells were infected for 24 h with the indicated control Ad-TA, Ad-FAK, or Ad-Pyk2 constructs before initiating growth analyses. Values in A–C are means ± SD of three experiments. (D) lysates from FAK−/− vector and Pyk2 shRNA MEFs infected with Ad-FAK or Ad-Pyk2 were resolved by SDS-PAGE and analyzed by HA-tag, Pyk2, and actin blotting. (E) FAK promotes FAK−/− MEF motility better than Pyk2. The indicated MEFs were analyzed for 10 μg/ml-FN–stimulated Boyden chamber motility over 4 h and values are expressed as a percentage of FAK−/− vector control (set to 100). As indicated, FAK−/− vector shRNA or FAK−/− Pyk2 shRNA MEFs were infected with Ad-FAK or Ad-Pyk2 for 48 h and analyzed for motility responses. Values ± SD are from three separate experiments.

Pyk2 contains less polymerized F-actin compared with control FAK−/− Scr shRNA MEFs (Fig. 1 H). The number and size of FAs in FAK−/− Pyk2 shRNA MEFs was equivalent to normal MEFs (unpublished data). Tyrosine phosphorylation of the FA-associated protein paxillin is elevated in FAK−/− compared with normal MEFs (Ilic et al., 1995), and this was reduced by Pyk2 shRNA expression in growing (Fig. 1 C) and FN-plated FAK−/− MEFs (Fig. 1 I). As these changes in FAK−/− Pyk2 shRNA MEFs were not associated with alterations in α5, β1, or αν integrin subunit surface expression (unpublished data), our data support the notion that Pyk2 facilitates enhanced FA formation and paxillin tyrosine phosphorylation in FAK−/− MEFs.
coordinated cell contraction associated with trailing-tail region release (Fig. 3A and Video 1). FAK

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/MEFs exhibit membrane ruffling in all directions and limited directional movement (Video 2). Although reduced Pyk2 expression prevents excessive FA formation (Fig. 1), when FAK

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/Pyk2 shRNA MEFs moved in one direction, cells became very stretched, pieces broke off, and movement occurred in a snapping fashion (Fig. 3B and Video 3). This snapping phenotype was rescued by FAK re-expression (unpublished data). These results are consistent with the inability of FAK

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/Pyk2 shRNA MEFs to facilitate trailing-edge FA release as FAK

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MEFs exhibit decreased FA turnover dynamics (Ren et al., 2000; Webb et al., 2004). Moreover, these results show that compensatory Pyk2 expression is not causal for FAK

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MEF motility defects.

The connection between Pyk2 and RhoA activation

The regulation of FA formation is, in part, associated with RhoA GTPase activation. MEF binding and spreading on FN results in...
elevated RhoA-GTP levels in suspension (Fig. 4 A). In FAK−/−/H11002 MEFs, RhoA-GTP levels remain high upon initial cell binding to FN (15 and 30 min), whereas RhoA-GTP levels are low when FAK+/+ MEFs bind to FN (Fig. 4, A and B). By 90 min, RhoA-GTP levels are equally elevated in FAK−/−/H11002 and FAK+/+ MEFs.

Plating FAK−/−/H11002 Pyk2 shRNA MEFs on FN revealed the transient inhibition of RhoA at 15–30 min, followed by an extended phase of RhoA activation associated with FA formation and maturation (Ren et al., 1999; Vicente-Manzanares et al., 2005). An affinity binding assay can be used to determine the amount of activated RhoA-GTP as a function of total RhoA (Ren et al., 1999). Both FAK−/− and FAK+/+ MEFs exhibit elevated RhoA-GTP levels in suspension (Fig. 4 A). In FAK−/− MEFs, RhoA-GTP levels remain high upon initial cell binding to FN (15 and 30 min), whereas RhoA-GTP levels are low when FAK+/+ MEFs bind to FN (Fig. 4, A and B). By 90 min, RhoA-GTP levels are equally elevated in FAK−/− and FAK+/+ MEFs. Plating FAK−/− Pyk2 shRNA MEFs on FN revealed the transient
inhibition of RhoA-GTP levels, which is similar to RhoA regulation in FAK−/− MEFs (Fig. 4, A and B). This result supports the notion that Pyk2 is facilitating aberrant RhoA activation in FAK−/− MEFs.

To determine whether Pyk2 signaling promotes RhoA activation, FAK−/− Pyk2 shRNA MEFs were transduced with either human Ad-Pyk2 wild type (WT), Ad-Pyk2 A457 (kinase dead), or Ad-Pyk2 F402 or Ad–tetracycline transactivator (TA; control), and RhoA activity was evaluated after FN replating (Fig. 4 C). Ad-Pyk2 WT promoted constitutively activated RhoA, which is similar to what occurs in FAK−/− MEFs (Fig. 4, A and C). Expression of Pyk2 kinase dead or Pyk2 F402 had slight inhibitory effects on RhoA regulation upon FN plating (Fig. 4, C and D). Collectively, these results show that Pyk2 kinase activity and Pyk2 Y402 phosphorylation are needed to facilitate RhoA activation in FAK−/− MEFs.

**Pyk2 regulates p190RhoGEF expression**

Integrin-induced RhoA regulation is compromised in Pyk2−/− macrophages (Okigaki et al., 2003) but the signaling linkage between Pyk2 and RhoA remains undefined. GEFs function as RhoGTPase activators (Rossman et al., 2005), and we previously identified p190RhoGEF as a FAK-binding partner involved in laminin-stimulated RhoA activation in neuronal cells (Zhai et al., 2003). p190RhoGEF is a ubiquitously expressed RhoA-specific GEF of unclear biological function (Gebbink et al., 1997; van Horck et al., 2001). By semiquantitative RT-PCR analyses, low levels of p190RhoGEF mRNA were detected in FAK−/− MEFs, whereas more than fivefold higher p190RhoGEF mRNA levels were present in FAK−/− MEFs (Fig. 4 E). By protein blotting, p190RhoGEF expression was three- to fourfold higher in FAK−/− compared with FAK+/− MEFs, and Pyk2 shRNA expression was associated with reduced p190RhoGEF levels in FAK−/− MEFs (Fig. 4 F). Transient Ad-Pyk2, but not Ad-FAK, expression increased p190RhoGEF mRNA (not depicted) and protein levels about twofold by 48 h in FAK−/− Pyk2 shRNA MEFs (Fig. 4 F). This increase in p190RhoGEF protein was dependent on intrinsic Pyk2 kinase activity and Y402 phosphorylation (Fig. 4 G). These results show that Pyk2 signaling controls p190RhoGEF expression in FAK−/− MEFs, and this linkage is also conserved in rat pheochromocytoma PC12 cells (unpublished data).

**p190RhoGEF promotes deregulated RhoA activity and growth of FAK−/− MEFs**

To determine the function of p190RhoGEF in FAK−/− MEFs, lentiviral-mediated shRNA was used to stably knock down p190RhoGEF expression >85% (Fig. 5 A). Strikingly, FAK−/− p190RhoGEF shRNA MEFs exhibited a conversion from an epithelial-like to a fibroblast morphology and formed fewer FAs than FAK−/− Scr shRNA MEFs upon FN plating (Fig. 5, B and C). Reduction in p190RhoGEF was also associated with the cessation of deregulated RhoA activity in FAK−/− MEFs (Fig. 5 D). Despite the relatively normal appearance of FAK−/− p190RhoGEF shRNA MEFs, Boyden chamber FN motility assays revealed severe motility defects (Fig. 5 E). Time-lapse microscopy showed that FAK−/− p190RhoGEF shRNA MEFs spread on FN and possessed leading-edge membrane–ruffling activity but exhibited limited directional movement upon serum stimulation (Fig. S2 and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200708194/DC1). These results support a role for p190RhoGEF in MEF motility. Additionally, as FAK−/− p190RhoGEF shRNA MEFs form FAs within 60 min on FN (Fig. 5 B) under conditions of low RhoA-GTP binding (Fig. 5 D), our results support the notion that FA formation can also occur through alternative pathways (Zaidel-Bar et al., 2004) and that low levels of RhoA activity in FAK−/− p190RhoGEF shRNA MEFs likely represent contributions of other GEFs downstream of integrins.

To determine whether p190RhoGEF contributes to FAK−/− growth, cell proliferation assays were performed. FAK−/− p190RhoGEF shRNA MEFs grew significantly (P < 0.01 at day 3) slower than FAK+/− Scr shRNA MEFs (Fig. 5 F). Rescue assays were inconclusive because of shRNA inhibition of mouse p190RhoGEF reexpression (unpublished data). Interestingly, Ad-Pyk2 overexpression did not significantly promote FAK−/− p190RhoGEF shRNA MEF growth (Fig. 5 F; P > 0.05) and only weakly facilitated RhoA-GTP binding upon FN plating (Fig. 5 G). This result contrasts with strong RhoA activation (Fig. 4 C) and increased cell proliferation (Fig. 2 C) upon Pyk2 reexpression within FAK−/− Pyk2 shRNA MEFs. This result supports the conclusion that a Pyk2-p190RhoGEF signaling linkage promotes FAK−/− MEF proliferation and that p190RhoGEF is an important target for Pyk2-mediated RhoA activation.

**p190RhoGEF functions downstream of integrins in normal MEFs**

It is well established that Rho GTPase activation and cell contractility contribute to FA formation (Wehrle-Haller and Imhof, 2002; Zaidel-Bar et al., 2003). However, it remains unclear which GEFs downstream of integrins participate in this process. Lentiviral p190RhoGEF shRNA was used to stably reduce p190RhoGEF levels in normal (FAK+/−) MEFs (Fig. 6 A), and this resulted in the inhibition of RhoA activation at 90 and 120 min after FN plating compared with parental and Scr shRNA–expressing MEFs (Fig. 6, B and C). FAK+/− MEFs with reduced p190RhoGEF did not show differences in binding to or spreading on FN. At 60 min on FN, FAK+/− p190RhoGEF shRNA MEFs were fully spread but contained greater than twofold fewer FAs per cell than control FAK+/− Scr MEFs (Fig. 6, D and E). This difference in FA formation upon p190RhoGEF reduction was also associated with the inhibition of FN-stimulated motility (Fig. 6 F). These results support the notion that p190RhoGEF functions as an important regulator of RhoA activity and FA formation downstream of integrins.

To evaluate the specificity of p190RhoGEF action, transient siRNA knockdowns were performed with a closely related (Rossman et al., 2005) RhoA-specific GEF termed GEFH1 (Ren et al., 1998). Efficient GEFH1 knockdown was accomplished in both FAK−/− and FAK+/− MEFs, but there were no differences in paxillin-stained FAs or actin structures upon loss of GEFH1 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200708194/DC1). Alternatively, transient GFP-GEFH1 expression revealed a circular microtubule distribution consistent with GEFH1 binding to microtubules (Ren et al., 1998),
Figure 5. p190RhoGEF contributes to aberrant FAK−/− MEF morphology, RhoA activity, and FA formation. (A) Stable p190RhoGEF knockdown. FAK−/− MEFs were infected with lentiviral Scr or p190RhoGEF shRNA and sorted for GFP expression, and lysates were analyzed by p190RhoGEF, Pyk2, actin, and GFP blotting. (B) Phase-contrast images show that FAK−/− p190RhoGEF shRNA MEFs exhibit more of a fibroblast phenotype than FAK−/− Scr shRNA control MEFs. Reduced numbers of FAs connected to F-actin after FN replating (60 min) upon p190RhoGEF knockdown as determined by anti-paxillin and Alexa Fluor 350 phalloidin costaining. Bars, 30 μm. (C) Decreased FAK−/− FA formation upon p190RhoGEF knockdown. Box-and-whisker plots of paxillin-positive-stained points within FAK−/− Scr shRNA or FAK−/− p190RhoGEF shRNA MEFs plated on FN for 60 min (n = 15 cells per point). (D) p190RhoGEF knockdown prevents deregulated FAK−/− MEF RhoA activation. GTP-bound RhoA was determined by GST-RBD pulldown assays from lysates of suspended and FN-replated FAK−/− Scr shRNA and FAK−/− p190RhoGEF shRNA MEFs plated on FN for 60 min (n = 3 cultures per point). (E) Loss of p190RhoGEF inhibits motility. 10 μg/ml-FN-stimulated FAK−/−, FAK−/− Scr, and FAK−/− p190RhoGEF shRNA MEF Boyden chamber motility over 4 h. Means ± SD were determined from three experiments and are a percentage of FAK−/− control (set to 100). (F) p190RhoGEF contributes to FAK−/− cell growth. (G) Loss of p190RhoGEF inhibits motility.
whereas expression of GFP-p190RhoGEF was cytoplasmic distributed and selectively enriched within the tips of cell projections in normal MEFs (Fig. S4). Both GEFs enhanced F-actin generation and this was inhibited by GEF-inactivating point mutations in the catalytic domains of GEFFH1 (Y393A) and p190RhoGEF (Y1003A; unpublished data).

To explore the similarities and differences of these GEFs, GFP fusions of GEFFH1 WT (or catalytically inactive Y393A GEFFH1) and p190RhoGEF (or catalytically inactive Y1003A or Δ1292-1301, a FAK-binding mutant) were stably expressed in normal MEFs via lentiviral transduction (Fig. 7 A). GFP–p190RhoGEF WT and Y1003A were expressed at levels equivalent to endogenous p190RhoGEF (not depicted), whereas GFP–GEFFH1 WT expression was low (Fig. 7 A) and associated with a rounded and multinucleated phenotype, which is consistent with a role for GEFFH1 in cytokinesis (Birkenfeld et al., 2007). GFP–GEFFH1 and GFP–p190RhoGEF facilitated elevated RhoA activation upon FN plating and these effects were inactivated by Y393A or by Y1003A or Δ1292-1301 mutations in GEFFH1 and p190RhoGEF, respectively (Fig. 7 B and not depicted). Interestingly, GFP–p190RhoGEF WT–expressing MEFs exhibited a rounded phenotype identical to FAK−/− MEFs (Fig. 7 C), with an approximately threefold increase in FA formation (Fig. 7 D) and inhibited motility on FN (Fig. 7 E) compared with normal GFP-expressing MEFs. As changes in p190RhoGEF expression either prevent or stimulate FA formation with corresponding negative effects on cell motility, these results point to the importance of p190RhoGEF in FA regulation for cell movement.

FAK regulation of p190RhoGEF FA localization and tyrosine phosphorylation

Many regulatory and signaling proteins that participate in integrin signaling events are transiently recruited to FAs upon FN plating (Vicente-Manzanares et al., 2005). Although localization studies for endogenous p190RhoGEF distribution were inconclusive because of high nonspecific staining (not depicted), GFP–p190RhoGEF WT partially colocalized with paxillin at FAs upon FN plating of MEFs (Fig. 8). Interestingly, GFP–p190RhoGEF A1292-1301 with a deletion in the FAK-binding region or catalytically inactive GFP–p190RhoGEF Y1003A did not exhibit strong FA localization (Fig. 8) and did not function to facilitate RhoA activation (Fig. 7 B) or alter FA formation (Fig. 7 D). This differs from GFP–GEFFH1, which was primarily cytoplasmic distributed (Fig. 8) and was actively promoting FA formation (Fig. 7 D). Although overexpression of GFP–p190RhoGEF A1292-1301 or GFP–p190RhoGEF Y1003A had weak but significant (P < 0.05) inhibitory effects on FN-stimulated motility (Fig. 7 E), this was not associated with alterations in cell morphology (Fig. 8) or FA formation (Fig. 7 D) and may reflect alterations in endogenous p190RhoGEF function. Collectively, these results show that although GEFFH1 was active but not FA localized, p190RhoGEF activity required FAK binding and FA association in normal MEFs.

To determine if FA localization of p190RhoGEF is essential for its effects on FA formation, GFP–p190RhoGEF was transiently expressed in FAK−/− MEFs. Surprisingly, GFP–p190RhoGEF was not FA localized but exhibited potent effects on enhancing FA formation (Fig. 9 A). By coimmunoprecipitation analyses, endogenous p190RhoGEF forms a complex with Pyk2 in FAK−/− MEFs (Fig. 9 B) and is tyrosine phosphorylated upon FN plating (Fig. 9 C). Importantly, Pyk2 also does not localize to FAs in FAK−/− MEFs (Sieg et al., 1998). However, as GFP–p190RhoGEF did not function to promote FA formation or localize to FAs in FAK−/− Pyk2 shRNA MEFs (unpublished data), these results are consistent with the importance of FAK in mediating p190RhoGEF FA localization and with potentially overlapping roles for Pyk2 or FAK in promoting p190RhoGEF tyrosine phosphorylation and activation. In vitro kinase assays using purified Pyk2 or FAK kinase domains or full-length Src showed that each of these PTKs could directly phosphorylate p190RhoGEF (Fig. 9 S, available at http://www.jcb.org/cgi/content/full/1292-1301/DC1). Analysis of GFP–p190RhoGEF–expressing FAK−/− MEFs revealed adhesion-dependent p190RhoGEF WT tyrosine phosphorylation and the formation of a FAK–paxillin–p190RhoGEF signaling complex (Fig. 9 D). FAK association and p190RhoGEF tyrosine phosphorylation upon FN plating were inhibited by p190RhoGEF catalytically inactivating (Y1003A) mutation or deletion of the FAK-binding region (Δ1292-1301). Together, these results support the notion that p190RhoGEF activity is regulated in part by FAK and or Pyk2 binding leading to enhanced p190RhoGEF tyrosine phosphorylation and the formation of an adhesion-dependent signaling complex.

Discussion

Many investigators have compared FAK−/− and FAK+/+ MEFs to define the role of FAK in various signaling pathways (Mitra et al., 2005). One complication of these analyses is that expression of the FAK-related PTK Pyk2 is elevated in FAK−/− MEFs. We show that increased Pyk2 expression occurs after conditional FAK inactivation within FAKloxPloxP MEFs and upon FAK knockdown by shRNA expression within human diploid fibroblasts. The same shRNA was used to knock down FAK within human MCF-7 and MDA-MB-231 breast carcinoma, PC3 prostate carcinoma, or NB8 neuroblastoma cells without detectable changes in Pyk2 expression (Mitra et al., 2006a,b). Thus, compensatory changes in Pyk2 levels are independent of the means to inactivate FAK and may reflect differences in cell origin or transformation.

We found that Pyk2 shRNA knockdown within FAK−/− MEFs slows cell growth that could be rescued by either transient FAK or Pyk2 reexpression. Previous studies have linked FAK

MEF proliferation. Cell growth assays over 5 d show that FAK−/− p190RhoGEF shRNA MEFs grow slower than FAK+/− Scr shRNA MEF controls and that Ad-Pyk2 overexpression does not function to enhance FAK−/− p190RhoGEF shRNA MEF cell proliferation. Cells were infected 24 h before initiating growth analyses. Values are means ± SD from two experiments. (G) Pyk2 overexpression weakly activates RhoA in FAK−/− p190RhoGEF shRNA MEFs. Cells were preinfected with Ad-Pyk2 WT, subjected to FN replating, and analyzed for Rho-GTP binding following by blotting for total RhoA and Myc-Pyk2 levels.
et al., 2006) and that a Pyk2-p190RhoGEF signaling linkage promotes FAK\(^{+/+}\)/H11002 MEF proliferation. Although stable Pyk2 knockdown in FAK\(^{+/+}\)/H11002 MEFs decreased paxillin tyrosine phosphorylation to normal levels, the most striking finding of our study was that aberrant FAK\(^{+/+}\)/H11002 MEF phenotypes of elevated FA formation, altered F-actin distribution, and a rounded epithelial-like morphology were linked to Pyk2-enhanced p190RhoGEF expression promoting constitutive signaling to MAPK activation and increased cell cycle progression (Zhao et al., 2003). Although Pyk2 overexpression in FAK\(^{-/-}\) MEFs enhances MAPK activation (Sieg et al., 1998), we found that Pyk2-enhanced FAK\(^{-/-}\) MEF proliferation was dependent on p190RhoGEF expression and was correlated with the ability of Pyk2 to stimulate RhoA activation. This result is consistent with the finding that Rho kinase inhibition restored adhesion-dependent control of FAK\(^{-/-}\) MEF proliferation (Pirone et al., 2006) and that a Pyk2-p190RhoGEF signaling linkage promotes FAK\(^{-/-}\) MEF proliferation.

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Figure 7. p190RhoGEF-stimulated RhoA activity and FA formation are dependent on FAK binding. (A) GFP-p190RhoGEF or GFP-GEFH1 overexpression in normal MEFs. (A) FAK−/+ MEFs were infected with a lentiviral expression vector for GFP or GFP fusions of p190RhoGEF WT, p190RhoGEF Y1003A (catalytically inactive), p190RhoGEF Δ1292-1301 (FAK-binding mutant), GEFH1 WT, or GEFH1 Y393A (catalytically inactive) and sorted for GFP expression. Shown is the flow cytometry profile of GFP expression (open peaks) compared with FAK−/+ autofluorescence (shaded peaks). (B) GFP-p190RhoGEF-mediated RhoA activation is dependent on FAK binding. GTP-bound RhoA was determined by GST-RBD pulldown assays from lysates of suspended and FN-replated FAK−/+ expressing GFP or the indicated GFP-p190RhoGEF constructs followed by blotting for RhoA expression. (C) FAK−/+ p190RhoGEF WT MEFs show enhanced FA formation and actin fibers similar to FAK−/− MEFs. Cells were plated onto 1-µg/ml FN–coated coverslips for 60 min and costained with antibodies to vinculin and Alexa Fluor 350 phalloidin for F-actin. Bar, 30 µm. (D) p190RhoGEF and GEFH1 promote FA formation in MEFs. Box-and-whisker plots of paxillin-positive–stained points within MEFs stably overexpressing the indicated GFP-p190RhoGEF or GFP-GEFH1 constructs upon FN plating for 60 min (n = 15 cells per point). (E) GEF-mediated increased FA formation is associated with decreased cell motility. MEFs overexpressing the indicated GFP-p190RhoGEF or GFP-GEFH1 constructs were evaluated for 10-µg/ml FN–stimulated Boyden chamber motility over 4 h. Means ± SD were determined from two experiments and are a percentage of FAK−/+ GFP MEFs (set to 100). Asterisks indicate that the difference between FAK−/+ GFP and FAK−/+ GFP–p190RhoGEF Δ1292-1301 or FAK−/+ GFP–p190RhoGEF Y1003A is significant (P < 0.05).
Y402-dependent signals from Pyk2, but not FAK, regulate p190RhoGEF expression in FAK
/H11002/HEFs and in PC12 cells. Although studies have identified p190RhoGEF as a protein
induced by CD40 stimulation of B cells (Lee et al., 2003), the important signaling pathways controlling either Pyk2- or CD40-induced p190RhoGEF expression remain to be defined.

In FAK
/H11002/HEFs, stable Pyk2 or p190RhoGEF knockdown restored normal FN-associated regulation of RhoA activity and FA formation but these reversals did not enhance cell motility. Therefore, deregulated RhoA activity likely contributes to but is not causal for FAK-null motility defects (Ren et al., 2000). Importantly, knockdown of GEFH1 that is closely related to p190RhoGEF did not reduce FA formation FAK
/H11002/HEFs, and this further supports the specificity of our findings. Importantly, Pyk2 or p190RhoGEF knockdown results in FAK
/H11002/MEFs with normal ruffling and lamellipodia formation but these cells exhibit tail retraction and severe motility defects. We speculate that either cell polarization (Tilghman et al., 2005) or rear FA-release defects (Ren et al., 2000) act to limit FAK
/H11002/Pyk2

RhoA activation (Fig. 10). Interestingly, FAK-null keratinocytes also display altered actin and FA formation. Pyk2 is expressed and activated in these cells but it remains unclear whether elevated Rho activity in the absence of FAK within keratinocytes is associated with a Pyk2-p190RhoGEF signaling linkage (Schober et al., 2007). p190RhoGEF is a ubiquitously expressed RhoA-specific GEF (Gebbink et al., 1997; van Horck et al., 2001). p190RhoGEF contains a central tandem Dbl-pleckstrin homology domain, an N-terminal leucine-rich region, a cysteine-rich zinc finger domain, a large C-terminal domain with a potential coiled-coil region shown to bind microtubules (van Horck et al., 2001), the 3’ untranslated region of neurofilament mRNA (Canete-Soler et al., 2001), and FAK (Zhai et al., 2003). In neuronal cells, p190RhoGEF possesses antiapoptosis activity (Wu et al., 2003), is implicated in the pathogenesis of motor neuron degeneration (Lin et al., 2005), and plays a negative role in axonal branching and synapse formation downstream of FAK (Rico et al., 2004). Through combined knockdown or transient overexpression studies, we found that kinase- and Y402-dependent signals from Pyk2, but not FAK, regulate p190RhoGEF expression in FAK
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GEFs are multidomain proteins regulated in a complex fashion (Bos et al., 2007). This can include protein–protein, protein–lipid, and second messenger binding and posttranslational modification of GEFs. These interactions or modifications can induce GEF translocation to sites of small G protein localization, autoinhibition release, or allosteric conformational activation of the catalytic region. The importance of FAK in p190RhoGEF regulation was supported by comparisons of MEFs overexpressing WT or the FAK-binding mutant (H90041292) of p190RhoGEF. Although p190RhoGEF WT partially localized to FAs, promoted RhoA activation, and enhanced FA formation, p190RhoGEF/H90041292 overexpression was cytoplasmic distributed and did not affect FN-associated RhoA activity or FA formation. Although it is also possible that the H90041292 deletion has global effects on catalytic domain folding, overexpressed GEFH1 was not FA localized but still functioned similar to p190RhoGEF WT in activating RhoA and promoting FA formation. These results support a model whereby p190RhoGEF binding to FAK facilitates its localization to and activation at FAs, whereas the related GEFH1 does not share this mode of regulation. Additionally, p190RhoGEF catalytic activity was also important in promoting FA localization through undefined mechanisms. As catalytically inactive (Y1003A) shRNA MEF motility. As transient FAK reexpression can rescue the motility defects of FAK−/− Pyk2 shRNA MEFs, stable reconstitution with WT FAK and various mutants will yield a powerful cell system to elucidate the molecular connections of FAK-mediated cell motility.

In normal MEFs, reduction in p190RhoGEF expression did not affect binding to or spreading on FN, but these cells formed fewer FAs under conditions of low RhoA activity. FAK+/− p190RhoGEF shRNA MEFs exhibited reduced motility on FN, supporting the notion that p190RhoGEF functions as an important regulator of RhoA activity and FA formation downstream of integrins and is needed for efficient cell movement. As FN-stimulated FAK and tyrosine phosphorylation was also reduced in FAK+/− p190RhoGEF shRNA MEFs (unpublished data) and contractility-associated mechanisms within cells can facilitate FAK recruitment to and activation at growing FAs (Kirchner et al., 2003), our results support the existence of a FAK−p190RhoGEF regulatory circuit controlling FA formation and cell motility (Fig. 10). Additionally, as FAK activation and paxillin phosphorylation can influence the distribution of these proteins at FAs (Zaidel-Bar et al., 2007), there may be other feedback mechanisms limiting FA formation upon p190RhoGEF knockdown in normal MEFs.

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p190RhoGEF could weakly colocalize with FA structures (unpublished data), we speculate that increased numbers of FAs formed by WT, but not p190RhoGEF Y1003A overexpression influences the equilibrium and distribution of both endogenous and exogenous p190RhoGEF localized to FAs in MEFs.

One additional level of p190RhoGEF regulation is that adhesion-dependent p190RhoGEF tyrosine phosphorylation was inhibited by both Δ1292-1301 and Y1003A mutations. Although it remains unclear how phosphorylation may affect p190RhoGEF function, this modification, as well as FAK binding, correlated with p190RhoGEF-mediated RhoA activation. Interestingly, p190RhoGEF WT did not localize to the many FAs in FAK−/− MEFs yet still functioned to enhance FAK−/− FA formation. As we find that p190RhoGEF binds to cytoplasmic-distributed Pyk2 within FAK−/− MEFs and is tyrosine phosphorylated upon FAK−/− MEF adhesion to FN, we hypothesize that p190RhoGEF activity is regulated in part by FAK and/or Pyk2 binding leading to enhanced p190RhoGEF tyrosine phosphorylation.

In summary, our studies have uncovered a novel role of p190RhoGEF as a mediator of aberrant FAK−/− MEF phenotypes and as an important regulator of FN-associated RhoA activation and FA formation in normal MEFs. As the formation of too many or too few FAs limit cell movement, future studies on the regulation of p190RhoGEF activity and the FAK−p190RhoGEF complex will yield important insights into the temporal balance of RhoA activity needed for cell migration.

Materials and methods

Antibodies and reagents

mAbs to phosphotyrosine (clone 4G10), vinculin (clone V284), and FAK (clone 4.47) were obtained from Millipore. mAbs to p190RhoGAP (clone 30), Pyk2 (clone 111), paxillin (clone M107), and phosphospecific paxillin pY3-118 (clone 30) were obtained from BD Biosciences. Rabbit polyclonal anti-RhoA (sc-179) was obtained from Santa Cruz Biotechnology, Inc. Texas red–phalloidin and Alexa fluor 350 phalloidin were obtained from Invitrogen. HA tag (clone 16B12), Myc tag (clone 9E10), GFP (clone B34), and Cre recombinase (clone 7.23) mAbs were obtained from Covance Research. Anti–β-actin (clone AC-17) mAb was obtained from Sigma-Aldrich. Rabbit polyclonal antibodies (7801/7802) were generated to mouse GEFH1, Cdc42, and Rac1 (University of California, San Diego). Antibodies were affinity purified using GST-p190RhoGAP (1457–1582) covalently coupled to glutathione agarose (Sigma-Aldrich) using dimethylimidelinate as described previously (Sieg et al., 1998). Antibody specificity was confirmed by Western blot analysis combined with soluble GST-p190RhoGAP (1457–1582) as a competitive inhibitor. pCDNA3-Y1003A p190RhoGAP was provided by J. Lee (Ewha Womans University, Seoul, Korea). Purified bovine plasma FN was obtained from EMD.

Lentiviral constructs

shRNA constructs were created using pLentiLox3.7 as previously described (Rubinson et al., 2003). Human FAK shRNA was created and used as previously described (Mitra et al., 2006a). For mouse Pyk2 and p190RhoGAP, sense oligos used were as follows: Pyk2, 5′-GAA AGT AGT ACG TCT AAG GAG GAA ACT TTT TC-3′; and p190RhoGAP, 5′-GGA ACA GAG ATC ATC TAG TAT TTT ACA TTA TTT TAC-3′. The 5′ end belongs to the U6 promoter and lowercase letters represent components of the shRNA loop. The 3′ end was added to generate a Xhol site. Antisense oligo used were as follows: Pyk2, 5′-ctg aga cgg aat gaa gaa tag tgc gtt gta gaa gaa aag agg att tgc gta gat tag gag aag gaa gag taa tag ttc gaa taa tag ttt c-3′; p190RhoGAP-1, 5′-ctg aga cgg aag agg aag gag gat gat att cag ttc tta gta ct-3′; p190RhoGAP-2, 5′-ctg aga cgg aag agg aag gag gat gat att cag ttc tta gta ct-3′; and p190RhoGAP-3, 5′-ctg aga cgg aag agg aag gag gat gat att cag ttc tta gta ct-3′.
aga aac aaG AAC CAG ACT GTA ATA TCT tct gaa AGA TAT TAC AGT CTG GTT Ca-3; and Scr, 5'-agc aga aac aaT CCT CGG AAC GTG TCA GTG tct ett aoa ACG TGA CAC GTT CGG AAG Ca-3. An additional four nucleotides at the 5' end were added to generate Xho site. pShuttle3.7 expresses the shRNA and GFP from distinct promoters, and virus production was achieved by 293T cell transfection as previously described (Mitra et al., 2006a). Media containing viruses was used to infect target cells after 48 h. To create N-terminal GFP fusions of p190RhoGEF WT, p190RhoGEF Y1003A and Δ129-1301 RhoGEF (Zhai et al., 2003) were digested with KpnI and XbaI and cloned into the same sites within pEGFP-C2. For lentiviral expression, GFP-p190RhoGEF was removed by NheI-XbaI digestion and cloned into NheI-Swal-digested pCDH1-MSC1 (System Biosciences). GFP-GEFH1 WT and catalytically inactive Y393A (Krendel et al., 2005) were removed from pCMV5 by EcoRI-Cla digestion and blunt-end cloned into pCDH1-MSC1. Viruses were produced by 293T cell transfection, concentrated by polyethylene glycol precipitation, and used to infect target cells.

RT-PCR
Total RNA was extracted with Trizol reagent (Invitrogen) and cDNA was synthesized from 5 μg using SuperScript first-strand synthesis system for RT-PCR (Invitrogen). A 1-μl aliquot of the reverse-transcription reaction was then used for subsequent PCR amplifications. Primers used were the following: p190RhoGEF forward, 5′-GAGCAAGGAACTGACAACTG-3′; p190RhoGEF reverse, 5′-CCGACTCGGACGATCTTG-3′; β-actin forward, 5′-TCGAAAGGTGAGGAGGATTG-3′; and β-actin reverse, 5′-TTTGATGTCAGCCAGTCTCC-3′. p190RhoGEF was amplified for 30 cycles and primers for actin were added after cycle 10.

Cell culture and viruses
FAK−/− mice (Beggs et al., 2003) were obtained from H. Beggs (University of California, San Diego, San Francisco, CA) and were crossed with p53-null mice (Jilic et al., 1995). Embryonic day–8.5 mouse embryos were isolated, placed within a drop of Matrigel (BD Biosciences), and cultured for 10 d. MEFs that grew out of embryos were disaggregated, plated onto 10-μg/ml FN-coated dishes, and expanded. At passage two, 1/3 of MEFs were used for genomic DNA isolation genotype confirmation, 1/3 were expanded, and 1/3 were transduced with Ad-Cre recombinase at 50 MOI. Cre protein expression was detected at 48 h of infection but was undetectable after 7 d and subsequent passages. FAK−/−, FAK−/+, and FAK-reconstituted (DP3) MEFs are p53-null and were maintained as described previously (Sieg et al., 1999). Human diploid fibroblasts and PC12 cells were obtained from American Type Culture Collection. Human fibroblasts were grown in the same media as mouse MEFs (Sieg et al., 1999). PC12 cells were grown in DME with 10% FBS. At 24-h intervals, two wells were trypsinized and individually enumerated using an automated hemocytometer. Each experiment was repeated three times.

Analysis of variance was used to determine statistical significance for proliferation and motility assays.

Immunofluorescent staining
Cells were plated onto 10-μg/ml FN-coated glass coverslips in the absence of serum for the indicated times. Cells were fixed in 3.7% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 10 min. Blocking was performed with 100 μg/ml ChromPure donkey IgG in PBS (Jackson ImmunoResearch Laboratories) at room temperature for 1 h. Vinculin (1:50) or paxillin (1:50) antibodies were diluted in PBS and incubated overnight at 4°C. After three washes in PBS, coverslips were incubated with anti–mouse IgG and fluorescein-conjugated phalloidin to visualize actin stress fibers. In cells expressing GFP or GFP fusion proteins, actin was visualized with Alexa Fluor 350 phalloidin. After washing, coverslips were mounted in Vectashield (Vector Laboratories). Images from wide UV (excitation, 330–385 nm; dichroic mirror, 400 nm; emission, 420 nm) and chloro-charge coupled device camera (ORCA ER; Hamamatsu), an in-duced confocal laser energy at 405 nm, and a monochromator were sequentially captured at 60× (UPLFL objective, 1.25 NA; Nikon TE300) with a heated and motorized stage. For each experiment, 12 individual cell fields were recorded. Cell trajectories were measured by tracking the position of the cell nucleus over time. Cells that did not spread or remain within the field of view during the experimental period were not evaluated. For cell proliferation assays, 5 × 103 cells were plated into 6-well plates precoated with 0.5% gelatin and grown in DME with 10% FBS. At 24-h intervals, two wells were trypsinized and individually enumerated using an automated hemocytometer. Each experiment was repeated three times. One-way analysis of variance was used to determine statistical significance for proliferation and motility assays.

Online supplemental material
Fig. S1 shows scratch-wound healing motility defects of FAK−/−/Pyk2 shRNA MEFs. Fig. S2 shows time-lapse video analyses of random MEF motility. Fig. S3 shows that GEFH1 knockdown does not affect FN-associated FA formation. After 48 h, GEFH1 knockdown was confirmed by blotting (Birkenfeld et al., 2007). Cells were starved overnight, trypsinized, suspended for 1 h, and plated onto coverslips coated with 10-μg/ml FN for 1 h. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained for actin and paxillin using standard methods.

Cell migration and proliferation assays
For cell migration assays, cells were used 48 h after Ad infection and serum starved in 0.5% FBS overnight. Millicell chambers with 8-μm pores were coated on the both sides with 10 μg/ml FN for 2 h at room temperature. Membranes were washed with PBS and air dried for 2 h. Cells were suspended by limited EDTA-trypsin treatment, washed by 0.25 mg/ml soybean trypsin inhibitor in DME, suspended in Migration medium (0.5% BSA in DME), and counted. 5 × 104 cells in 0.3 ml held in suspension for 1 h was added to each MilliCell chamber and incubated for 4 h at 37°C. Cells were fixed and stained with crystal violet. Each data point represents three individual chambers enumerated as previously described (Hisia et al., 2005). For scratch-wound assays, cells were seeded in six-well plates and starved for 24 h. Cells were wounded by scratching with pipette tip, washed with PBS, photographed (0-h point), and placed into growth media containing 0.5 μg/ml mitomycin-C to block cell division. After 16 h, matched-pair wound regions were photographed. For phase-contrast, cells were microwaved by DME with 10-g/ml FN-coated glass coverslips in growth media. After 3 h, images were collected with a charge coupled device camera (Orcia 285; Hamamatsu) at 5-min intervals over 4 h with a 20× lens on an automated (Metamorph 6.3; Universal Imaging Corporation) microscope (Nikon TE300) with a heated and motorized stage. For each experiment, 12 individual cell fields were recorded. Cell trajectories were measured by tracking the position of the cell nucleus over time. Cells that did not spread or remain within the field of view during the experimental period were not evaluated. For cell proliferation assays, 5 × 103 cells were plated into 6-well plates precoated with 0.5% gelatin and grown in DME with 10% FBS. At 24-h intervals, two wells were trypsinized and individually enumerated using an automated hemocytometer. Each experiment was repeated three times. One-way analysis of variance was used to determine statistical significance for proliferation and motility assays.

Cell lysis, immunoprecipitation, blotting, and pulldown assays
Cells were washed in ice-cold PBS and solubilized in modified Ripa lysis buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS (Sieg et al., 1998). For immunoprecipitations, 2.5 μg of antibodies were incubated with lysates (each sample containing 500–1,000 μg of total protein) for 3 h at 4°C and collected by binding to protein G plus or protein A–agarose beads. SDS-PAGE, antibody blotting, and sequential membrane rephrasing was performed as previously described (Sieg et al., 1998). Rho activity was measured by pulldown assays using GST–Rhotekin Rho binding domain (GST-RBD) (Ren et al., 2000). Clarified Ripa cell lysates were incubated with 20 μg GST-RBD beads at 4°C for 45 min and washed four times with buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml PMSF) Bound proteins were eluted using a solution of 50 μg/ml glyceraldehyde and phosphocyanin A. Blotting Image J version1.37 (National Institutes of Health) was used to obtain densitometric values from blots, and Rho activity was determined as the amount of RBD-bound Rho versus total Rho in the lysate.


