FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts

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Summary

A key step in cell migration is the dynamic formation and disassembly of adhesions at the front and the concomitant movement and release of adhesions in the rear of the cell. Fibroblasts maintained in the absence of serum have stable adhesions within the rear of the cell and exhibit reduced trailing-edge retraction resulting in an elongated cell phenotype. Addition of lysophosphatidic acid (LPA) induced the movement of adhesions and retraction of the trailing edge, thus mimicking tail retraction in a migrating cell. Focal adhesion kinase (FAK), guanine nucleotide exchange factors (GEF) for Rho and the Rho effector Rho kinase II (ROCKII) are crucial for the regulation of adhesion movement and trailing-edge retraction. Downregulation of FAK by small interfering RNAs or small hairpin RNAs blocked LPA-induced adhesion movement and restoration of cell shape. This phenotype was rescued by the ectopic expression of PDZ-RhoGEF or a RhoA-effector-domain mutant that activates ROCK. Knockdown of PDZ-RhoGEF or ROCKII inhibited LPA-induced trailing-edge retraction and adhesion movement. Moreover, overexpressed PDZ-RhoGEF co-immunoprecipitated with FAK and localized to FAK-containing adhesions. These studies support a model in which FAK and PDZ-RhoGEF cooperate to induce Rho/ROCKII-dependent focal adhesion movement and trailing-edge retraction in response to LPA.

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Introduction

Fibroblast migration is a highly regulated process that leads to a distinct migratory polarity (Ridley et al., 2003). Protrusion of the leading edge and the establishment of new peripheral adhesions coupled with the maintenance of stable attachments at the cell rear create the typical asymmetrical profile of a migrating cells with a broad lamellipodia (front) and narrow long trailing edge (rear) (Regen and Horwitz, 1992). As the lamellipodia advances and the trailing edge stretches the cell becomes elongated. Inward movement and subsequent release of adhesions at the rear leads to trailing-edge retraction and cell body advance whereupon the cell becomes more rounded (symmetrical) and the cycle is repeated (Rid et al., 2005; Smilenov et al., 1999; Webb et al., 2005). Fibroblast movement requires spatial regulation of cell attachment to extra cellular matrix (ECM). Adhesions are formed at sites of lamellipodial protrusion. Once formed, these adhesions must be strong enough to generate traction forces necessary to support translocation of the cell body. However, the trailing edge of the cells must be able to detach from the substrate to permit cell translocation (Regen and Horwitz, 1992). Detachment of the trailing edge requires continued myosin-driven cytoskeletal tension that results in movement and ultimate release (disassociation/degradation) of adhesions within the trailing edge (Palecek et al., 1998; Palecek et al., 1996; Rid et al., 2005; Smilenov et al., 1999). The nature of the signals that promote dissociation/degradation of the adhesions in the trailing edge is poorly understood.

Lysophosphatidic acid (LPA) stimulation of several cell types induces tyrosine phosphorylation and activation of focal adhesion kinase (FAK) (Bian et al., 2006; Luttrell et al., 1997; Seufferlein and Rozengurt, 1994). FAK is a non-receptor tyrosine kinase that is associated with adhesions (Hildebrand et al., 1993; Schaller et al., 1992), sites of close contact between the cell and the extracellular matrix. FAK is activated in response to integrin engagement and its phosphorylation leads to the recruitment and activation of Src, and the induction of several downstream signaling pathways (Parsons, 2003; Parsons et al., 1994). In addition FAK serves as a scaffold for the recruitment of the linker protein p130Cas (BCAR1) (Burnham et al., 1996), and the two GTPase-activating proteins ASAP1 (ARF GTPase-activating protein 1, DDEF1) (Liu et al., 2002) and GRAF (GTPase regulator associated with focal adhesions) (Hildebrand et al., 1996). Thus, FAK functions to recruit proteins that contribute to the regulation of adhesion signaling and promotes the dynamic remodeling of adhesions by triggering adhesion disassembly through a Src- and MEK-dependent cascade (Webb et al., 2004). Cells deficient for FAK exhibit defects in adhesion disassembly (Webb et al., 2004) and Rho regulation upon integrin (Ren et al., 2000) or LPA receptor stimulation (Palazzo et al., 2004)

In the present study, we investigate the role of LPA, FAK and PDZ-RhoGEF [PDZ-domain-containing Rho guanine nucleotide exchange factor; PDZ RhoGEF (human); GTRAP48 (rat)] in the regulation of adhesion movement, trailing-edge retraction and cell-shape change. We observed that maintaining fibroblasts in the absence of serum results in stabilization of adhesions within the rear of the cell, and significantly reduced trailing-edge retraction resulting in an elongated cell phenotype. LPA but not PDGF was sufficient to restore trailing-edge retraction and to increase inward movement of adhesions, and to restore cell shape. Thus, the response of serum-deprived cells to LPA parallels events that lead to tail retraction in a migrating cell. Using this paradigm, we provide evidence that LPA induces dynamic movement of adhesions present in the trailing edge, and this process requires function of FAK, PDZ-RhoGEF and Rho/Rho kinase II (ROCKII). This study provides evidence for the spatial activation of an LPA-receptor–FAK–PDZ-RhoGEF signaling complex and suggests that modulation of adhesion movement within the trailing edges of fibroblasts is linked to Rho/ROCKII-dependent retraction.

Results

LPA stimulates trailing-edge retraction and rear-adhesion dynamics

NIH3T3 cells, cultured on fibronectin and deprived of serum for 6 hours, acquired a distinct morphology characterized by a protrusive lamellipodia and an elongated tail-like structure, which was approximately four times longer than control cells deprived of serum but then treated with LPA (Fig. 1A). Interestingly, the rate of lamellipodia protrusion (as visualized by actin-rich structures in βactin-expressing cells) was the same in cells kept without serum and stimulated with LPA (Fig. 1A). Cells that had been serum-starved overnight (12-16 hours) also displayed an elongated phenotype with substantially less protrusive activity (Fig. 1B). Stimulation of serum-starved NIH3T3 cells with 2 μM LPA induced the retraction of the elongated tail and restored normal cell morphology, whereas addition of 100 nM PDGF was much less effective at inducing normal morphology (Fig. 1B). Time-lapse imaging of serum-starved cells stimulated with 2 μM LPA revealed that restoration of the normal rounded morphology occurred with a $t_{1/2}$ of 90-120 minutes (Fig. 1C and supplementary material Movie 1). To assess whether the LPA induced trailing-edge retraction was reflected in changes in the dynamic behavior of adhesions, TIRF microscopy was used to monitor focal adhesions labeled with paxillin conjugated to GFP (paxillin-GFP) in cells serum starved overnight and in LPA-stimulated cells. As shown in Fig. 2A and supplementary material Movie 2, adhesions present in the rear of serum-starved cells did neither exhibit a change in fluorescence intensity nor in position, indicating that these adhesions were relatively stable. By contrast, LPA stimulation substantially increased the dynamics of adhesions, as measured by the inward

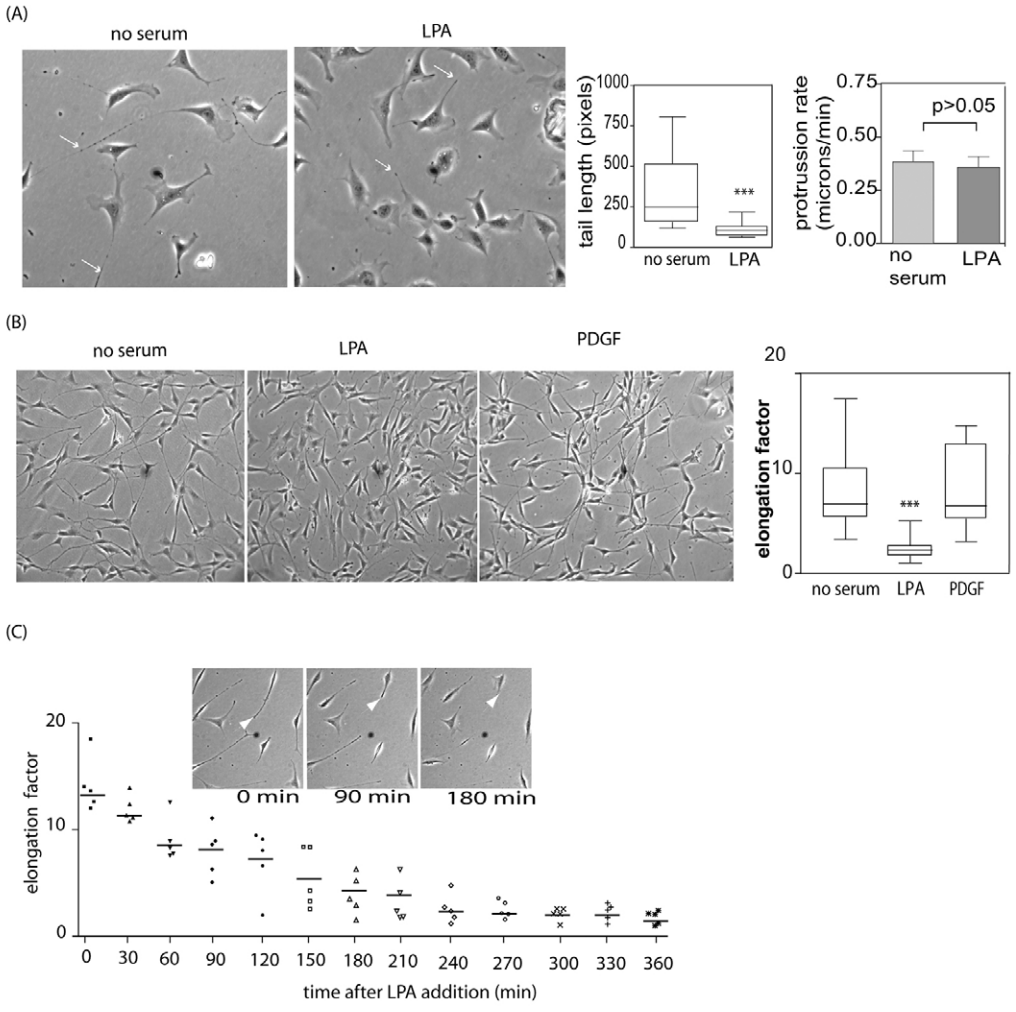

Fig. 1. Serum starvation of NIH3T3 cells results in failure of trailing-edge retraction. (A) Length of the trailing edge and protrusion rates in NIH3T3 cells stably expressing βactin-mRFP cultured without serum or without serum but plus 2 μM LPA. For trailing-edge measurement, cells were filmed for 6 hours immediately after serum removal with images acquired at 5-minute intervals, using phase-contrast microscopy. Images acquired at the end of the sixth hour were quantified are shown. Data represent the analysis of 60 cells from two different experiments. For protrusion of the lamellipodia, cells were starved for 6 hours and at the end of sixth hour, images of βactin-mRFP in protruding lamellipodia were obtained for 20 minutes at 20-second intervals. The data represent analysis of six protruding lamellipodia per experimental condition. (B) Phase-contrast images and quantification of cell elongation of serum-starved cells, serum-starved cells treated with 2 μM LPA or 100 nM PDGF. Data represent the analysis of 50-72 cells per condition from two different experiments. (C) Kinetics of changes in cell elongation following LPA stimulation of serum-starved fibroblasts.

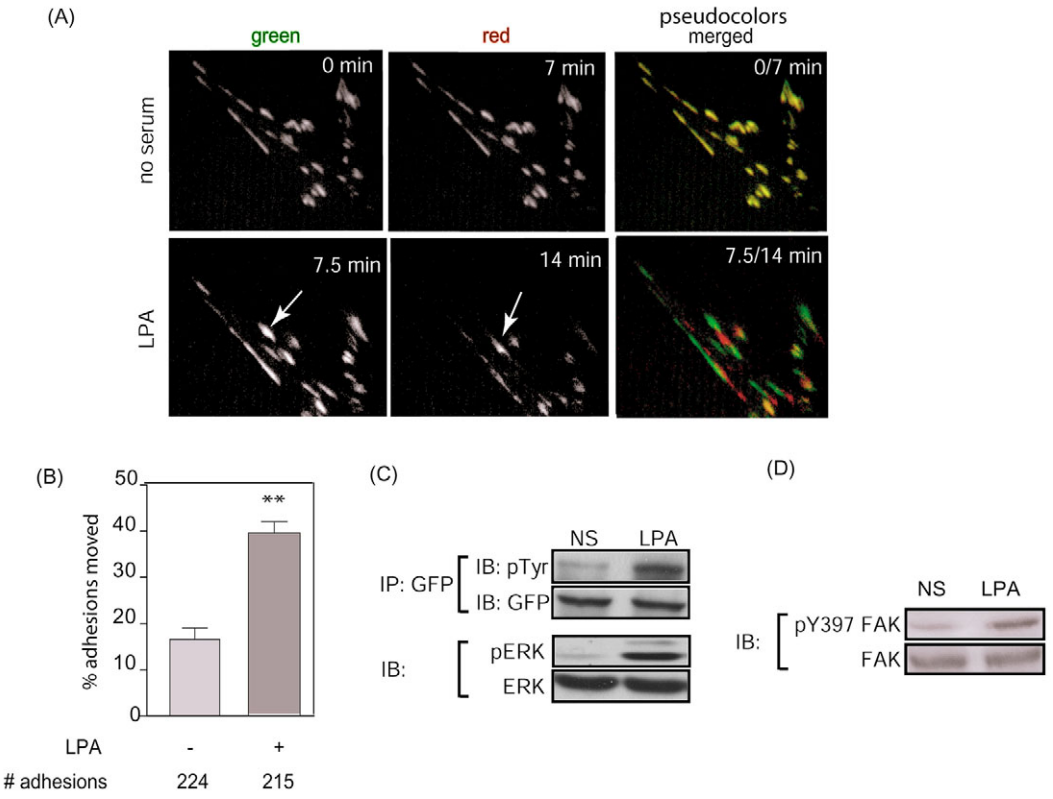

Fig. 2. LPA stimulation of adhesion movement. Rat2 or NIH3T3 cells stably expressing GFP-FAK or paxillin-GFP were plated in the presence of serum-containing (10%) medium on dishes coated with 1 μg/ml fibronectin. The following day, cells were placed in serum-free medium for 12 hours and subsequently stimulated with 2 μM LPA. (A) Analysis of adhesion movement in response to LPA. Serum starved NIH3T3 cells stably expressing paxillin-GFP were stimulated with 2 μM LPA and the movement of adhesions was analyzed using TIRF microscopy as described in Materials and Methods. Cells were filmed for 7 minutes before addition of LPA and 7 minutes after addition of LPA (arrows indicate representative adhesions). Adhesions present at time 0 were pseudo-colored green (left panels) and adhesions present at 7 minutes were colored red (middle panels). Movement of the individual adhesions was revealed using the merged images (right panels) and analyzed as described in Materials and Methods. (B) Quantification of adhesion movement by LPA in NIH3T3 cells expressing paxillin-GFP was determined as described in Materials and Methods. The experiment was performed three times, and five to seven cells were analyzed per experimental condition. (C) Western blot analysis of FAK activation by LPA in Rat2 cells stably expressing GFP-FAK. Cells were stimulated with LPA for 5 minutes and GFP-FAK phosphorylation was assessed using antibody against phosphorylated tyrosine (pTyr) as described in Materials and Methods. The increase in activation of MAP kinase was determined by immunoblotting using antibodies against phosphorylated and total ERK (pERK and ERK, respectively). (D) LPA activation of endogenous FAK in Rat2 cells. Cells were stimulated with LPA for 5 minutes and western blots were probed with antibody against phosphorylated Tyr397 (pY397) or anti-FAK antibody as described in Materials and Methods.

movement of the adhesions over a 7-minute time period (Fig. 2A,B and supplementary material Movie 3). As previously reported the addition of LPA to serum starved cells induced a rapid increase in the tyrosine phosphorylation of FAK (Fig. 2C,D). These data indicate that LPA signaling is sufficient to induce changes in the movement of mature adhesions found in the trailing edge and that the activation of FAK is concomitant with this process.

Lack of FAK expression in fibroblasts results in an elongated phenotype in the presence of serum, and failure to induce adhesion movement and trailing-edge retraction

The knockdown of FAK using either small interference RNA (siRNA) designed to specifically target rat FAK (Tilghman et al., 2005) or small hairpin RNA (shRNA) to specifically target mouse FAK resulted in an elongated cell morphology (Fig. 3A,B) when cells were grown in the presence of serum. To confirm that the observed elongation of the cells was due to the loss of FAK expression, a Rat2 cell line was created that constitutively expressed (at ~20% the level of endogenous FAK) chicken FAK tagged to GFP (GFP-FAK), which was not knocked down by siRNA targeting endogenous FAK (Fig. 3C). The morphology of cells expressing GFP-FAK was indistinguishable from control cells expressing a LacZeo transgene (Fig. 3D). Whereas FAK siRNA treatment (targeting rat FAK) increased cell elongation in control Rat2 cells, the GFP-FAK-expressing cells retained their more rounded morphology (Fig. 3D). FAK immunostaining revealed that, in control cells, FAK silencing resulted in the absence of endogenous FAK in adhesions (Fig. 3D). By contrast, FAK siRNA treatment of Rat2 cells expressing GFP-FAK did not decrease the expression of GFP-FAK or alter its localization to adhesions (Fig. 3D). Therefore, the changes in cellular morphology were a consequence of reduced FAK expression and correlated with the loss of FAK from cell matrix adhesions.

To examine whether FAK functions downstream of LPA to stimulate adhesion dynamics and trailing-edge retraction, Rat2 cells that stably expressed paxillin-GFP were treated with control or FAK siRNA. Cells were serum starved for twelve hours and stimulated with 2 μM LPA, and adhesion dynamics were measured using TIRF microscopy (Fig. 4A). LPA readily induced dynamic movement of adhesions in control cells but failed to do so in FAK-siRNA-treated cells (Fig. 4B). This result is consistent with the failure of FAK-siRNA-treated cells to retract trailing edge in response to LPA.

Consistent with these observations, addition of serum or LPA to serum-starved control cells induced restoration of cell shape whereas serum-starved FAK-knockdown cells largely maintained their elongated morphology in response to either serum or LPA (Fig. 5). These observations are consistent with LPA controlling trailing-edge retraction and changes in cell shape through a receptor pathway that requires FAK activation and downstream signaling.

Overexpression of specific RhoA-pathway components rescues the FAK-knockdown phenotype

To determine the downstream effectors for FAK that contribute to adhesion movement and trailing-edge retractions, we assessed the role of RhoGEFs and putative Rho effectors. The RhoGEF, PDZ-RhoGEF has been shown to be an important effector of the LPA receptor signaling pathway (Yamada et al., 2005). To investigate the role of this GEF, Rat2 cells treated with FAK siRNA to block endogenous FAK expression were transfected with Myc-tagged PDZ-RhoGEF (Myc-PDZ-RhoGEF), Myc-tagged PDZ-RhoGEF lacking Rho Dbl homology (DH) and pleckstrin homology (PH) domains [Myc-PDZ-RhoGEF(1-585)] and Myc-tagged full-length p115RhoGEF (Myc-p115RhoGEF). The expression level of each of these three Rho GEFs was similar (Fig. 6A) and the localization of GEFs was consistent with previously published data (Banerjee and Wedegaertner, 2004). Both PDZ-RhoGEF and PDZ-RhoGEF(1-585) but not p115RhoGEF localized to the actin cytoskeleton (data not shown). As shown in Fig. 6B, the expression of wild-type PDZ-RhoGEF but not PDZ-RhoGEF(1-585) or full-length p115RhoGEF restored changes in cell shape induced by treatment of Rat2 cells with FAK siRNA. These data indicate that Rho activation by PDZ-RhoGEF is required downstream of FAK

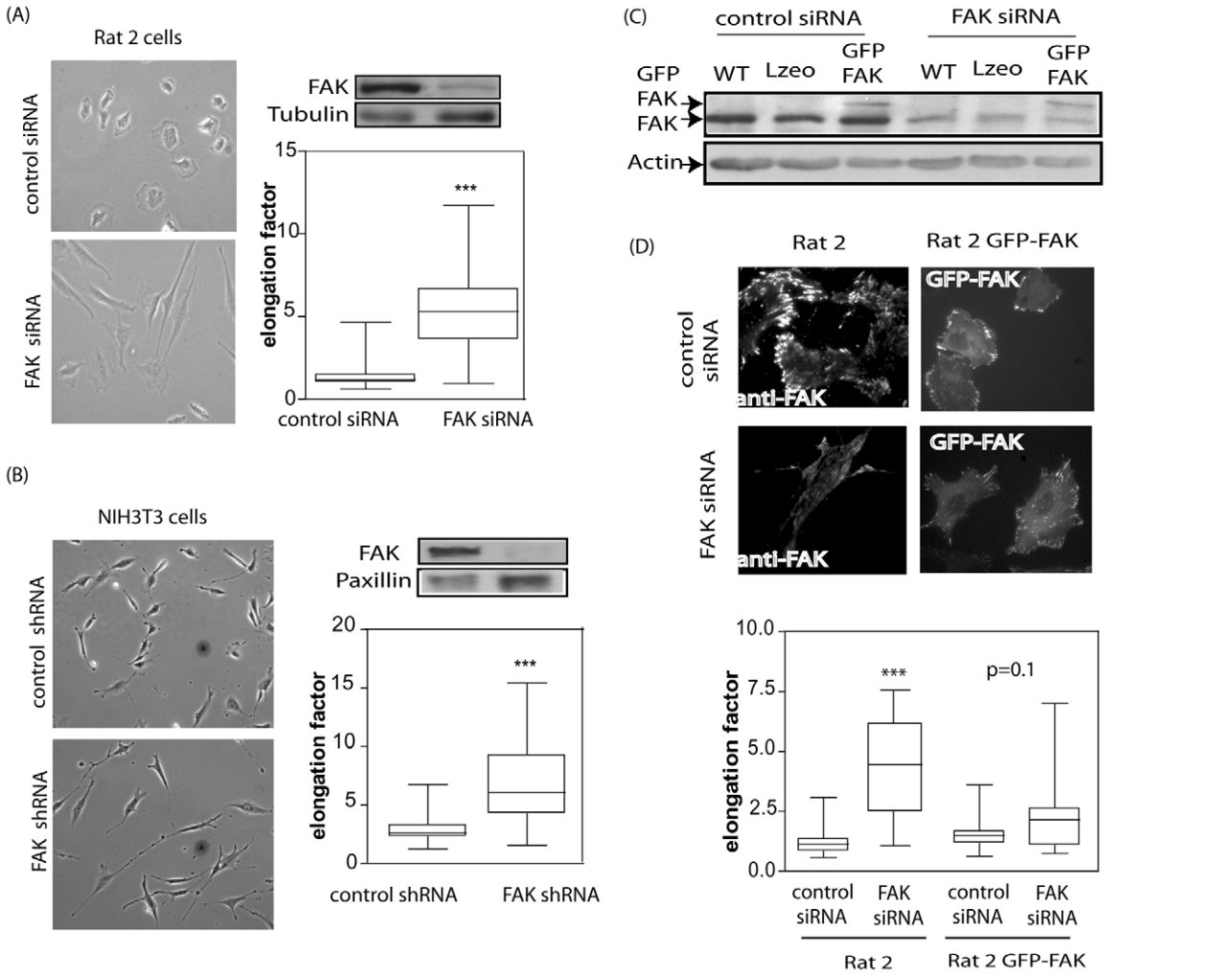

Fig. 3. Knockdown of FAK expression induces cell elongation. (A,B) Phase-contrast images, western blot analysis of FAK protein expression and quantification of cell elongation in control cells, FAK-siRNA treated Rat2 cells (A) or FAK-shRNA treated NIH3T3 cells (B) . The extent of FAK knockdown was determined by western blotting and compared with expression of paxillin. (C) Western blot analysis of FAK expression in wild-type Rat2 cells, Rat2 cells expressing LacZeo and Rat2 cells expressing GFP-tagged chicken FAK. Endogenous FAK expression was substantially reduced in wild-type (wt), control-transfected cells (Lzeo) and in cells expressing GFP-tagged chicken FAK (GFP-FAK) following treatment with FAK siRNA (right three lanes) as compared with control siRNA-treated cells (left three lanes).The level of expression of GFP-tagged chicken FAK remained unchanged upon treatment with either control or FAK siRNA. Actin levels indicate the equal loading of sample in each lane. (D) Expression of GFP-FAK decreases cell elongation following FAK knockdown using siRNA. The images on the left illustrate the reduced immunostaining of FAK observed in cells treated with FAK siRNA. The images on the right illustrate the continued expression of GFP-tagged chicken FAK (GFP-FAK) in cells treated with control and FAK siRNA. Graphs panels A, B and D show cell-shape measurements that were assessed by determining the elongation factor for cells treated with either siRNAs or shRNAs.

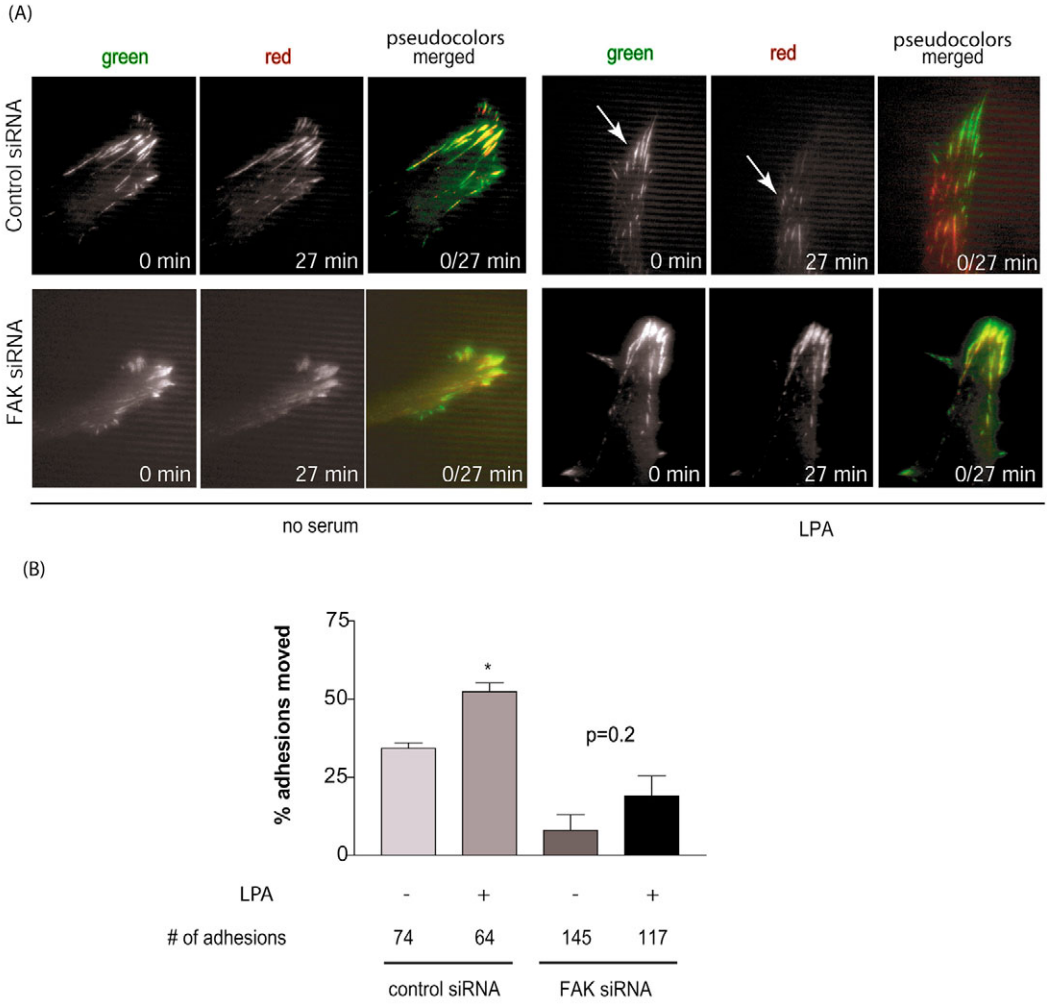

Fig. 4. LPA-induced adhesion movement is FAK dependent. (A) Analysis of adhesion movement in response to 2 μM LPA. Control or FAK-siRNA-treated Rat2 cells stably expressing paxillin-GFP were plated on fibronectin-coated glass T dishes i medium containing 10% serum. The following day, cells were serum-starved for 12 hours and stimulated with 2 μM LPA. Adhesion movement was analyzed using TIRF microscopy as described in Fig. 3. Cells were filmed for 27 minutes before the addition of LPA and 27 minutes after the addition of LPA. (B) Quantification of dynamic adhesions was assessed as described in Materials and Methods (arrows indicate representative adhesions). The experiment was performed three times, six to eight cells were analyzed per experimental condition.

in regulating adhesion dynamics that are necessary for rear retraction and restoration of cell shape. To assess which putative Rho effectors might be important for the restoration of cell shape in FAK-deficient NIH3T3 cells, we expressed two different active Rho-effector-domain mutants (Palazzo et al., 2001; Tosello-Trampont et al., 2003) in NIH3T3 cells whose FAK expression was downregulated (Fig. 6C). As shown in Fig. 6D, expression of a GFP-tagged RhoA effector mutant (RhoAG14VF39V) that can activate both mammalian diaphanous (DIAPH1; hereafter referred to as mDia) and ROCK efficiently rescued the elongated phenotype induced by FAK deficiency. By contrast, the negative control GFP-tagged PDZ-RhoGEF(561-585) [GFP-PDZ-RhoGEF(561-585)], the actin-binding domain of PDZ-RhoGEF fused to GFP or a Rho effector mutant that activates only mDia (RhoAG14VF39A), all failed to rescue the elongated phenotype in FAK-deficient cells (Fig. 6D). These data indicate that FAK and PDZ-RhoGEF cooperate to activate the Rho/ROCK pathway and induce trailing-edge retraction.

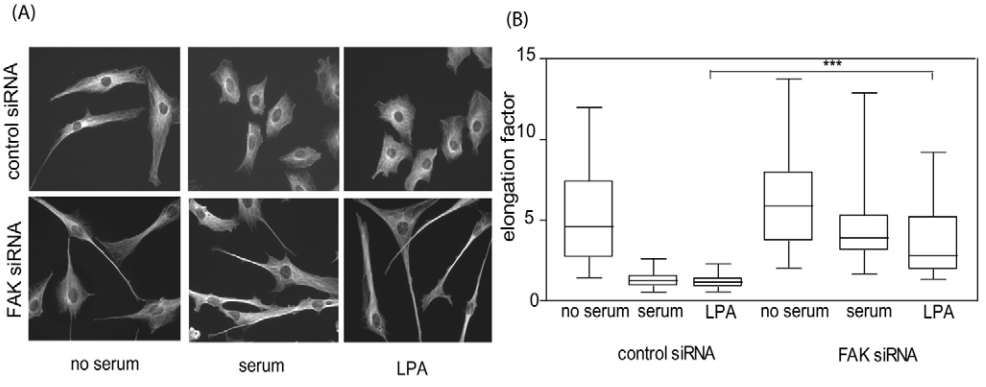

Fig. 5. FAK is required for LPA-mediated induction of trailing-edge retraction and the restoration of normal morphology. (A) Representative images of and α-tubulin staining of control and FAK-siRNA-treated cells that were either serum starved, or serum starved and stimulated with 10% serum or 2 μM LPA. (B) The elongation factor for siRNA-treated cells was determined after treating cells that had been serum starved overnight with serum or with LPA for 7 hours. The experiment was repeated twice and 50-60 cells were analyzed per condition.

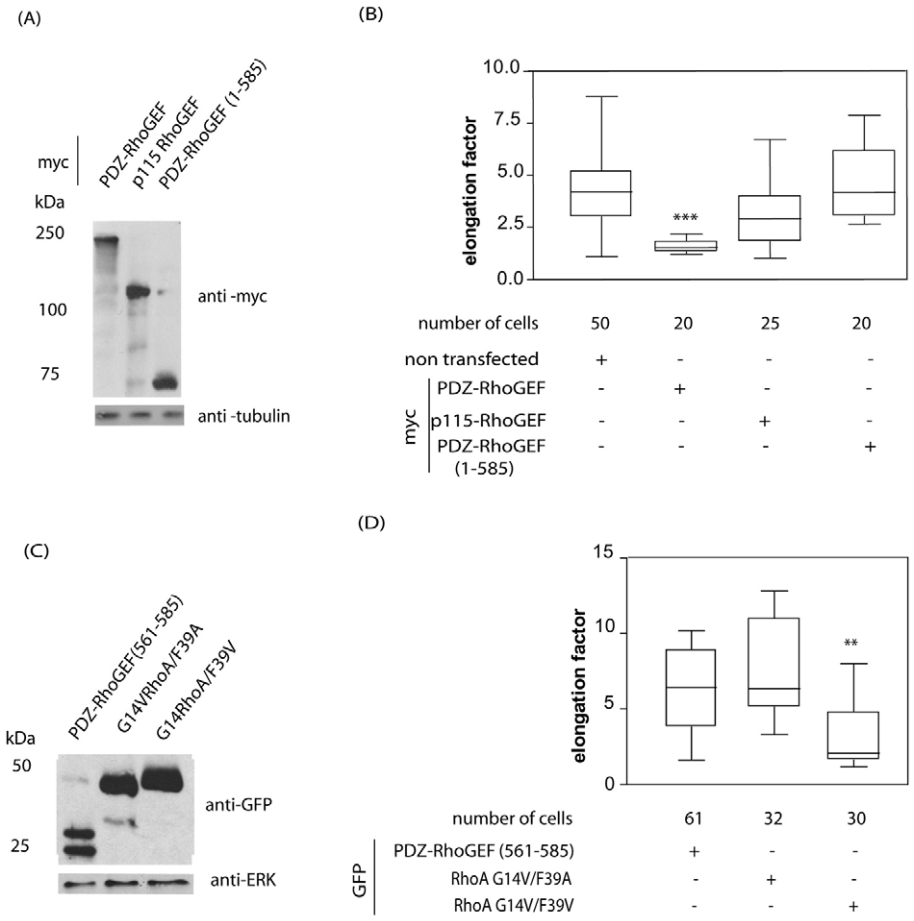

Fig. 6. Ectopic expression of PDZ-RhoGEF and RhoA-effector-domain mutant rescues elongation induced by knockdown of FAK expression. (A) NIH3T3 cells were transiently transfected with Myc-PDZ-RhoGEF, Myc-p115RhoGEF and Myc-PDZ-RhoGEF(1-585). Twenty-four hours after transfection, lysates were prepared and blotted with anti-Myc antibody. (B) Quantification of cell elongation. The shape of FAK-siRNA-treated cells that express different RhoGEF constructs was determined by measuring the elongation factor for individual cells. (C) FAK-shRNA-treated cells were transiently transfected with control non-functional PDZ-RhoGEF(561-585)-GFP , G14VRhoA/F39A-GFP or G14VRhoA/F39V-GFP. Twenty-four hours after transfection, lysates were prepared and blotted using anti-GFP antibody. The expression of GFP-tagged proteins was determined by immunoblotting individual lysates with anti-GFP and with anti-ERK antibodies to ensure equal loading of lysate samples. (D) Quantification of cell elongation in FAK-shRNA-treated cells expressing different RhoA-effector-domain mutants.

PDZ-RhoGEF–Rho/ROCK signaling is necessary for LPA-induced trailing-edge retraction and stimulation of adhesion dynamics

We next sought to determine whether PDZ-RhoGEF forms a complex with FAK and localizes to FAK-containing adhesions. Myc-PDZ-RhoGEF(1-585) or Myc-p115RhoGEF was overexpressed in HEK293T cells, as was GFP-FAK. As shown in Fig. 7A, GFP-FAK was readily detected in immune complexes with Myc-PDZ-RhoGEF(1-585) but not in immune complexes with Myc-p115RhoGEF. To test whether FAK and PDZ-RhoGEF co-localized in adhesions we overexpressed Myc-PDZ-RhoGEF, Myc-PDZ-RhoGEF(1-585) and Myc-p115RhoGEF in Rat2 cells stably expressing GFP-FAK. We observed that Myc-PDZ-RhoGEF, Myc-PDZ-RhoGEF(1-585) but not Myc-p115RhoGEF localized to FAK containing adhesions (Fig. 7B). To confirm the requirement for PDZ-RhoGEF in a signaling pathway that controls cell elongation downstream of the LPA receptor, Rat2 cells were treated with PDZ-RhoGEF siRNA to attenuate PDZ-RhoGEF expression (Fig. 7C). PDZ-RhoGEF siRNA-treated cells exhibited elongated phenotype even in the presence of serum (Fig. 7C, phase images); a phenotype comparable to that observed in FAK-siRNA-treated cells. Stimulation of serum-starved control and PDZ-RhoGEF-siRNA-treated cells with 2 μM LPA revealed that the PDZ-RhoGEF-siRNA-treated cells remained elongated (Fig. 7D). TIRF microscopy was used to measure adhesion dynamics in LPA-stimulated and serum-starved control and PDZ-RhoGEF-siRNA-treated Rat2 cells stably expressing paxillin-GFP (Fig. 8A). As shown in Fig. 8B LPA stimulation of control cells but not PDZ-RhoGEF-siRNA-treated cells induced adhesion dynamics (Fig. 8B). These data provide additional support for the role of PDZ-RhoGEF in a FAK-dependent pathway that contributes to the regulation of rear retraction and adhesion movement.

Since, the Rho-effector-domain mutant that can not activate ROCK failed to rescue the FAK knockdown phenotype, we reasoned that shRNA-mediated knockdown of ROCKI and or ROCKII would prevent LPA from stimulating adhesion movement and trailing-edge retraction in response to LPA. As shown in Fig. 9A, shRNA-mediated knockdown of ROCKII expression (but not ROCKI expression) induced cell elongation in the presence of serum. The phenotype of ROCKII-knockdown cells, however, was distinct from that observed for FAK-knockdown cells (shown in Fig. 3A,B), in that the cell bodies exhibit a somewhat flatter morphology. By contrast, the knockdown of ROCKI substantially impaired formation of mature adhesions and actin stress fibers (Fig. 9B and data not shown), which is consistent with recent observations (Totsukawa et al., 2004; Yoneda et al., 2005). We observed that LPA failed to induce adhesion movement in cells lacking either ROCKI or ROCKII (Fig. 9B,C), indicating that both ROCKI and ROCKII contribute to the regulation of adhesion mobility. As shown in Fig. 9D, knockdown of ROCKII prevented the LPA-dependent stimulation of trailing-edge retraction and the concomitant decrease in cell elongation. These results support a model in which LPA activation of Gα12 and Gα13 (members heterotrimeric G protein family) leads to activation of FAK–PDZ-RhoGEF–Rho/ROCKII pathway that regulates the dynamics of mature adhesions, which – in turn – lead to trailing-edge retraction and concomitant decrease in cell elongation in serum-starved cells treated with LPA.

Discussion

In this report we show that serum deprivation of rodent fibroblasts markedly reduced trailing-edge retraction without having a significant effect on lamellipodia protrusive activity at the leading edge. The perturbation in trailing-edge retraction results in an elongated cell morphology following overnight serum starvation. Addition of LPA to serum-starved cells stimulates rear retraction and decreases cell elongation, and provides a model that parallels events within the trailing edge of a migrating fibroblast. Importantly, trailing-edge retraction requires LPA-induced stimulation of the dynamic movement of mature adhesions. Using this model, we have identified specific signaling components that contribute to trailing-edge retraction in fibroblasts in response to LPA. LPA-induced retraction of the trailing edge requires FAK. The siRNA-mediated attenuation of FAK expression blocks LPA induction of adhesion dynamics, trailing-edge retraction and restoration of cell shape, suggesting that dynamic remodeling of adhesions is necessary for trailing-edge retraction. LPA induced changes in adhesion dynamics; trailing-edge retraction and cell elongation also required PDZ-RhoGEF. The siRNA-mediated knockdown of PDZ-RhoGEF blocked LPA-induced adhesion dynamics and prevented the restoration of cell shape. Furthermore, expression of PDZ-RhoGEF

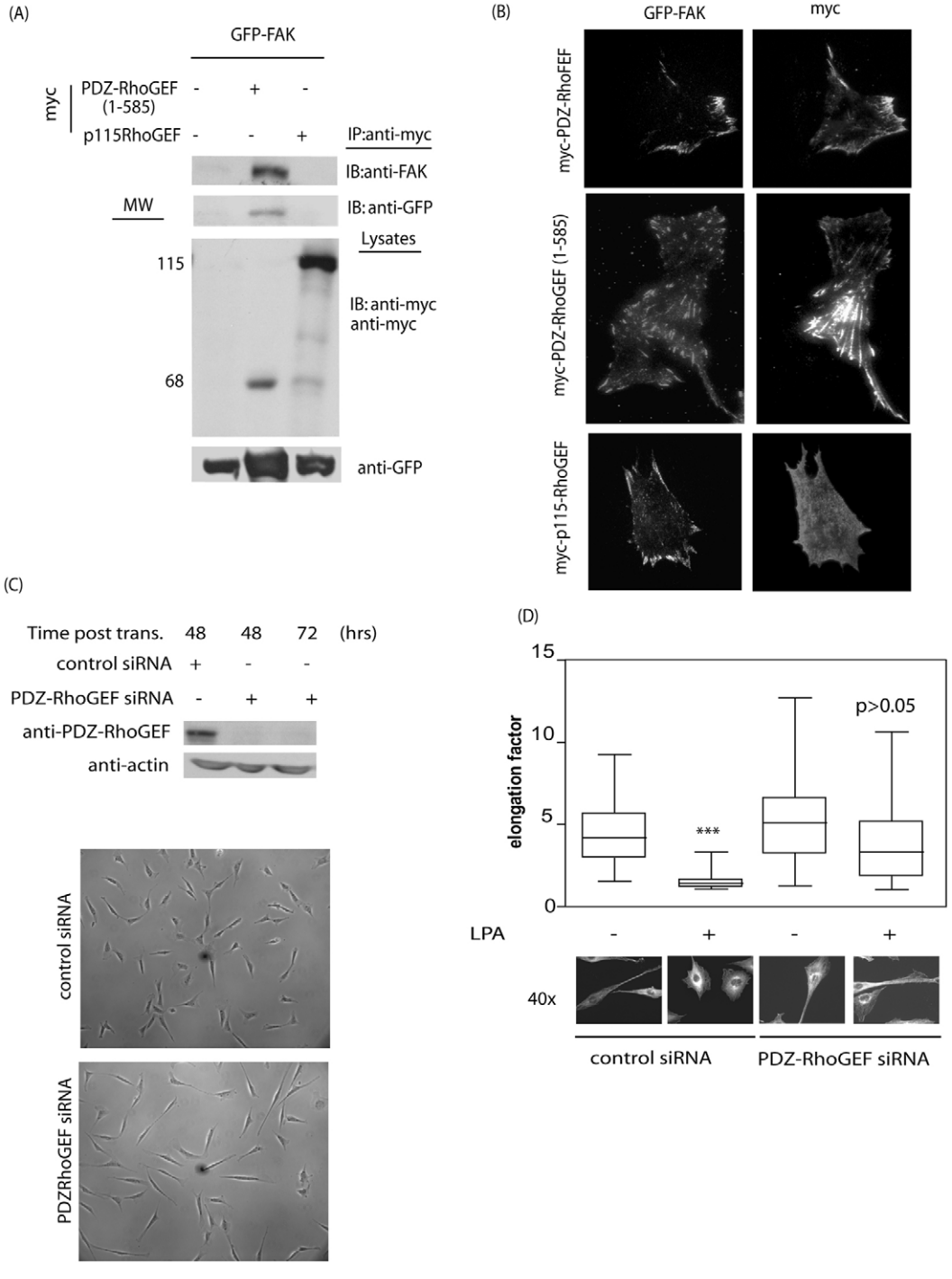

Fig. 7. GFP-FAK co-immunoprecipitates and localizes with co-expressed Myc-PDZ-RhoGEF and Myc-PDZ-RhoGEF(1-585). (A) HEK293T cells were transfected with with 0.5 μg GFP-FAK and Myc-PDZ-RhoGEF(1-585) or Myc-p115RhoGEF. To measure the amount of FAK in complex with the individual GEF proteins, immune complexes were isolated from lysates using anti-Myc antibody, and subjected to SDS-PAGE and western blotting with anti-FAK (top panel) or anti-GFP (second panel) antibodies. The expression of GEF proteins and GFP-FAK in lysates was determined by SDS-PAGE and immunoblotting lysates directly with anti-Myc or anti-GFP antibodies (bottom two panels). (B) Rat2 cells stably expressing GFP-FAK were transiently transfected with Myc-PDZ-RhoGEF, Myc-PDZ-RhoGEF(1-585) or Myc-p115RhoGEF, detached and seeded on fibronectin coated glass T dishes, fixed and Myc stained visualized using TIRF microscopy. Images show the colocalization of GFP-FAK and Myc-tagged PDZ-RhoGEF (top and middle panels), and the lack of colocalization of GFP-FAK and Myc-tagged p115RhoGEF. (C) Western blot analysis of endogenous PDZ-RhoGEF expression and phase-contrast images of control Rat2 cells and Rat2 cells treated with PDZ-RhoGEF siRNA. (D) Fluorescent images and cell elongation quantification of control Rat2 cells and Rat2 cells treated with PDZ-RhoGEF siRNA .

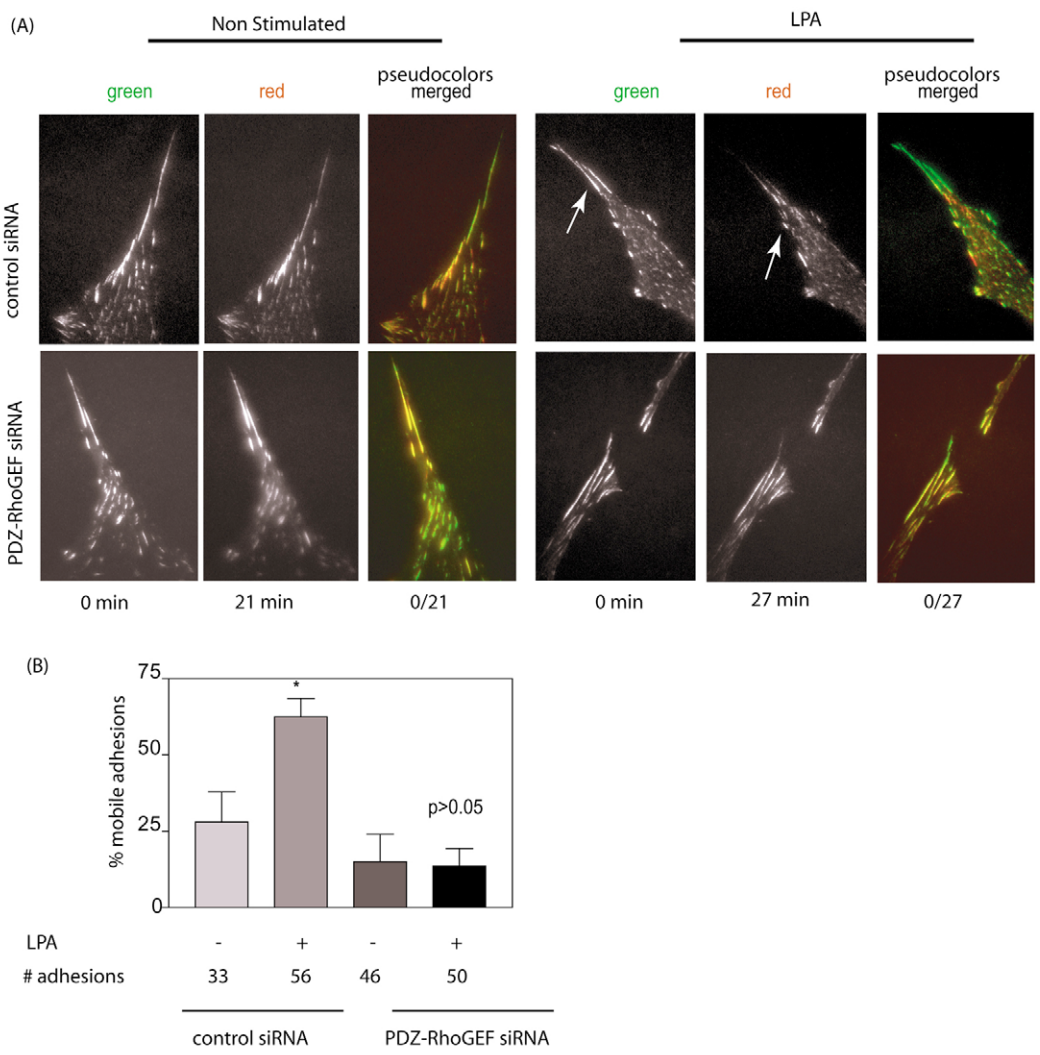

Fig. 8. PDZ-RhoGEF is required for LPA induction of adhesion movement. (A) Analysis of adhesion movement in response to LPA. Control Rat2 cells and Rat2 cells treated with PDZ-RhoGEF siRNA that stably expressed paxillin-GFP were plated on fibronectin-coated dishes in the presence of medium containing 10% serum. The following day, cells were serum starved for 12-16 hours and then stimulated with 2 μM LPA. Adhesion dynamic was analyzed using TIRF microscopy as described in Materials and Methods. Cells were filmed for 21 minutes before the addition of 2 μM LPA and 27 minutes after the addition of 2 μM LPA. (B) Quantification of dynamic adhesions (arrows indicate representative adhesions). The experiment was performed twice with five to seven cells analyzed per condition.

rescues the FAK-siRNA-induced phenotype with respect to cell elongation, i.e. cells are less elongated. In addition, PDZ-RhoGEF co-immunoprecipitated with FAK and colocalizes with FAK-containing adhesions, which suggests that activation of FAK and PDZ-RhoGEF are closely coupled. Finally, two lines of evidence suggest that ROCKII is one of the major targets of activated Rho in response to LPA. Expression of a Rho effector mutant that preferentially activates mDia failed to rescue the elongated phenotype of FAK knockdown cells; whereas a mutant that activates both mDia and ROCK pathways rescued the elongated phenotype of FAK-siRNA-treated cells. In addition, knockdown of ROCKII but not ROCKI induced a phenotype that parallels that observed in FAK-deficient cells with respect to cell elongation, and retention of stress fibers and adhesions structures. Our observations are consistent with a model in which LPA binding to its receptor stimulates activation of Gα12 and Gα13, FAK, and the closely coupled activation of PDZ-RhoGEF, Rho and ROCKII, leading to spatial (trailing edge) stimulation of adhesion dynamics through a localized increase in contractility, retraction of the cell rear and decrease in cell elongation.

There is abundant evidence that G-protein-coupled receptors (GPCRs), such as those for LPA, activate Rho and Rho-dependent signaling pathways through the Gα12 and Gα13 family of heterotrimeric G proteins (Barr et al., 1997; Gohla et al., 1998; Majumdar et al., 1999; Offermanns et al., 1994). The RhoGEFs, p115RhoGEF, PDZRhoGEF and leukemia-associated RhoGEF (LARG) contain a regulator of G-protein-signaling (RGS) domain that binds activated Gα12/13 resulting in RhoGEF activation (Wang et al., 2004). Thus, the RGS-RhoGEFs are thought to serve as effectors of activated Gα12/13 and as molecular bridges between heterotrimeric G proteins and the Rho network (Fukuhara et al., 1999; Reuther et al., 2001; Togashi et al., 2000; Zheng et al., 1995).

A number of studies show that activation of tyrosine kinases by GPCRs is required for the efficient stimulation of Rho and Rho signaling (for a review, see Sah et al., 2000). Indeed, many GPCRs are potent activators of FAK, suggesting a close link between FAK activation and Rho signaling (Hordijk et al., 1994; Luttrell et al., 1997; Seufferlein and Rozengurt, 1994). Furthermore, it has been reported that stimulation of Gα12 and Gα13 induces FAK-dependent PDZ-RhoGEF tyrosine phosphorylation leading to enhanced activation of Rho (Chikumi et al., 2002). How FAK is participating in Gα12 and Gα13 signaling to Rho remains unclear. The observation that FAK forms a complex with PDZ-RhoGEF and the report that PDZ-RhoGEF forms oligomers (Chikumi et al., 2004), lead us to speculate that LPA-mediated activation of Gα12 and Gα13 may induce oligomerization of PDZ-RhoGEF and concomitant dimerization of FAK, then leading to transphosphorylation and activation of

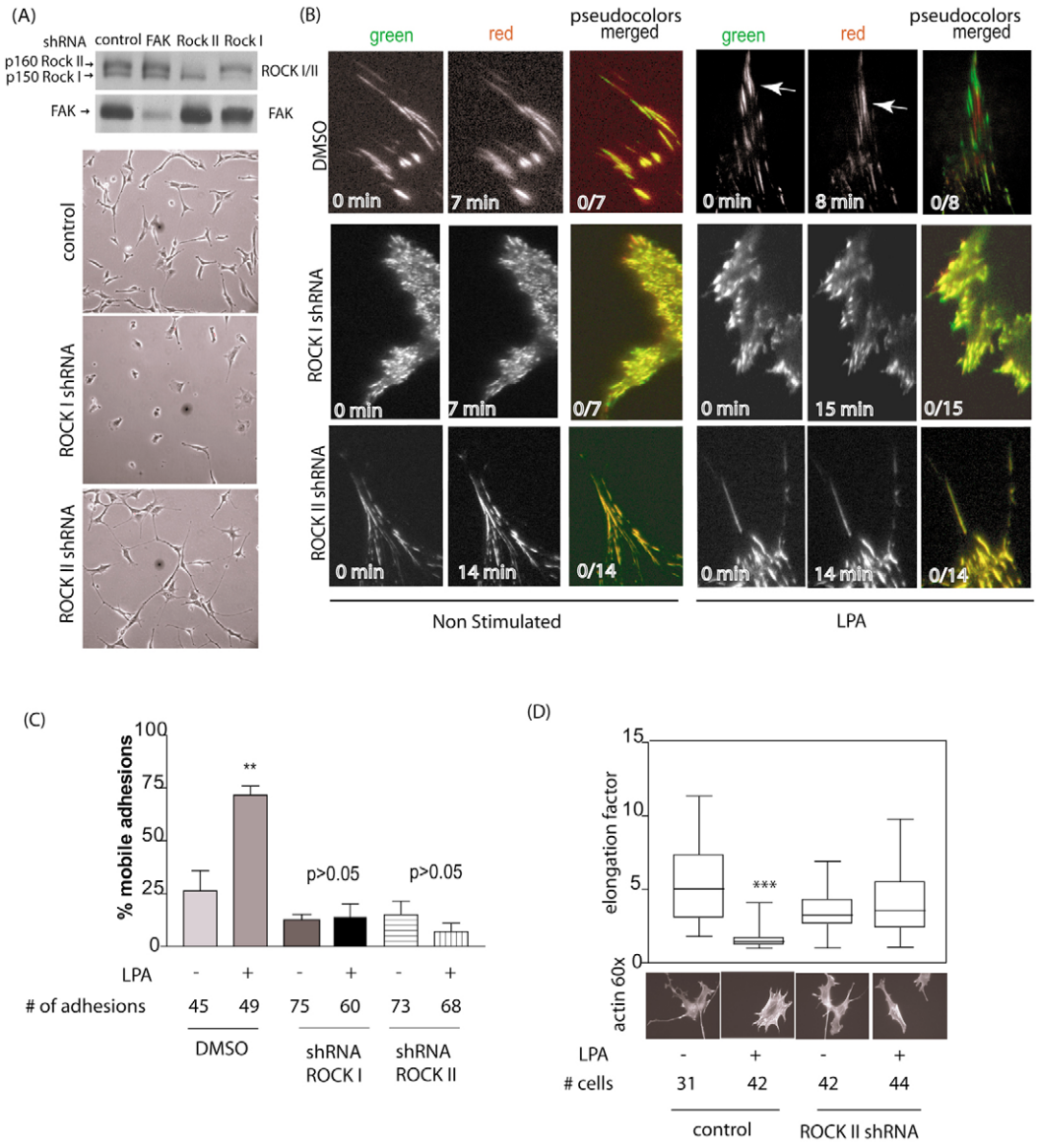

Fig. 9. LPA induced adhesion movement requires ROCKI and ROCKII. (A) Western blot analysis of ROCKI and ROCKII protein expression in control NIH3T3 cells and NIH3T3 cells treated with shRNA targeting FAK, ROCKI or ROCKII (top panel). Micrographs show phase-contrast images of control NIH3T3 cells and NIH3T3 cells treated with shRNA targeting ROCKI or ROCKII. (B) Control NIH3T3 cells and NIH3T3 cells treated with shRNA targeting ROCKI or ROCKII were serum starved for 12 hours. Following starvation, cells were stimulated with 2 μM LPA and adhesion movement was visualized by TIRF microscopy. (C) Quantification of adhesion movement (as described in Materials and Methods) in unstimulated serum-starved cells and in serum-starved cells stimulated with LPA (arrows indicate representative mobile adhesions). The experiment was performed twice with six to eight cells analyzed per experimental condition. (D) Quantification of cellular elongation in LPA-stimulated control cells and ROCKII-shRNA-treated cells. The experiment was performed twice.

FAK within such complexes. Alternatively, LPA stimulation of acidic phospholipids (such as PtdIns(4,5)P_2) might activate FAK directly, as recently demonstrated (Cai et al., 2008). We speculate that activated FAK phosphorylates PDZ-RhoGEF, thereby stimulating GEF activity and GTP binding to Rho, and that this localized activation of Rho leads to activation of effectors at or proximal to adhesion sites.

It has been shown previously that LPA stimulation of serum-starved neuronal cells that had been treated with ROCK inhibitor resulted in the failure of LPA to induce cell shape changes, rapid neurite retraction and cell rounding (Jalink et al., 1993; Kranenburg et al., 1999). However, our study did not delineate the contribution of ROCKI and ROCKII in the process of LPA-induced neurite retraction. More relevant to our study are the observations that knockdown of ROCKII but not ROCKI yields a cellular phenotype similar to that observed in response to FAK knockdown with respect to cell shape, stress fiber augmentation and loss of dynamic adhesions (Yoneda et al., 2005). In addition, recent studies have shown that Rho-dependent breakdown of cell-cell adhesions requires ROCKII but not ROCKI (Samarin et al., 2007). Therefore, the studies by Jalink et al. and Kranenburg et al. indicate that ROCKII plays a role in the induction of adhesion-complex dynamics and adhesion-complex breakdown. Recent evidence indicates that ROCKI rather than ROCKII is important for stress fiber formation (Yoneda et al., 2005). ROCKII appears to function in the regulation of microfilament bundling at the focal adhesion site and is involved in phagocytosis of matrix-coated beads, a function not sensitive to ROCKI depletion.

The effects of ROCKII on adhesion movement in the trailing edge of fibroblasts could be attributed to the induction of localized activation of myosin. We speculate that ROCKII-dependent tension at the trailing-edge adhesions during LPA stimulation contributes to the force that promotes inward movement of focal adhesions and, ultimately, their disassembly. In cells with attenuated expression of FAK, PDZ-RhoGEF or ROCKII adhesions remained stable even after stimulation with LPA, indicating that this pathway is important in regulating localized changes in contractility. This observation suggests that adhesion turnover requires localized modulation of myosin activity that is provided by the localized activation of the FAK-GEF-Rho/ROCKII pathway.

Materials and Methods

Cell culture, plasmids and siRNAs

Rat2 and HEK293T cells were maintained in Dulbecco's modiefied Eagle's medium (DMEM) plus 10% fetal calf serum (FCS; GibcoBRL) at 37°C in 5% CO_2. NIH3T3 cells were maintained in DMEM plus 10% calf serum (GibcoBRL), at 37°C in 5% CO_2. For FAK- and PDZ-RhoGEF-knockdown experiments, cells were transfected with siRNA oligonucleotides specifically targeting rat FAK, PDZ-RhoGEF (available as GTRAP48; Dharmacon pool) or with control siRNA oligonucleotides using Ca^{2+} phosphate as previously described (Tilghman et al., 2005). FAK, ROCKI and ROCKII shRNA expressing plasmids were obtained from Sigma. For transient expression of Myc-PDZ-RhoGEF, Myc-PDZ-RhoGEF(1-585), Myc-p115RhoGEF and GFP-FAK, Rat2 or HEK293T cells were plated in the presence of 10% serum in 100 mm^2 plastic culture dishes at densities of 4×10^5 and 1.5×10^6 cells per dish, respectively. The next day cells were transfected using Polyfect reagent (Qiagen) with 0.25 μg or 0.5 μg of the designated plasmid. Twenty-four hours later cells were processed for experiments.

The Flp-In System (Invitrogen) was used to create stable cell lines expressing GFP-tagged or red fluorescent protein (mRFP)-tagged proteins. Briefly, NIH3T3-LacZeo or RAT2-LacZeo cell lines with a flp recombinase target (FRT) site integrated in the genome were created. cDNAs encoding either paxillin-GFP, GFP-FAK or βactin-mRFP fusion proteins were cloned into the *Nhe*I-*Bam*HI or *Hind*III-*Not*I sites of the pcDNA5/FRT vector. The host cells were then co-transfected with pOG44 plasmid encoding Flp recombinase and either pcDNA5/FRT-paxillin-GFP, pcDNA5/FRT-GFP-FAK or pcDNA5/FRT βactin-mRFP and recombinant cell lines were selected according to the manufacturer's instructions. Stable clones were analyzed for expression of paxillin-GFP, GFP-FAK and βactin–mRFP by fluorescent microscopy and western blotting.

Myc-PDZ-RhoGEF, Myc-p115RhoGEF and GFP-PDZ-RhoGEF(561-585) plasmids were engineered as described elsewhere (Banerjee and Wedegaertner, 2004). Paxillin-GFP was a generous gift from Rick Horwitz (University of Virginia, Charlottsville, VA) GFP-FAK was generated by cloning FAK from pCMV-Myc –FAK vector (Xiong and Parsons, 1997) into vector pEGFP-C1 (Clontech, CA). βactin–mRFP was a generous gift from the laboratory of Frank Getler (MIT, Cambridge, MA). RhoA-effector-domain mutants were a generous gift from Kodi Ravichandran (University of Virginia). FAK, ROCKI and ROCKII shRNA plasmids were obtained from the Sigma shRNA library. Lentiviral packaging plasmids pMDG and pCMVδR 8.2 were generous gift from David Rekosh (University of Virginia). Lentiviral production was performed according to Sigma protocol. The organization of adhesions in cells whose expression of FAK, PDZ-RhoGEF, ROCKI and ROCKII was knocked down were assessed by immunostaining for FAK and paxillin (supplementary material Fig. S1).

Serum starvation and LPA-stimulation experiments

Serum deprivation is defined as removal of serum up to 6 hours. Serum starvation is defined as removal of serum for 12-16 hours. Transfected and untransfected cells were plated overnight on glass T dishes or plastic dishes coated with fibronectin (1 μg/ml) in the presence of medium containing 10% serum. The following day, culture medium was changed to medium containing (10%) serum or not, or to serum-free medium plus LPA (2 μM).

Western blotting

Cells were lysed in 200 μl of RIPA buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.1 M NaCl, 1 mM sodium orthovanadate, 0.1 M sodium pyrophosphate, 100 mM NaF and 1 mM PMSF). Lysates were clarified by centrifugation at 13,000 ***g*** for 10 minutes. Clarified lysates were boiled in 1× sample buffer (0.04 M Tris-HCl pH 6.8, 1% SDS, 1% β-mercaptoethanol and 10% glycerol) for 10 minutes and resolved by SDS-PAGE. Proteins were transferred to Immobilon membrane (Whatman) and blocked with 5% BSA in PBS (140 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4\cdot7H_2O$, 0.14 mM KH_2PO_4 pH 7.3), 0.1% Tween 20, pH 7.2 for 1 hour at room temperature. Membranes were incubated overnight at 4°C with one of the following antibodies: anti-actin (Zymed, 1:5000), anti-FAK polyclonal antibody (UpstateBiochemicals, 1:1000), anti-pY397 FAK polyclonal antibody (Biosource, 1:1000), anti-MAPK monoclonal antibody (1:2000, Sigma), anti-phosphorylated-MAPK polyclonal antibody (1:2000, gift from Michael J. Webber, University of Virginia), anti-GTRAP 48 (against PDZRhoGEF) monoclonal antibody (1:1000, BD Transduction), anti-GFP polyclonal antibody (1:1000, Cell Signaling), anti tubulin monoclonal antibody (1:5000, Sigma), anti-phosphorylated-tyrosine monoclonal antibody (4G10; 1:1000, UpstateBiochemicals) or anti-Myc monoclonal antibody 9E10 (SantaCruz, 1:1000), polyclonal anti-ROCKII antibody (1:1000, Upstate), monoclonal anti ROCKI/II (1:1000, Upstate) and monoclonal anti-Paxillin antibody (1:1000, BD Transduction). Membranes were subsequently probed with secondary antibodies linked to horserasish peroxidase (HRP; Amersham). Western blot membranes were developed using enhanced chemiluminescent substrate for detection of HRP (Amersham). Western blot results were prepared using AlphaEaseFC (FluorChem 8800) for Windows software.

Immunoprecipitation experiments

Twenty-four hours after transient transfection with indicated expression vectors cells were washed with ice-cold PBS, and lysed in immuno-precipitation (IP) buffer (50 mM Tris-HCl pH 7.6, 160 mM NaCl, 0.5 mM EDTA/EGTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF and 1 μg/ml leupeptin). Cell extracts were clarified by centrifugation at 13,000 ***g*** and cell supernatants were applied to agarose beads (10% of total volume) conjugated to anti-Myc (4A6) antibody (UpstateBiochemicals Inc.). Immunoprecipitation was carried out for 2 hours at 4°C; immune complexes were washed three times with IP buffer and boiled in 1× sample buffer. Samples were processed for SDS-PAGE and western blotting as described above.

Analysis of adhesion movement

Adhesion movements were visualized using TIRF microscopy in serum-deprived NIH3T3 or Rat2 cells that stably expressed paxillin-GFP, before and after the addition of 2 μM LPA. Cells were maintained at 37° with a Bioptechs Delta T heated chamber system. Cells were imaged using a Nikon Eclipse TE2000-E inverted microscope equipped with a TIRF illuminator, a 488 argon laser and a 60× DIC TIRF objective (NA1.45) equipped with a Bioptechs objective heater. Images were acquired with a Retiga 1300i CCD camera and the QCapture Pro software (Q Imaging). Adhesion movement was quantified as described before (Smilenov et al., 1999) briefly; the first frame of a movie was pseudo-colored green, the last frame pseudo-colored red and the two images were merged. Adhesions that contained both red and green, and thus appeared yellow, were scored as poorly dynamic. The fraction of adhesions that move was scored to determine the percentage of red adhesions relative to total adhesions in the first frame.

Phase microscopy

NIH3T3 cells stably expressing βactin-mRFP or Rat2 cells were serum-deprived in the absence or presence of 2 μM LPA and filmed using a Zeiss Axiovert 135 TV microscope equipped with a CCD camera MTI 176.

Immunostaining

Control cells, or FAK- or PDZ-RhoGEF-siRNA-treated Rat2 fibroblasts were plated overnight on fibronectin-coated coverslips (1 μg/ml, Sigma) in medium containing 10% serum. Cells were either left untreated or serum-starved overnight and then stimulated with 2 μM LPA with 0.1% carrier BSA or 10% fetal bovine serum (FBS) for indicated times. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS and blocked with 20% goat serum in PBS. Cells were washed three times with PBS and immunostained with the following antibodies: anti-tubulin B2-11 (Sigma, 1:5000) and anti-Myc 9E10 (1:250, SantaCruz). To visualize actin cells were treated with Alexa-Fluor-488-conjugated phalloidin (Invitrogen, Inc.). To visualize microtubules or Myc-tagged GEF constructs, cells were treated with secondary goat anti-mouse antibodies conjugated to Alexa-Fluor-594 (1:2000, Invitrogen, Inc.). Fluorescent images were acquired using a Nikon Eclipse E600 upright fluorescence microscope equipped with a Hamamatsu ORCA CCD camera. Images were captured and analyzed with OpenLab (Improvision) software.

Analysis of trailing-edge length and cell elongation

To measure the length of the trailing edge, a line was drawn from the tip of the trailing edge towards the base of the nucleus. To measure the cell length, a line was drawn from the center of the nucleus to the furthest visible microtubule tip. To measure the cell width, a second line was drawn across the cell passing through the center of the nucleus at a 90° angle to the first line. To calculate the 'elongation factor', the cell length was divided by cell width.

Analysis of lamellipodia protrusion

NIH3T3 cells stably expressing βactin-mRFP or Rat2 cells were plated in medium containing 10% serum on fibronectin-coated (1 μg/ml) glass T dishes at the density of 3×10^4 cells per dish. The next day, serum-containing medium was changed to serum-free medium or to serum-free medium containing 2 μM LPA. After 6 hours cells were filmed for a total of 15-20 minutes, collecting one image every 20 seconds using TIRF (for NIH3T3 cells) or DIC (for Rat2 cells) microscopy. Protrusion rates were calculated using Image J software (Wayne Rasband, National Institute of Health, MA).

Statistics

Statistical analysis was done using non-parametric, non-assuming Gaussian distribution Mann-Whitney *t*-test using Prism software. ***P=0.001, **P=0.01 and *P=0.05 denote statistical differences.

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