A novel role for Lsc/p115 RhoGEF and LARG in regulating RhoA activity downstream of adhesion to fibronectin

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Summary

Adhesion of cells to extracellular matrix proteins such as fibronectin initiates signaling cascades that affect cell morphology, migration and survival. Some of these signaling pathways involve the Rho family of GTPases, such as Cdc42, Rac1 and RhoA, which play a key role in regulating the organization of the cytoskeleton. Although significant advances have been made in understanding how Rho proteins control cytoskeletal architecture, less is known about the signals controlling activation of the GTPases themselves. The focus of this study was to determine which guanine nucleotide exchange factor(s) are responsible for activation of RhoA downstream of adhesion to fibronectin. Using an affinity pulldown assay for activated exchange factors, we show that the RhoA-specific exchange factors Lsc/p115 RhoGEF and LARG are activated when cells are plated onto fibronectin, but not other exchange factors such as Ect2 or Dbl. Knockdown of Lsc and LARG together significantly decreases RhoA activation and formation of stress fibers and focal adhesions downstream of fibronectin adhesion. Similarly, overexpression of a catalytically inactive mutant of Lsc/p115 RhoGEF inhibits RhoA activity and formation of stress fibers and focal adhesions on fibronectin. These data establish a previously uncharacterized role for the exchange factors Lsc/p115 RhoGEF and LARG in linking fibronectin signals to downstream RhoA activation.

Key words: Lsc, p115 RhoGEF, Leukemia-associated RhoGEF, RhoA, Fibronectin, Guanine nucleotide exchange factors, Extracellular matrix, Stress fibers, Focal adhesions, Cell spreading

Introduction

The ability of most cells to survive, proliferate and migrate is regulated in part by the adhesive interactions they make with different components of the surrounding extracellular matrix (ECM), such as fibronectin (FN) (Boudreau and Jones, 1999). Attachment to FN is mediated through two different types of adhesion receptors, integrins and syndecans (Bass and Humphries, 2002; Hynes, 2002). Integrins are transmembrane heterodimers comprising α and β subunits. Different αβ combinations show specificity for different ECM ligands, with αvβ3 and α5β1 being some of the major integrin receptors that bind to FN (Hynes, 2002). Syndecans are transmembrane heparan sulfate proteoglycans, characterized by the presence of long glycosaminoglycan chains attached to the extracellular domain of the proteins. Specifically, syndecan-4 has been implicated in signaling processes downstream of adhesion to FN (Bass and Humphries, 2002; Bass et al., 2007).

The mature FN molecule is a dimer of two disulfide-linked chains. Each monomer chain contains multiple repeat domains, and distinct regions serve as binding sites for the different adhesion receptors. The tripeptide RGD sequence in FN repeat III_{10}, part of the cell-binding domain (CBD), is the central recognition sequence required for most FN-binding integrins (Hynes, 2002; Pankov and Yamada, 2002). A different region containing FN repeats III_{12-14} is the major heparin-binding domain (HBD) and serves as the attachment site for syndecans (Bass and Humphries, 2002).

Attachment of cells to FN activates different members of the Rho family of small GTPases, allowing cells to spread and migrate efficiently by means of dynamic rearrangements of the actin cytoskeleton. Formation of filopodia, lamellipodia and small focal complexes, controlled by Cdc42 and Rac1, allows cells to attach to and spread on the matrix. Activation of RhoA causes the formation of stress fibers and focal adhesions, which tends to counter spreading and results in stable adhesion to the matrix (Burridge and Wennerberg, 2004; Defilippi et al., 1999; Nobes and Hall, 1995). Although it is clear that RhoA is activated downstream of adhesion to FN, the specific contributions of integrins and/or syndecans to this increase in activity remains more controversial (Saoncella et al., 1999; Wang et al., 2005).

Rho proteins are activated when they bind to GTP and inactivated when the nucleotide is hydrolyzed to GDP. This regulatory cycle is controlled by different protein families. GTPase-activating proteins (GAPs) decrease Rho protein activity by stimulating their intrinsic GTP hydrolysis activity (Moon and Zheng, 2003). Guanine nucleotide exchange factors (GEFs) increase the activity of Rho proteins by promoting the exchange of GDP for GTP. Specifically, GEFs for Rho proteins

perform this function by binding to the GTPase and destabilizing the nucleotide-binding pocket, allowing for dissociation of the bound GDP. Owing to the high GTP:GDP ratio in the cytoplasm, the lost GDP is quickly replaced by GTP (Rossman et al., 2005).

The Dbl family of proteins is a large group of exchange factors for the Rho GTPases, which are characterized by tandem Dbl homology (DH) and pleckstrin-homology (PH) domains. DH domains are responsible for the catalytic activity of the proteins. Functions for the PH domain range from assisting in the exchange reaction to membrane anchorage or protein binding (Rossman et al., 2005). Of the approximately 70 known members of the Dbl GEF family, very few have been extensively studied. Little is known about which specific GEFs are responsible for linking different ECM signals (such as adhesion to FN) to Rho proteins. Considering the major regulatory function of GEFs in controlling the function of Rho proteins, this represents a major gap in our understanding of this signaling pathway. Several lines of evidence have indicated that activation of Rac by ECM adhesion occurs through the Dock180-ELMO complex. Dock180 is a member of a second family of unconventional GEFs that lack DH domains (Meller et al., 2005). The exchange factor Vav1 might also be responsible for transmitting ECM signals to Rac, but such a role for Vav1 is restricted mainly to cells of the hemopoietic lineage (Hornstein et al., 2004).

The major goal of this project was to identify the GEF(s) responsible for FN-induced RhoA activation. Using a nucleotide-free mutant of RhoA (which has a high affinity for activated GEFs), we identified Lsc/p115 RhoGEF and leukemia-associated RhoGEF (LARG) as candidate GEFs for activation of RhoA downstream of FN. Lsc (the murine homolog of p115 RhoGEF) belongs to a family of RhoA-specific GEFs known as RGS-GEFs, which also includes PDZ-RhoGEF. The RGS-GEFs have been best characterized as exchange factors responsible for RhoA activation by stimulation of G-protein-coupled receptors (GPCRs) (Fukuhara et al., 2001). However, in this study, we demonstrate that Lsc/p115 RhoGEF and LARG also play an important role in the activation of RhoA downstream of FN.

Results

The RhoA GEFs Lsc/p115 RhoGEF and LARG are activated upon adhesion of fibroblasts to FN

The spreading of cells on FN is a dynamic process. When suspended REF52 fibroblasts are re-plated onto FN, coordinated control of cytoskeletal remodeling by several members of the Rho family of GTPases causes the cell morphology to change from round to flattened, with extensive adhesions to the matrix. Initially, a typical Rac-induced morphology dominates, with the cells displaying highly active lamellipodia in all directions and punctate focal complexes. Later on, RhoA activity causes the formation of stress fibers and focal adhesions (Fig. 1A). Previous work from our laboratory and other groups has shown that, when cells are plated on FN, RhoA activity follows a biphasic pattern, where significant activation of RhoA occurs after a transient inhibition caused by Src-mediated p190 RhoGAP activation (Arthur and Burridge, 2001; Arthur et al., 2000; Ren et al., 1999). In this study, we used a modified experimental system focused on the activation phase of RhoA on FN. NIH 3T3 fibroblasts were held in suspension for 2 hours, causing a very efficient reduction in RhoA activity. Once plated on FN, instead of the biphasic pattern described previously, a steady increase in activity of RhoA is observed (Fig. 1B).

To identify the GEF(s) responsible for RhoA activation downstream of FN, we performed pulldown assays with the nucleotide-free RhoA mutant RhoA(17A). Previous work from our laboratory has validated the use of RhoA(17A) in affinity precipitations for activated GEFs. We have shown that RhoA(17A) preferentially binds to GEFs, and not Rho effectors or GAPs. Furthermore, this interaction is specific as RhoA(17A) can only precipitate RhoA-specific GEFs, and not Rac- or Cdc42-specific GEFs (Arthur et al., 2002; Garcia-Mata et al., 2006; Noren et al., 2003). Initially, an unbiased proteomics approach was employed. Suspended and FN-adherent fibroblasts were lysed, and pulldowns performed with purified RhoA(17A). Silver-stained samples were then examined for protein bands whose association with RhoA(17A) increased upon adhesion to FN (data not shown). The most consistent and reproducible increase in interaction with RhoA(17A) was observed for an approximately 110-kDa band, which was identified by mass spectrometry as the RhoA-specific GEF Lsc (also known as ‘Lbc’s second cousin’).

To confirm these data, identical experiments were performed and samples blotted with an antibody against Lsc. Specifically, mouse fibroblasts were serum starved for 3 hours, held in suspension for 2 hours in serum-free media and plated onto FN-coated dishes for various periods of time. The association between endogenous Lsc and RhoA(17A) increased upon plating of cells on FN, indicative of an increase in activity of the GEF (Fig. 1C). Thus far, activation of Lsc/p115 RhoGEF by FN has been seen in all cell lines we have tested, including REF52 and HeLa cells (data not shown). We were also able to detect FN-induced activation of the closely related GEF LARG (Fig. 1C). Unlike the related RGS-GEFs Lsc and LARG, other RhoA GEFs such as Dbl or Ect2 were not activated by attachment to FN (Fig. 1C). These experiments therefore suggested that the activation of the related RGS-GEFs Lsc/p115 RhoGEF and LARG by FN is a specific process. Previous work with the RGS-GEFs has focused on their established function downstream of GPCR stimulation. These experiments demonstrate for the first time the ability of FN to activate Lsc/p115 RhoGEF and LARG and therefore suggest a novel role for these members of the RGS-GEF family.

Lsc/p115 RhoGEF and LARG can increase formation of stress fibers and partially colocalize with focal adhesions on FN

As a first step to determine whether Lsc/p115 RhoGEF could affect RhoA function downstream of adhesion to FN, we overexpressed a construct containing GFP-tagged wild-type p115 RhoGEF [GFP-p115(FL)] in REF52 fibroblasts (Fig. 2A). Interestingly, at early time points during spreading on FN, compared with non-expressing cells, cells overexpressing GFP-p115(FL) demonstrated an increased formation of cortical actin bundles known as arcs (Fig. 2B, top panel, arrow). Although not a commonly studied actin structure, arcs have been shown to be dependent on RhoA activity and have been described as precursors to stress fibers (Hotulainen and

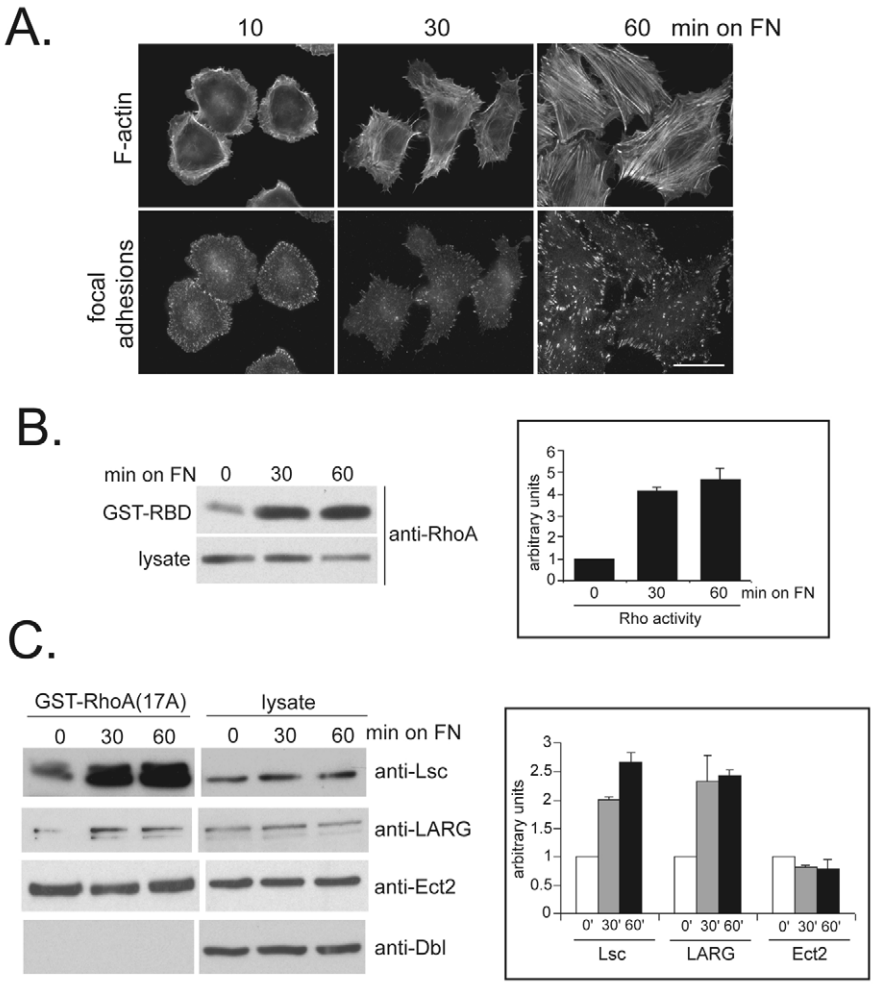

Fig. 1. Adhesion to FN causes activation of RhoA and the RhoA-specific GEFs Lsc/p115 RhoGEF and LARG. Mouse fibroblasts were starved in serum-free media and then held in suspension in the same media for 2 hours. (A) Cells were plated onto FN-coated coverslips for the times indicated, then fixed and stained with phalloidin to visualize F-actin, and antibodies against phosphotyrosine to visualize focal adhesions. Bar, 40 μm. (B,C) Cells were plated onto FN-coated dishes for the times indicated, lysed and (B) pulldowns performed with GST-RBD and samples blotted with an antibody against RhoA, or (C) pulldowns performed with GST-RhoA(17A) and samples blotted with antibodies against the indicated GEFs. Quantification of all blots was performed as described in Materials and Methods.

Lappalainen, 2006; Zhang et al., 2003). As expected, at later time points, cells overexpressing GFP-p115(FL) demonstrated an increase in formation of stress fibers when compared with control cells (Fig. 2B). Identical results were obtained with overexpression of a GFP-tagged LARG construct (Fig. 2B).

Over the time course of FN attachment, localization of GFP-p115(FL) was observed to be mainly cytoplasmic, with increased staining intensity visible in discrete 'patches' proximal to the periphery of the cells (Fig. 2B,C). Confocal images taken at the ventral surface of the cells revealed that these discrete patches of GFP-p115(FL) can partially colocalize with focal adhesions, as visualized by immunostaining for paxillin (Fig. 2C, arrows). Once again, identical results were obtained with overexpression of a GFP-tagged LARG construct (Fig. 2C). Therefore, these overexpression experiments provided initial evidence that Lsc/p115 RhoGEF and LARG can increase RhoA signaling and partially colocalize to focal adhesions upon adhesion to FN.

Knockdown of Lsc/p115 RhoGEF and LARG together inhibits formation of stress fibers and RhoA activity downstream of adhesion to FN

To evaluate the role of Lsc and LARG in the function of RhoA downstream of FN, we knocked down expression of Lsc using RNAi. REF52 fibroblasts were transiently transfected with knockdown (KD) oligonucleotides against Lsc and LARG, individually or together. At 48 to 72 hours post transfection, the cells were serum starved, held in suspension and plated onto FN-coated coverslips. No significant difference in formation of stress fibers was observed when each GEF was knocked down alone (data not shown). However, cells transfected with both KD oligonucleotides against Lsc and LARG demonstrated a significant decrease in formation of stress fibers, as compared with cells transfected with a control siRNA oligonucleotide (Fig. 3A, top panel). Furthermore, while double-KD Lsc-LARG cells were able to form small peripheral focal complexes, they were defective in the ability to form Rho-induced focal adhesions (Fig. 3A, bottom panel).

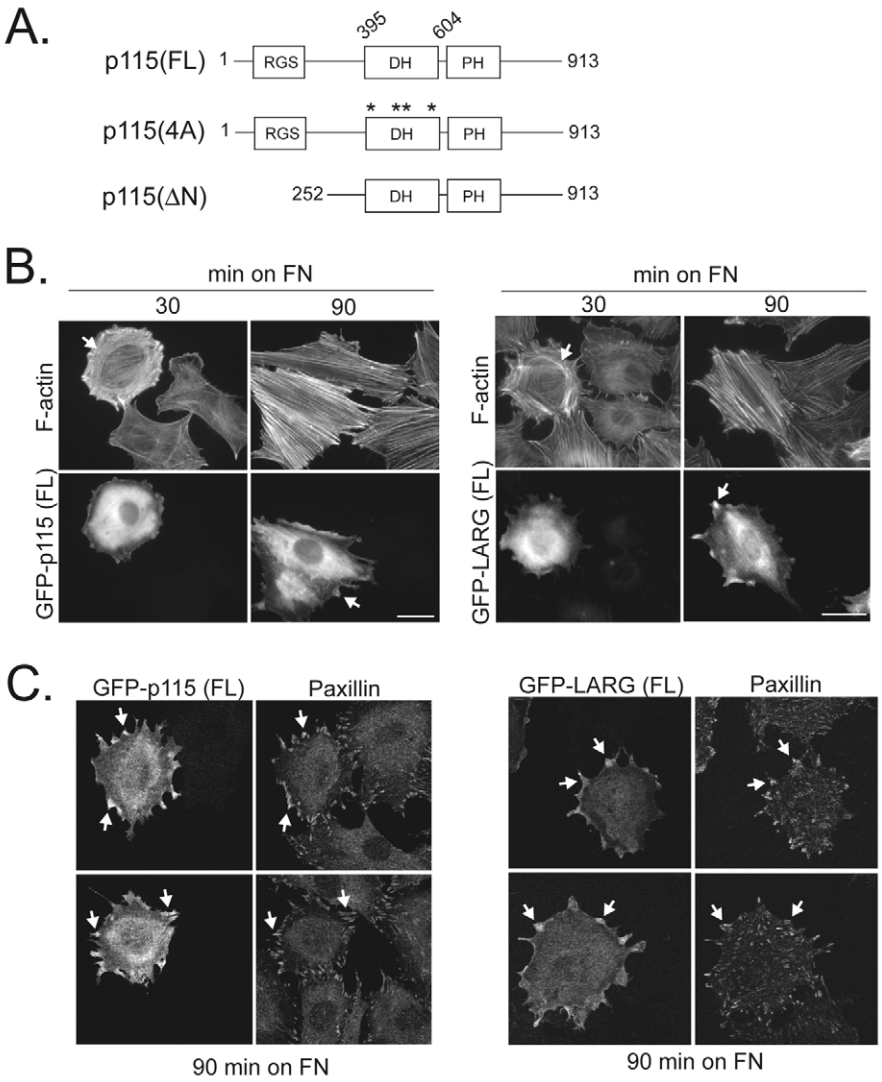

Fig. 2. Lsc/p115 RhoGEF and LARG increase stress fibers and localizes to focal adhesions on FN. (A) Domain structure of full-length p115 RhoGEF [p115(FL)] and the different mutants used in this study. The DH-dead p115(4A) mutant contains alanine point substitutions (*) of four residues (E423, K567, L570, N603) in the DH domain that are important for the catalytic exchange reaction. The p115(ΔN) mutant lacks the N-terminus of the protein containing the RGS domain. All constructs were cloned into N-terminal GFP- or V5-tagged vectors. (B,C) REF52 fibroblasts were transfected with vector encoding either GFP-p115(FL) or GFP-LARG(FL). 24 hours post transfection, cells were serum-starved, held in suspension for 2 hours and plated onto FN-coated coverslips for the times indicated. (B) The cells were then fixed and stained with phalloidin to visualize F-actin. Arrows in the top panels point to the tight cortical actin bundles known as arcs. Arrows in the bottom panels point to the discrete patches of p115 RhoGEF or LARG localization. Bar, 40 μm. (C) The cells were fixed and stained with antibody against paxillin to visualize focal adhesions. The images represent 0.3 μm confocal sections at the ventral surface of the cells. Arrows point to areas of colocalization between paxillin-containing focal adhesions and the discrete patches of p115 RhoGEF or LARG localization.

The efficiency of knockdown of Lsc and LARG is demonstrated in Fig. 3B. Expression of the RhoA GEF Lfc was unaffected in these knockdown cells, demonstrating the specificity of the siRNA (Fig. 3B).

When assayed for RhoA activity, double-KD Lsc-LARG cells demonstrated a dramatic decrease in the ability to activate RhoA by adhesion to FN compared with control cells (Fig. 3C). The inability of the Lsc-LARG siRNA to inhibit RhoA activation completely is probably due to incomplete knockdown of the GEFs. Importantly, the defect in RhoA activation in double-KD Lsc-LARG cells can be rescued by re-expression of wild-type human p115 RhoGEF (Fig. 3D). These experiments showed that the RGS-GEFs Lsc/p115 RhoGEF and LARG are both involved in regulating RhoA downstream of FN.

Formation of stress fibers and focal adhesions downstream of adhesion to FN is inhibited by a DH-dead mutant of Lsc/p115 RhoGEF

To explore further the role of Lsc/p115 RhoGEF in RhoA activation by FN, and to determine whether the GEF activity of the protein is necessary for this function, we decided to use a mutant of p115 RhoGEF that lacks the ability to exchange nucleotide on RhoA. To create a catalytically inactive form of p115 RhoGEF [p115(4A)], we mutated four different residues in the DH domain of p115 RhoGEF (Fig. 2A). These residues are conserved in different RhoA GEFs and have been shown to be important for GTPase binding and catalytic activity (Kristelly et al., 2004; Rossman and Sondek, 2005; Worthylake et al., 2000). As expected, p115(4A) showed significantly decreased binding to RhoA(17A) compared with the wild-type protein (Fig. 4A). We hypothesized that overexpression of the p115(4A) construct would act in a dominant-negative fashion, sequestering FN-induced signals away from the endogenous GEFs involved in RhoA activation by FN (such as Lsc/p115 RhoGEF and LARG), thus causing a loss of signaling to RhoA and a subsequent loss of formation of stress fibers and focal adhesions.

To test this hypothesis, REF52 cells transfected with an N-terminally GFP-tagged p115(4A) construct were plated onto FN, and stress fibers and focal adhesions visualized by immunofluorescence staining. Compared with control cells, formation of stress fibers and focal adhesions was either reduced or completely inhibited in cells overexpressing GFP-p115(4A) (Fig. 4B). To exclude the possibility that the loss of

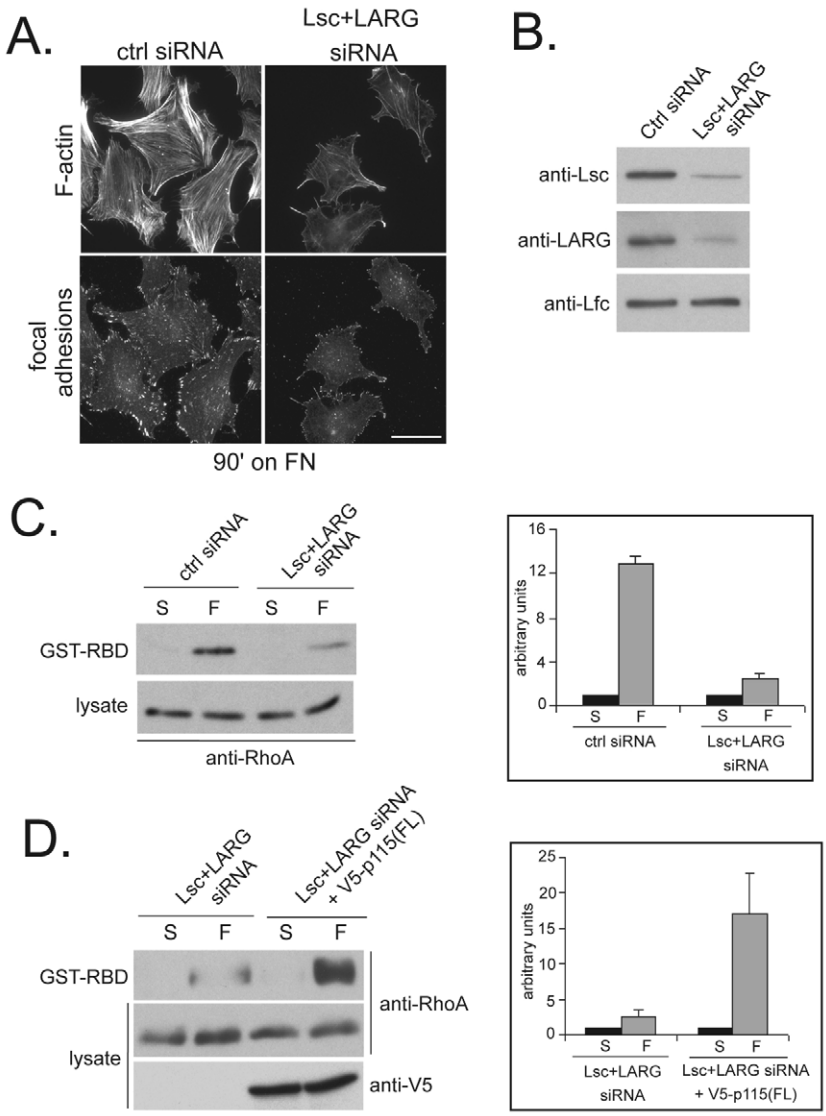

Fig. 3. Knockdown of Lsc/p115 RhoGEF and LARG decreases formation of stress fibers and focal adhesions and RhoA activity downstream of FN. (A) REF52 cells were transfected with either control or siRNA oligonucleotides against Lsc and LARG as described in Materials and Methods. 72 hours post transfection, the cells were serum starved, held in suspension for 2 hours and plated onto FN-coated coverslips for 90 minutes. The cells were fixed and stained with phalloidin and antibody against phosphotyrosine to visualize stress fibers and focal adhesions. Bar, 40 μm. (B) Control cells or cells transfected with siRNA against Lsc and LARG were lysed and samples blotted with antibodies against Lsc and LARG to demonstrate the efficiency of knockdown. Identical blots with an antibody against Lfc show that protein levels of the closely related GEF Lfc are unaffected, demonstrating the specificity of the knockdown. (C) Fibroblasts were transfected with either control or siRNA oligonucleotides against Lsc and LARG. 72 hours post transfection, the cells were serum-starved, held in suspension for 2 hours and plated onto FN-coated dishes for 60 minutes. The cells were then lysed, GST-RBD pulldowns performed and samples blotted with an antibody against RhoA to visualize the levels of RhoA activity. (D) Fibroblasts were transfected with siRNA oligonucleotides against Lsc and LARG. 48 hours post transfection, the cells were re-transfected with either a vector control or a V5-tagged full-length p115 RhoGEF construct. 72 hours post transfection, the cells were processed for Rho activity assays, as described in (C) above. Lysates were also blotted with an antibody against V5 to show the expression levels of V5-p115(FL).

stress fibers is a result of sequestration of RhoA by p115(4A) caused by overexpression, cells transfected with vector encoding p115(4A) were plated onto FN and treated briefly with nocodazole. Depolymerization of microtubules by nocodazole has been shown to activate RhoA through the RhoA GEF Lfc (Krendel et al., 2002). As expected, nocodazole treatment was able to rescue formation of stress fibers in p115(4A)-overexpressing cells, suggesting that the loss of stress fibers seen in cells overexpressing p115(4A) is not due to sequestration of endogenous RhoA (Fig. 4C). Furthermore, in agreement with the effect on formation of stress fibers and focal adhesions, overexpression of p115(4A) was also able to inhibit activation of RhoA by FN (Fig. 4D). These experiments with a DH-dead mutant of Lsc/p115 RhoGEF further support a role for Lsc/p115 RhoGEF in regulating RhoA signaling downstream of adhesion to FN.

Activation of Lsc/p115 RhoGEF by FN involves integrin receptors but not GPCRs

In addition to tandem DH-PH domains, the members of the RGS-GEF family all contain an N-terminal RGS domain. The RGS domain is so named because of its similarity to the RGS box of the regulators of G-protein signaling (RGS) proteins. In response to serum factors such as lysophosphatidic acid (LPA), RGS-GEFs bind to activated $G\alpha_{12/13}$ proteins through their RGS domain and stimulate the intrinsic GTPase activity of the $G\alpha_{12/13}$ proteins. In turn, interaction with the RGS domain allows $G\alpha_{12/13}$ proteins to activate these GEFs (Rossman et al., 2005).

As activation of Lsc/p115 RhoGEF and LARG by LPA is a well-documented event, all of the experiments conducted in this study were performed in the absence of serum to ensure that the cells were not exposed to signals other than adhesion to FN. Our experiments have therefore suggested that the ability of FN to activate Lsc is independent of GPCR signaling. However, to further exclude the possibility of GPCR involvement in Lsc/p115 RhoGEF activation by FN, we made use of a mutant of p115 RhoGEF that lacks the N-terminal region of the protein containing the RGS domain [p115(ΔN), depicted in Fig. 2A]. It has been previously shown that, unlike full-length protein, p115(ΔN) is incapable of binding to constitutively active $G\alpha_{13}$ and will not translocate to the

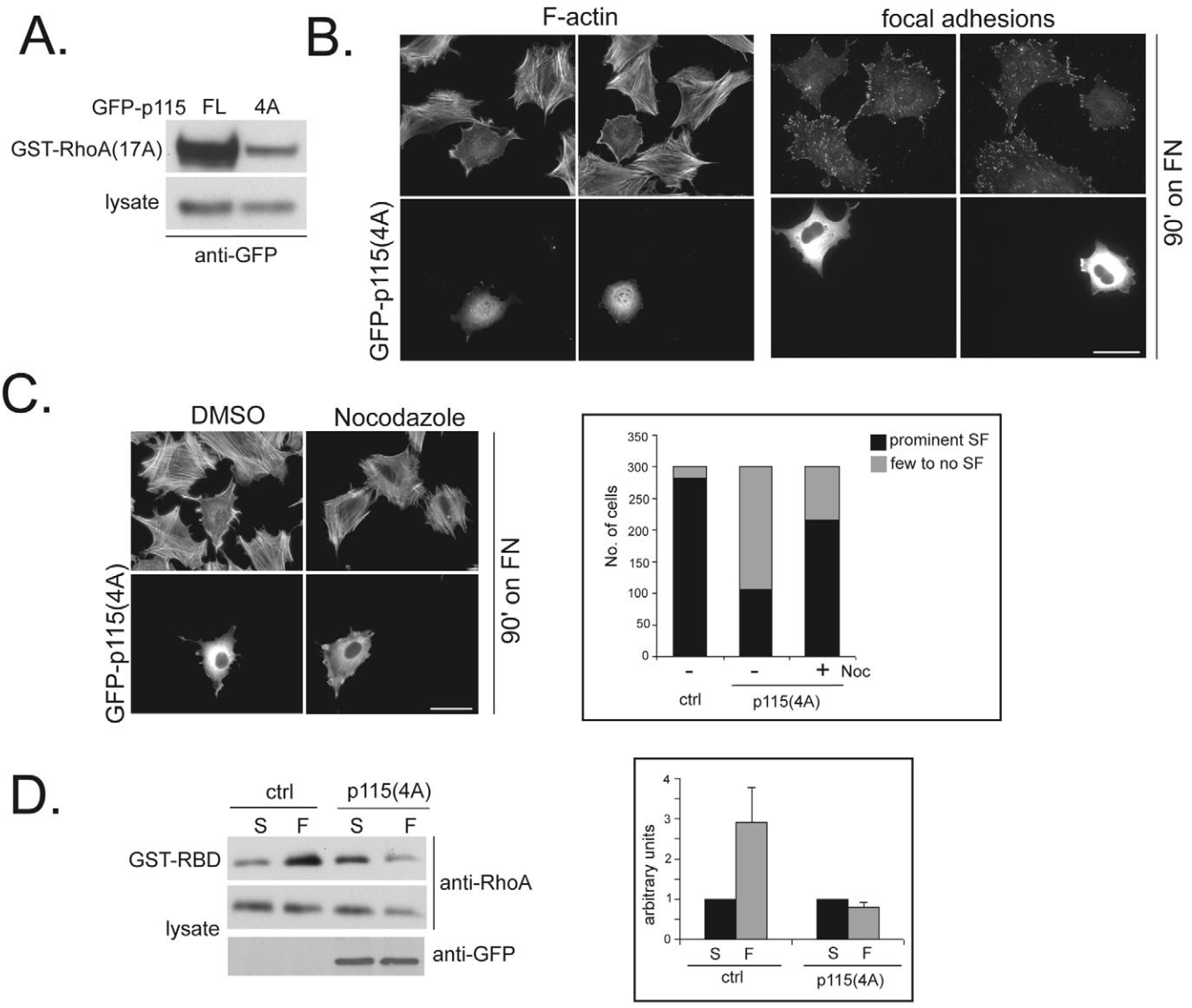

Fig. 4. DH-dead p115 RhoGEF inhibits formation of stress fibers and focal adhesions and RhoA activity on FN. (A) REF52 fibroblasts were transfected with vector encoding GFP-p115(FL) or GFP-p115(4A). 24 hours post transfection, the cells were lysed, pulldowns performed with RhoA(17A) and samples blotted with an antibody against GFP. (B) REF52 cells transfected with vector encoding GFP-p115(4A) were serum-starved, held in suspension for 2 hours and plated onto FN-coated coverslips. The cells were then fixed and stained with phalloidin to visualize F-actin and an antibody against phosphotyrosine to visualize focal adhesions. Bar, 40 μm. (C) REF52 cells overexpressing p115(4A) were plated onto FN-coated coverslips for 60 minutes. The cells were then treated with either DMSO or 10 μM nocodazole for 30 minutes and fixed and stained with phalloidin to visualize stress fibers. Cells were scored according to whether they had prominent stress fibers versus few to no stress fibers. (D) Cells were transfected with GFP-p115(4A), serum-starved and held in suspension for 2 hours. The cells were then plated onto FN-coated dishes, and GST-RBD pulldowns performed, and samples blotted with an antibody against RhoA to visualize the levels of RhoA activity. Lysates were also blotted with an antibody against GFP to show the levels of expression of GFP-p115(4A).

membrane when these proteins are coexpressed, suggesting that p115(ΔN) cannot be activated by LPA (Bhattacharyya and Wedegaertner, 2003a; Bhattacharyya and Wedegaertner, 2003b). To confirm these data in our experimental system, mouse fibroblasts were transfected with vector encoding either V5-tagged full-length p115 RhoGEF [p115(FL)] or RGS-deleted p115 RhoGEF [p115(ΔN)]. At 24 hours post transfection, the cells were serum starved for 16 hours, treated with 5% serum and GEF activity assayed by RhoA(17A) pulldowns. Fig. 5A demonstrates that, unlike p115(FL), p115(ΔN) was not significantly activated by serum treatment.

Next, cells overexpressing p115(ΔN) were serum starved, plated onto FN, and RhoA(17A) assays performed. Importantly, unlike serum treatment, adhesion to FN was able to stimulate activation of p115(ΔN) (Fig. 5B). In addition, overexpression of p115(ΔN) in double-KD Lsc-LARG cells was able to rescue the defect in RhoA activation when the cells are plated onto FN (Fig. 5C). Therefore, both these

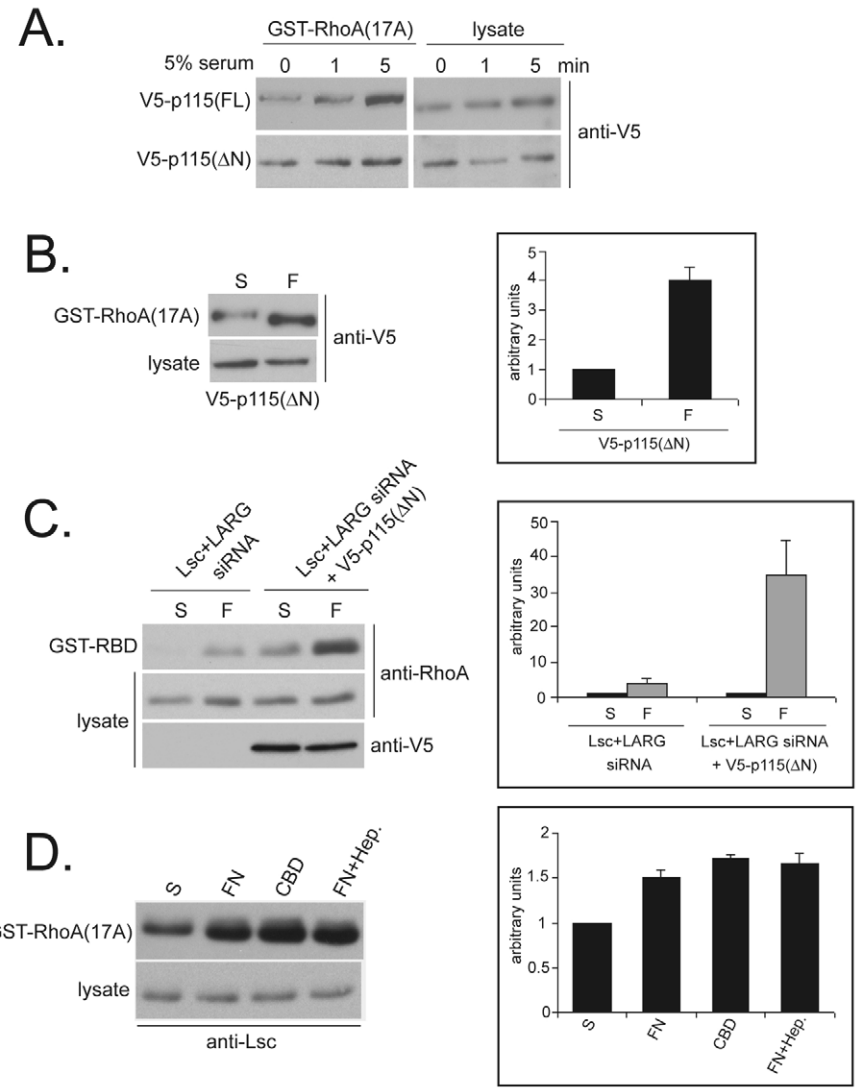

Fig. 5. Activation of Lsc/p115 RhoGEF by adhesion to FN involves integrins but is independent of GPCRs. (A) Fibroblasts were transfected with constructs expressing either V5-p115(FL) or V5-p115(ΔN). 24 hours post transfection, the cells were serum starved for 16 hours, treated with 5% fetal bovine serum for the times indicated and pulldowns performed with RhoA(17A). (B) V5-p115(ΔN)-transfected fibroblasts were serum starved, held in suspension for 2 hours, plan ted onto FN-coated dishes and RhoA(17A) pulldowns performed. (C) Fibroblasts were transfected with siRNA oligonucleotides against Lsc and LARG. 48 hours post transfection, the cells were re-transfected with either a vector control or V5-p115(ΔN). The next day, all the cells were serum starved, held in suspension for 2 hours and plated onto FN-coated dishes for 60 minutes. The cells were then lysed, GST-RBD pulldowns performed and samples blotted with an antibody against RhoA to visualize the levels of RhoA activity. Lysates were also blotted with an antibody against V5 to show the levels of expression of V5-p115(ΔN). (D) Fibroblasts were serum starved and held in suspension for 2 hours. To prevent the production and secretion of endogenous FN by the fibroblasts, 25 μg/ml cycloheximide was included in the media during starvation and suspension. The cells were then plated onto dishes coated with FN, CBD or FN plus heparin. Samples were lysed, incubated with RhoA(17A) and processed for SDS-PAGE and blotting with an antibody against Lsc.

experiments suggest that activation of Lsc/p115 RhoGEF by FN is independent of GPCR signaling.

To resolve further the mechanism of Lsc/p115 RhoGEF activation by FN, we decided to investigate whether integrins and/or syndecans were involved. To do this, we plated serum-starved mouse fibroblasts onto either full-length FN, the CBD fragment of FN alone or full-length FN treated with heparin (to block syndecan adhesion). Compared with suspended cells, activation of Lsc was observed upon adhesion in all cases, indicating that integrin adhesion alone is sufficient for activation of Lsc (Fig. 5D). The experiments above demonstrate that activation of the RhoA GEF Lsc/p115 RhoGEF by adhesion to FN occurs through a mechanism that involves integrins but is independent of GPCRs.

Discussion

Adhesion to the ECM has long been known to influence many characteristics of cells, including their growth, survival, morphology and migratory properties. With the discovery that members of the Rho family of GTPases regulate the organization of the cytoskeleton (Ridley and Hall, 1992; Ridley et al., 1992), it was logical to ask whether the effects of the ECM on morphology and migration of cells are mediated by Rho proteins. Using the experimental model of plating suspended cells (typically fibroblasts) on surfaces coated with ECM proteins such as FN, rapid activation of Rac1 and Cdc42 was demonstrated (Price et al., 1998). Adhesion to FN was shown to induce a biphasic RhoA activity pattern, where a transient dip in activity was followed by a sustained rise in activity (Ren et al., 1999). Our laboratory has been interested in the signaling events that regulate the different stages of Rho activity in response to adhesion to FN. In previous work, we demonstrated that integrin engagement stimulated activation of Src kinase activity, leading to the phosphorylation and activation of p190RhoGAP, and a transient decrease in Rho GTP levels (Arthur et al., 2000). In the present study, we wanted to identify the specific GEF(s) responsible for the activation phase of RhoA in response to adhesion to FN.

Several previous studies have investigated the role of specific RhoA GEFs in different adhesion signaling pathways. For example, it was demonstrated that p190 RhoGEF is phosphorylated by focal adhesion kinase (FAK) in response to laminin engagement in neuronal cells (Zhai et al., 2003). Also, involvement of Lsc in a FN adhesion

pathway was implied by work in Lsc-knockout neutrophils, which demonstrate a reduction in the ability to bind to FN when stimulated with formyl-peptide (fMLP) (Francis et al., 2006). In the current study, we took an unbiased proteomics approach to identify the specific RhoA GEF(s) responsible for regulating RhoA signaling in response to adhesion to FN. Initially, Lsc/p115 RhoGEF was identified as a GEF whose activity [as assessed by RhoA(17A) binding] was stimulated upon adhesion to FN. Further investigation using western blots demonstrated that, although the related RGS-GEF LARG is also activated upon adhesion of fibroblasts to FN, other GEFs such as Ect2 or Dbl are not (Fig. 1). We have established that both Lsc/p115 RhoGEF and LARG are responsible for the ability of cells to activate RhoA downstream of FN, as knockdown of both these GEFs in fibroblasts greatly diminishes FN-induced RhoA activation as well as formation of stress fibers and focal adhesions (Fig. 3). The residual RhoA activity present in the double-KD Lsc-LARG fibroblasts is probably due to incomplete knockdown of these GEFs. Although we cannot rule out the possibility that other GEFs play a secondary role in this pathway (such as the third RGS-GEF family member, PDZ RhoGEF), considering the drastic effect of double KD of Lsc and LARG on FN-induced RhoA signaling in fibroblasts, the contributions of other GEFs, if any, are probably minor.

Considering the well-described function of the RGS-GEF family in mediating LPA-induced RhoA activity, we were initially surprised to have identified Lsc/p115 RhoGEF and LARG as being involved in FN-induced RhoA activity as well. Consistent with a role for these GEFs in RhoA signaling downstream of matrix adhesion, Lsc/p115 RhoGEF and LARG are present in discrete patches at the periphery of the cell that colocalize with paxillin-containing focal adhesions (Fig. 2C). Furthermore, experiments using an RGS-deleted mutant of p115 RhoGEF confirmed that activation of Lsc/p115 RhoGEF by FN is unrelated to the GPCR pathway (Fig. 5). Several lines of evidence have indicated that RGS-GEFs can function in diverse signaling pathways unrelated to GPCR signaling. LARG has been shown to bind to the insulin-like growth factor 1 (IGF-1) receptor and potentially is responsible for transducing signals from IGF-1 to RhoA (Taya et al., 2001). Plexin-B1, a member of a family of receptors that mediate axonal guidance by responding to repulsive cues, has also been shown to bind to PDZ-RhoGEF and LARG and to signal to RhoA through them (Swiercz et al., 2002). Interestingly, previous studies have shown that CD44, a receptor for the ECM component hyaluronan, can bind to both Lsc/p115 RhoGEF and LARG, and this interaction increases RhoA signaling (Bourguignon et al., 2003; Bourguignon et al., 2006).

The specific roles of integrins and syndecans in the downstream activation of RhoA are a matter of controversy in the field. Saoncella and colleagues determined that, although stress fibers and focal adhesions will not form on cells plated onto the cell binding domain (CBD) of FN alone, they will form upon addition of an antibody against syndecan-4, which suggests that syndecan-4 is required for the activation of RhoA (Saoncella et al., 1999). However, a recent paper demonstrated that CBD is sufficient for formation of stress fibers and focal adhesions, suggesting that integrins alone can induce RhoA activity (Wang et al., 2005). Furthermore, it was shown that, although the heparin-binding domain (HBD) alone cannot induce stress fibers and focal adhesions, it can contribute to the formation of these structures when the CBD is present at suboptimal concentrations (Wang et al., 2005). Therefore, having determined that the GEFs Lsc/p115 RhoGEF and LARG are activated by adhesion to FN, we wanted to investigate which adhesion receptors were involved in this process. Our results show that Lsc can be activated by the plating of cells on CBD alone or on heparin-treated FN (which blocks syndecan-4 binding to HBD), which suggests that integrins are sufficient for the activation of the GEF in our experimental system (Fig. 5D).

Previous studies on RGS-GEFs have explored the mechanisms by which these GEFs are activated. Specifically, it has been shown that all the RGS-GEFs can dimerize through a C-terminal homo-oligomerization domain, and that dimerization inhibits the GEF activity of the proteins (Chikumi et al., 2004). It has also been shown that RGS-GEFs are substrates for several different kinases. Although the effects of phosphorylation on RGS-GEFs have not been determined, there is some evidence to suggest that phosphorylation causes an increase in their exchange activity. Protein kinase C α (PKCα) can be activated by adhesion to FN (Disatnik et al., 2002; Dovas et al., 2006) and has been shown to phosphorylate p115 RhoGEF in response to thrombin treatment (Holinstat et al., 2003). Furthermore, LARG is phosphorylated by both FAK and Tec kinase (Chikumi et al., 2002; Suzuki et al., 2003). The exact processes through which Lsc/p115 RhoGEF and LARG are activated by adhesion to FN, whether by phosphorylation or some other mechanism, remain to be explored in detail.

Materials and Methods

Cell lines, reagents and constructs

NIH 3T3 and REF52 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% bovine calf serum or fetal bovine serum (Sigma), respectively, and antibiotic-antimycotic solution (Sigma). Nocodazole was purchased from Calbiochem, and cycloheximide was purchased from Sigma. A construct expressing full-length p115 RhoGEF (residues 1-913, corresponding to IMAGE clone #3451036) was obtained from Invitrogen. A construct expressing full-length LARG was obtained from Alexander Belyavsky (Engelhardt Institute of Molecular Biology, Russia). The DH-dead mutant (with residues E423, K567, L570 and N603 mutated to alanine) of p115 RhoGEF was made using the QuikChange Mutagenesis Kit according to the manufacturer's instructions (Stratagene). The mutant of p115 RhoGEF lacking the RGS-domain-containing N-terminus (residues 1-252 deleted) was amplified by PCR using the appropriate primers. All full-length and mutant DNA fragments mentioned above were cloned into GFP and V5 N-terminally tagged vectors using Gateway Technology (Invitrogen), according to the manufacturer's instructions. Accuracy of all constructs was verified by DNA sequencing.

Purification of recombinant proteins

FN was purified from blood plasma as described previously (Engvall and Ruoslahti, 1977) or obtained from Invitrogen. A construct containing the cell-binding domain (CBD) of FN (containing FN repeats III_{7-10}) in a His-tagged pET 15b vector was obtained from Ikramuddin Aukhil (UNC Chapel Hill). Expression and purification of CBD was performed as described previously (Aukhil et al., 1993; Hashimoto-Uoshima et al., 1997). Construction of the pGEX4T-1 prokaryotic expression constructs containing RhoA(G17A) and the Rho-binding domain (RBD) of Rhotekin have been described previously (Liu and Burridge, 2000; Reuther et al., 2001). Briefly, expression of the fusion proteins in *Escherichia coli* was induced with 100 μM IPTG for 12-16 hours at room temperature. Bacterial cells were lysed in buffer containing 50 mM Tris pH 7.6 (for GST-RBD) or 20 mM HEPES pH 7.6 [for GST-RhoA(17A)], 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 μg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and the proteins purified by incubation with glutathione-sepharose 4B beads (GE Healthcare) at 4°C.

FN plating

Petri dishes or coverslips were coated overnight at 4°C with a 30 μg/ml solution of FN in phosphate-buffered saline (PBS; Invitrogen), or with a 12.5 μg/ml solution

of CBD in PBS (Fig. 5D). The next morning, the plates were washed with PBS, and blocked for 1 hour at 37°C in a solution of DMEM supplemented with 0.5% delipidated bovine serum albumin (BSA; Sigma). As indicated in Fig. 5D, 100 μg/ml heparin (Sigma) was added to a subset of FN-coated dishes during the delipidated BSA incubation. Before all experiments conducted in this study, fibroblasts were completely deprived of serum by extensive washing with PBS, followed by incubation for 3-16 hours in 0.5% delipidated BSA-DMEM. The cells were then held in suspension for 2 hours in the same media, plated onto FN-coated dishes or coverslips for various times and processed for pulldown experiments or immunofluorescence, respectively.

RBD and nucleotide-free (NF) RhoA pulldowns

Active RhoA pulldown experiments were performed as described elsewhere (Arthur and Burridge, 2001). Briefly, suspended and adherent fibroblasts were lysed in 50 mM Tris (pH 7.6), 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 mM $MgCl_2$, 200 μM orthovanadate and protease inhibitors. Lysates were clarified by centrifugation, equalized for total volume and protein concentration, and rotated for 30 minutes with 30 μg of purified GST-RBD bound to glutathione-sepharose beads. The bead pellets were washed in 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 200 μM orthovanadate, with protease inhibitors, and subsequently processed for SDS-PAGE. Affinity precipitation of exchange factors with the nucleotide-free RhoA mutant (G17A) has been described in detail in previous work from our laboratory (Arthur et al., 2002; Garcia-Mata et al., 2006; Noren et al., 2003). Briefly, cells were lysed in 20 mM HEPES (pH 7.6), 150 mM NaCl, 1% Triton X-100, 5 mM $MgCl_2$, 200 μM orthovanadate plus protease inhibitors. Equalized and clarified lysates were incubated with 20 μg of purified RhoA(17A) bound to glutathione-sepharose beads for 60 minutes at 4°C. Samples were then washed in lysis buffer and processed for SDS-PAGE. For the mass spectrometric analysis, the gel was stained with Coomassie Blue, bands of interest analyzed by MALDI-TOF-MS, and selected tryptic peptides were sequenced by nano-ESI-MS/MS at the UNC Proteomics Facility.

Transfections and immunofluorescence

Transfection of NIH 3T3 and REF52 cell lines was performed using Lipofectamine and Plus Reagent, according to the manufacturer's instructions (Invitrogen). For immunofluorescence, coverslips were fixed for 15 minutes in 3.7% formaldehyde (Sigma) and permeabilized for 10 minutes in 0.2% Triton X-100 (Sigma). Primary and secondary antibody incubations were performed for 1 hour at room temperature. Anti-Paxillin was from BD Biosciences, and anti-phosphotyrosine (PY99) was from Santa Cruz Biotechnology. Alexa Fluor 594 phalloidin and goat anti-mouse Alexa Fluor 488 and 594 were obtained from Molecular Probes. Immunofluorescence images were taken with a Zeiss axiovert 200M microscope equipped with a Hamamatsu ORCA-ERAG digital camera and Metamorph Workstation (Universal Imaging Corp.). Confocal images were taken with a Zeiss 510 Meta laser scanning confocal microscope located in the Michael Hooker Microscopy Facility at UNC-Chapel Hill.

Western blotting

Cell lysates subjected to SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). For western blotting, membranes were incubated with primary and secondary antibodies for 1 hour at room temperature. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and visualized using Kodak BioMax film (Kodak). For quantification of western blots, intensity values of bands were measured from three different repeats for each experiment using Image J software (NIH). The graphs for all experiments are plotted as the fold increase over the suspension sample, and the error bars represent the standard error of the mean (s.e.m.). RhoA and Lsc antibodies were purchased from Santa Cruz Biotechnology. Anti-V5 was obtained from Invitrogen, and anti-GFP was from Roche. The antibodies against LARG and Lfc were kind gifts of Kozo Kaibuchi (Nagoya University, Japan) and Robert Rottapel (Ontario Cancer Institute, Canada), respectively. Peroxidase-conjugated goat anti-mouse and bovine anti-goat antibodies were from Jackson ImmunoResearch Laboratories.

siRNA oligonucleotides

Control siGLO oligonucleotides and those specific for knockdown of mouse Lsc (targeted sequence: 5′-GGGCTGAGCAGTATCCTAG-3′) and LARG (targeted sequence: 5′-GGACGGAGCTGTAATTGCA-3′) were purchased from Dharmacon. The Lsc oligonucleotide used displayed 100% homology to both mouse and rat Lsc nucleotide sequence, and thus was able to knockdown Lsc protein levels in both NIH 3T3 and REF52 fibroblasts. The Lsc oligonucleotide did display base-pair mismatches to the human p115 RhoGEF nucleotide sequence and thus did not inhibit the re-expression of human p115 RhoGEF in the knockdown fibroblasts. Transfection of oligonucleotides was performed with the TransIT-siQUEST reagent, obtained from Mirus Corporation, according to the manufacturer's instructions. Efficiency and specificity of knockdown for each experiment was assayed by western blot.

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