Filamin links cell shape and cytoskeletal structure to Rho regulation by controlling accumulation of p190RhoGAP in lipid rafts

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Summary

Cytoskeleton-dependent changes in the activity of the small GTPase Rho mediate the effects of cell shape on cell function; however, little is known about how cell spreading and related distortion of the cytoskeleton regulate Rho activity. Here we show that rearrangements of the actin cytoskeleton associated with early phases of cell spreading in human microvascular endothelial (HMVE) cells suppress Rho activity by promoting accumulation of p190RhoGAP in lipid rafts where it exerts its Rho inhibitory activity. p190RhoGAP is excluded from lipid rafts and Rho activity increases when cell rounding is induced or the actin cytoskeleton is disrupted, and p190RhoGAP knockdown using siRNA prevents Rho inactivation by cell spreading. Importantly, cell rounding fails to prevent accumulation of p190RhoGAP in lipid rafts and to increase Rho activity in cells that lack the cytoskeletal protein filamin. Moreover, filamin is degraded in spread cells and cells that express a calpain-resistant form of filamin exhibit high Rho activity even when spread. Filamin may therefore represent the missing link that connects cytoskeleton-dependent changes of cell shape to Rho inactivation during the earliest phases of cell spreading by virtue of its ability to promote accumulation of p190RhoGAP in lipid rafts.

Key words: Actin cytoskeleton, Rho, p190RhoGAP, Filamin, Extracellular matrix

Introduction

Formation of tissue patterns in the embryo and during wound healing requires that spatial differentials in cell growth, motility and differentiation be established in microenvironments that contain multiple soluble mitogens (Huang and Ingber, 1999). Cells can be switched between these different cell fates in the presence of soluble factors based on local mechanical interactions between cells and their extracellular matrix (ECM) adhesions that alter cell shape and cytoskeletal structure. Various adherent cells, including endothelial, epithelial, fibroblast, and smooth muscle cells, proliferate when spread on rigid ECM substrates, whereas they differentiate when cultured on flexible ECM gels, low ECM coating densities or microfabricated ECM islands substrates that restrict cell spreading (Chen et al., 1997; McBeath et al., 2004). Directional cell motility (Jiang et al., 2005b; Parker et al., 2002) and cell contractility (Polte et al., 2004; Tan et al., 2003) similarly vary as a function of cell shape and ECM mechanics under conditions in which integrin ligation remains constant. Thus, the modulation of intracellular signaling pathways by mechanical stress-induced cytoskeleton rearrangements plays a central role in regulation of cell behavior, via pathways that are, in part, independent of direct integrin signaling.

The small GTPase Rho has emerged as a pivotal control point through which cells sense changes in ECM mechanics and cytoskeletal organization, and thereby translate the 'cell shape signal' to downstream effectors that mediate these behaviors. For example, cell shape-dependent control of cell cycle progression is mediated by cytoskeletal distortion-dependent changes in Rho activity (Mammoto et al., 2004; Welsh et al., 2001) that modulate the biochemical machinery responsible for the G1-S checkpoint (Huang and Ingber, 1999). Rho is also involved in the mechanisms by which cell shape influences contractility (Tan et al., 2003) and cell lineage switching (McBeath et al., 2004; Sordella et al., 2003). At the same time, Rho plays a central role in the control of actin cytoskeleton remodeling and is required for formation of stress fibers and focal adhesions, as well as polarized cell spreading, motility, and cytokinesis (Burridge and Wennerberg, 2004; Etienne-Manneville and Hall, 2002). Thus, Rho is both a sensor and an actuator of changes in cytoskeletal organization.

Although many studies have examined how Rho induces cytoskeletal rearrangements, little is known about how changes in cell shape and cytoskeletal organization themselves regulate Rho. This mechanism is complicated because Rho activity is subjected to non-linear feedback control mechanisms that produce complex changes in Rho activity dynamics in response to ECM binding to integrins and associated cell shape distortion. For example, cell adhesion and spreading on fibronectin substrates trigger a triphasic response: there is a rapid transient inhibition of Rho activity, followed by an activation phase, and then a subsequent gradual decrease down to a low basal level (Ren et al., 1999). A multi-phasic time

course of this type often results from multiple feedback control loops. In fact, once activated, Rho's ability to increase myosin light chain phosphorylation through activation of Rho-associated kinase (ROCK) may feed back to further activate Rho by increasing the level of tension in the cytoskeleton (Paszek et al., 2005; Riveline et al., 2001).

Rho activity can be suppressed by any one of a variety of different RhoGAP proteins that return it to its inactive GDP-bound state. p190RhoGAP has been shown to be phosphorylated by Src tyrosine kinases when cells first attach to ECM substrates and integrin receptors become ligated; this allows p190RhoGAP to exert its RhoGAP activity, leading to inactivation of Rho (Arthur and Burridge, 2001; Arthur et al., 2000). Cell detachment and rounding in mitosis also have been reported to suppress p190RhoGAP and increase Rho activity (Maddox and Burridge, 2003). However, several lines of evidence suggest that cytoskeletal distortion can convey distinct signals from those triggered by integrin engagement alone (Chen et al., 1997; Huang and Ingber, 1999; Jiang et al., 2005b; Matthews et al., 2006; McBeath et al., 2004; Parker et al., 2002; Polte et al., 2004; Tan et al., 2003), and these signals may be required for the changes in Rho activity that drive cell cycle progression, stem cell lineage switching and other physiological cellular responses (Huang and Ingber, 1999; Mammoto et al., 2004; McBeath et al., 2004; Tan et al., 2003). Thus, some signaling mechanisms triggered by integrin binding that are important for control of Rho activity may be modulated through subsequent mechanical changes in the cytoskeleton. Importantly, p190RhoGAP associates with the actin cytoskeleton, raising the possibility that p190RhoGAP may connect Rho regulation to the physical state of the cytoskeleton, in addition to mediating effects elicited by direct integrin engagement. However, the molecular basis for this interaction between the cytoskeleton, p190RhoGAP and Rho remains unknown (Brouns et al., 2001).

p190RhoGAP exhibits its GAP activity specifically when localized in lipid rafts (Sordella et al., 2003). Lipid rafts are membrane microdomains enriched in saturated phospholipids, sphingolipids and cholesterol that participate in a wide range of biological processes, including signal transduction, apoptosis, adhesion, migration, protein sorting, and cytoskeletal remodeling (Brown and London, 1998; Fullekrug and Simons, 2004; Harris and Siu, 2002; Simons and Toomre, 2000). The observation that Rho GTPases and p190RhoGAP both localize to lipid rafts (del Pozo et al., 2004; Kawamura et al., 2003; Michaely et al., 1999; Sordella et al., 2003), and that p190RhoGAP mediates integrin ligation-dependent inactivation of Rho (Arthur and Burridge, 2001; Arthur et al., 2000), raise the possibility that these specialized signaling complexes also may play a role in the inhibition of Rho observed during cell spreading on ECM.

Filamin A (filamin) is an actin-binding protein with cross-linking activity that binds to Rho and many other related proteins, including ROCK and β1 integrin, as well as to the lipid raft-associated protein caveolin-1 (Calderwood et al., 2001; Ohta et al., 1999; Stahlhut and van Deurs, 2000; Ueda et al., 2003). Filamin acts as a scaffold for actin cytoskeleton reorganization and plays a role in signal transduction and cell migration (Feng and Walsh, 2004; Stossel et al., 2001). Here we show in human microvascular endothelial (HMVE) cells that filamin links cell spreading on ECM and associated changes of cytoskeletal structure to Rho regulation based on its ability to control p190RhoGAP accumulation in lipid rafts where it inhibits Rho activity.

Results

Cytoskeleton-dependent control of Rho activity

Fibroblasts have been reported to exhibit an initial transient suppression of Rho activity within approximately 15-30 minutes after they attach to fibronectin-coated substrates (Arthur and Burridge, 2001; Ren et al., 1999). When Rho activity was measured in HMVE cells using the rhotekin pull-down assay (Ren et al., 1999) before and after plating on fibronectin-coated dishes, a similar repression of RhoA activity was observed within 30-60 minutes after plating, that subsequently returned to starting levels by 1.5 hour (Fig. 1A). To explore whether changes of cytoskeletal structure that are associated with cell spreading contribute to this response, cells were plated on fibronectin dishes in the presence of cytochalasin D (cytoD; 500nM), a drug that disrupts the physical continuity of the actin cytoskeleton and prevents cell extension (Fig. 1B) without producing a net change in total F-actin polymer (Schliwa, 1982). In contrast with control cells, Rho activity was not suppressed when the cytoD-treated cells adhered to the fibronectin substrates; instead, activated Rho accumulated to more than threefold higher levels by 1.0-1.5 hour after plating (Fig. 1A). Similarly, when cells were placed in suspension to prevent spreading without use of pharmacological modifiers, a similar fourfold increase in Rho activity was observed relative to cells on fibronectin by 1.0-1.5 hour (Fig. 1A). Rho activity in suspended cells reached a maximal and stable level within 0.5 hours after detachment (not shown). Thus, cell shape and cytoskeletal distortion appear to control Rho activity.

Cytoskeleton-dependent control of p190RhoGAP localization

p190RhoGAP has been shown to mediate the inhibition of Rho activity induced by integrin engagement associated with the initial formation of cell-ECM adhesions (Arthur and Burridge, 2001; Arthur et al., 2000). However, since mechanical distortion of the cytoskeleton that is associated with cell spreading on ECM can elicit signals that are distinct from those elicited by integrin binding alone (Chen et al., 1997), we examined whether global restructuring of the actin cytoskeleton induced by cytoD can modulate p190RhoGAP's ability to alter Rho activity. We found that suppression of p190RhoGAP protein expression by more than 95% using siRNA resulted in a twofold increase in activated Rho in spread cells, whereas it had no effect in cytoD-treated cells that already exhibited optimal levels of Rho activity (Fig. 2A), and it did not alter the morphology of control or drug-treated cells (data not shown). Thus, p190RhoGAP appears to be required for the inhibition of Rho that is induced by the cytoskeletal restructuring that accompanies early phases of cell spreading on ECM.

Next we explored whether lipid rafts contribute to this response because p190RhoGAP has been reported to exhibit its GAP (GTPase activating protein) activity at these signaling sites (Sordella et al., 2003). In our initial studies, we extracted HMVE cells spread on FN with the detergent Triton-X-100 (TrX) and found that p190RhoGAP was present in both the

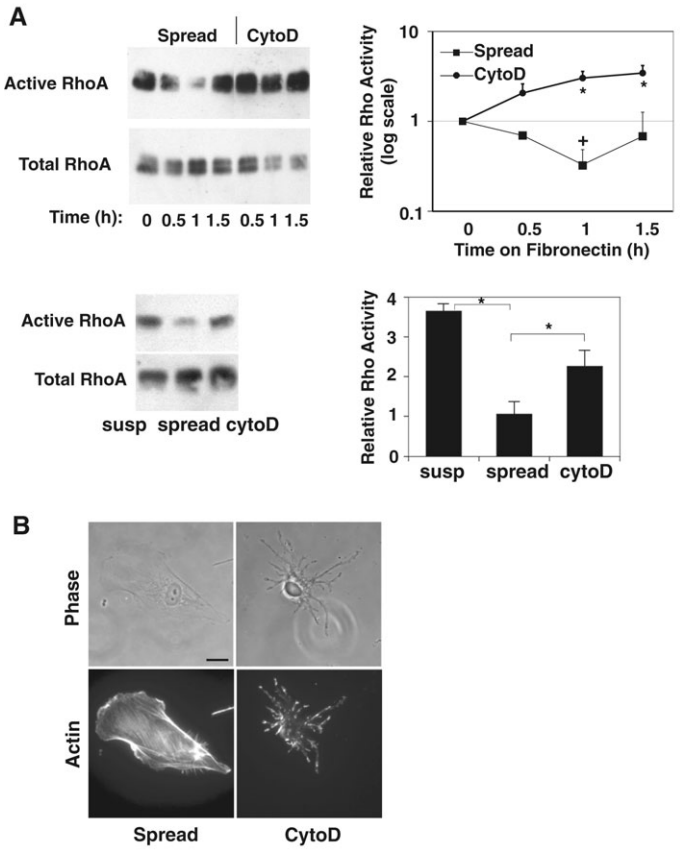

Fig. 1. Actin cytoskeleton configuration regulates Rho activity in HMVE cells. (A) Time course of Rho activity in cells plated on fibronectin-coated dishes in the presence or absence of cytoD (upper panel), and comparison of Rho activity in spread cells versus round cells 1.5 hours after replating (lower panel). Rho activity is presented as the ratio of active RhoA (determined using the rhotekin-RBD bead pull down assay) to total RhoA protein in each cell lysate (upper panel, **P*<0.05 for Rho activity in spread versus cytoD-treated cells; ^{+}P<0.02 for Rho activity at 1 hour after plating versus time zero. Lower panel, **P*<0.01). (B) Phase contrast and fluorescence images of cells stained for F-actin showing cell morphology and the actin cytoskeleton of HMVE cells in the presence or absence of cytoD (scale bar, 5 μm).

soluble and detergent-insoluble fractions (Fig. 2B). The insoluble fraction obtained in this manner has been previously shown to be enriched for lipid rafts in other cells (Simons and Toomre, 2000). We also found this fraction to be enriched for caveolin-1 (Fig. 2B), which is a defining component of lipid rafts (Smart et al., 1995; Song et al., 1996). Quantification of these results revealed that about 10% of total p190RhoGAP protein was present in the TrX-insoluble fraction, which was significantly enriched (more than threefold higher) relative to the focal adhesion protein paxillin, making it unlikely that the presence of p190RhoGAP in this fraction is due to non-specific contamination during the extraction procedure. By contrast, this enrichment was not seen in cytoD-treated cells as the TrX insoluble fraction contained approximately 3% of both total p190RhoGAP and paxillin (Fig. 2B); this low level of expression may represent baseline non-specific background for this technique. In any case, these initial findings suggested that p190RhoGAP is enriched in the lipid raft fraction in spread cells.

To confirm that p190RhoGAP localizes within lipid rafts in HMVE cells, we used the more rigorous, detergent-free sucrose gradient method for raft fractionation (Smart et al., 1995; Song et al., 1996) because detergents can disturb protein interactions with lipid rafts (Simons and Toomre, 2000; Smart et al., 1995; Song et al., 1996). Western blot analysis of the different sucrose gradient fractions revealed that the lipid raft marker, calveolin-1 (Smart et al., 1995; Song et al., 1996), was enriched in fractions 4 and 5 when HMVE cells spread on fibronectin (boxed lanes in Fig. 2C). Fractions free of lipid rafts or caveolae were identified by the absence of caveolin-1 and by the presence of paxillin (Fig. 2C) and RhoGDI (not shown), which have been previously shown not to be present in raft fractions (Kawamura et al., 2003; Smart et al., 1995). In contrast to caveolin-1, paxillin (Fig. 2C) and RhoGDI exhibited distinct distribution patterns in that they were completely absent from fractions 4 and 5, and instead were concentrated in fractions 6-12.

Since p190RhoGAP was present in fraction 5 in spread cells but not fraction 4, these experiments show that a small but detectable amount of p190RhoGAP co-distributed with caveolin in the lipid raft fraction that contains heavier membranes, while the remainder co-distributed with paxillin (Fig. 2C). By contrast, when cells were treated with cytoD, p190RhoGAP was almost completely excluded from the lipid raft fraction (fraction 5), and instead redistributed to the paxillin-containing fractions 6-12, even though the distribution of caveolin-1 and paxillin remained unchanged (Fig. 2C). Similar results also were obtained when cells were prevented from spreading by either placing them in suspension, or adding latrunculin B (latB) that induces depolymerization of F-actin, and thus alters cytoskeletal organization through a different mechanism than cytoD (Spector et al., 1989) (Fig. 2C). Densitometric analysis of the blots revealed that after treatment with cytoD or culture in suspension, the ratio of p190RhoGAP in the lipid raft fraction to total p190RhoGAP protein distributed throughout the entire gradient decreased to one-fifth of that observed in spread control cells. In latB-treated cells, the fraction of p190RhoGAP in rafts was also significantly reduced to about one-third of that in control cells (Fig. 2C). Treatment of spread cells with the lipid raft-disrupting agent, methyl-β-cyclodextrin, decreased the level of p190RhoGAP level in the lipid raft membrane fraction to one-fourth of that of the untreated, spread control cells (Fig. 2C). HMVE cells, unlike fibroblasts or cell lines, are a highly heterogeneous primary cell population, and thus, their response to perturbations tends to be moderate and gradual rather than all-or-none. Thus, we always performed at least three independent measurements with different cell batches, and these all yielded consistent results. Thus, these findings suggest that the ability of p190RhoGAP to localize to lipid rafts is regulated by the structural configuration of the actin cytoskeleton.

Phosphorylation status of p190RhoGAP

p190RhoGAP is a substrate of Src tyrosine kinases and its activity is modulated by tyrosine phosphorylation (Arthur et al., 2000; Brouns et al., 2001). Integrin engagement has been

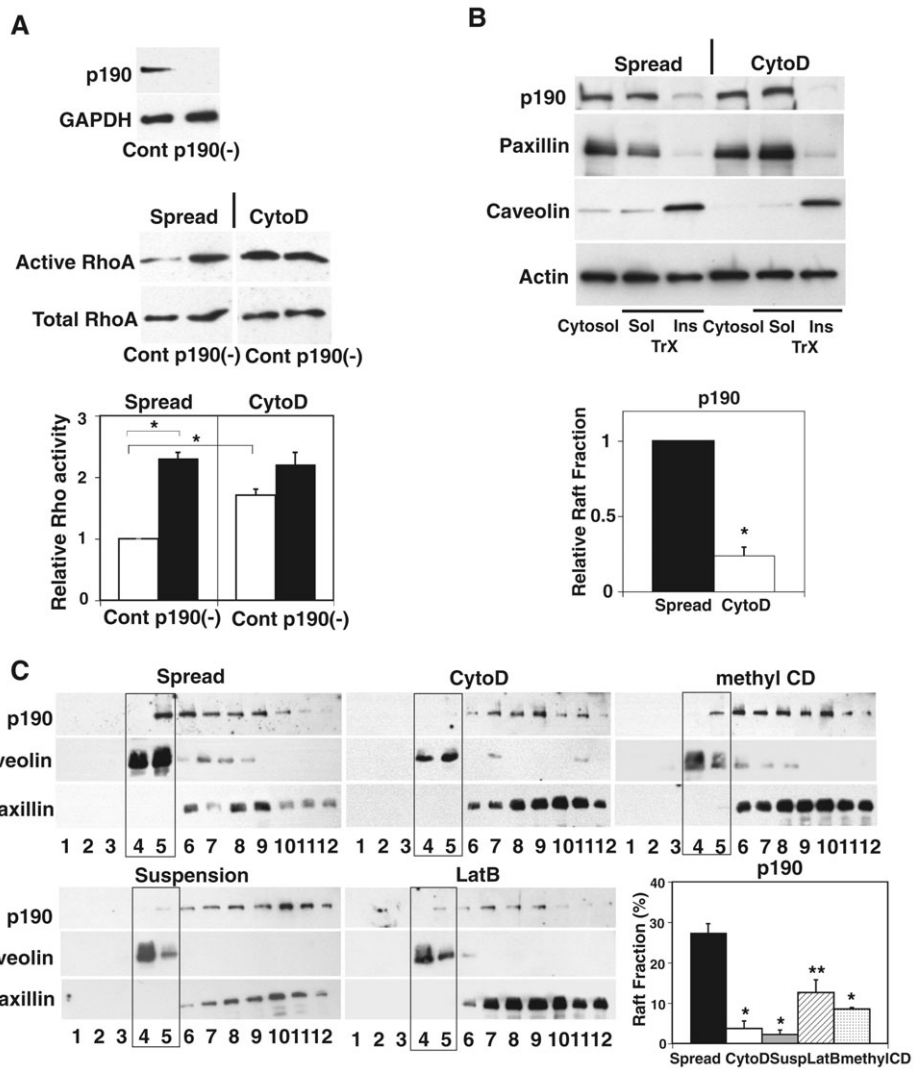

Fig. 2. Actin cytoskeleton configuration regulates p190RhoGAP localization. (A) Levels of active RhoA in p190RhoGAP knockdown HMVE cells in the presence or absence of cytoD 1 hour after replating on fibronectin-coated plates. Quantitative results were normalized to spread control cells without cyto D (**P*<0.01). (B) Immunoblots showing lipid raft localization of p190RhoGAP in spread versus cytoD-treated cells based on the detergent insoluble membrane purification method. Data are presented as the ratio of p190RhoGAP in the insoluble TrX fraction to the total protein level (**P*<0.01). (C) Localization of p190RhoGAP in spread, suspended, and cytoD-, lat B-, or methyl β-cyclodextrin treated cells based on the detergent-free sucrose gradient floating assay. Data are presented as the ratio of p190RhoGAP in the lipid raft fractions (fractions 4 and 5) to total p190RhoGAP protein distributed throughout the entire gradient (**P*<0.01; ***P*<0.02).

reported to activate p190RhoGAP through this Src-dependent phosphorylation mechanism (Arthur et al., 2000), although a contribution by the cyctoskeleton was not excluded. Here, we examined whether perturbation of cell shape and cytoskeletal structure regulate p190RhoGAP through this mechanism (i.e. by altering its tyrosine phosphorylation state), and if so, whether this is responsible for the effects on lipid raft localization we observed. The total level of tyrosine phosphorylation of p190RhoGAP observed in cells spread on fibronectin was suppressed by cytoD treatment to a degree similar to that produced by treatment with the Src-tyrosine kinase inhibitor PP2 (10 μM) (Fig. 3A). When the membrane subfractions were analyzed in spread cells, tyrosine phosphorylated p190RhoGAP was found in both the soluble fraction and in the TrX-insoluble lipid raft fraction (Fig. 3B). The level of tyrosine-phosphorylated p190RhoGAP was decreased in both the soluble and TrX-insoluble fractions of cytoD-treated cells relative to similar fractions in control spread cells (Fig. 3B); however, it was difficult to analyze p190RhoGAP phosphorylation in the TrX-insoluble fraction of drug-treated cells because protein levels were very low (Fig. 2B, Fig. 3B). Importantly, use of the detergent-free sucrose gradient assay revealed that although treatment of spread cells with PP2 (10 μM) almost completely inhibited p190RhoGAP phosphorylation, it did not inhibit p190RhoGAP accumulation in lipid rafts (Fig. 3A,C). The tyrosine kinase, Src, was also present both in lipid raft and non-lipid raft fractions, regardless of whether its activity was inhibited with PP2 (Fig. 3C). Thus, in these HMVE cells, p190RhoGAP phosphorylation by Src tyrosine kinases may occur inside or outside of the lipid rafts. Thus, changes of cell shape and associated cytoskeletal distortion appear to influence Rho by controlling accumulation of p190RhoGAP in lipid rafts which is a distinct mechanism from the one that is activated by initial integrin binding and involves Src-dependent phosphorylation of p190RhoGAP (Arthur et al., 2000).

Filamin regulates p190RhoGAP translocation and Rho activation

These findings raise the central question of how p190RhoGAP accumulation in lipid rafts is influenced by changes in cytoskeletal structure that accompany cell spreading. Filamin is a large cytoskeletal protein that mediates actin filament crosslinking, and binds to caveolin-1 as well as Rho effectors

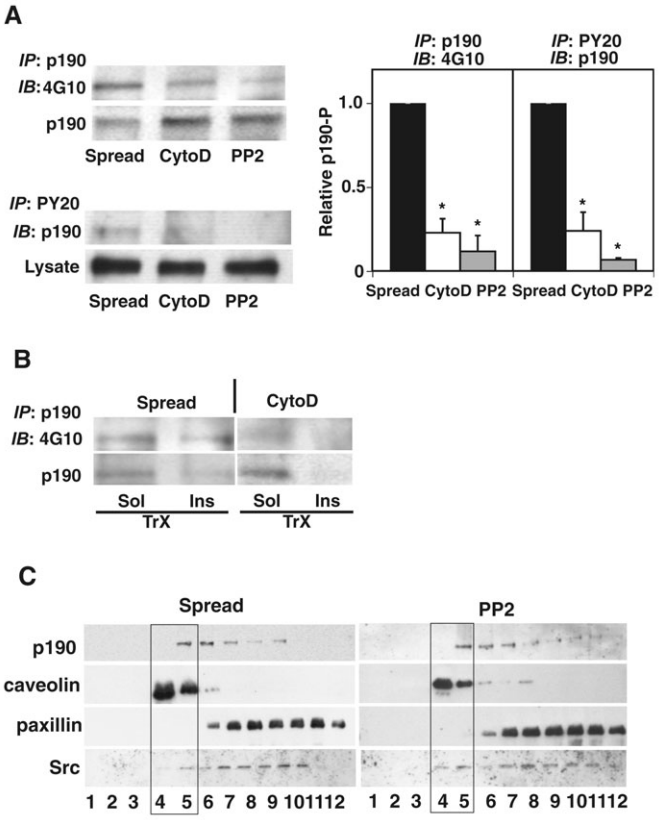

Fig. 3. Cell shape effects on the phosphorylation status of p190RhoGAP. (A) Immunoblots showing (a) tyrosine phosphorylated p190RhoGAP detected by phosphotyrosine antibody (4G10) or (b) p190RhoGAP immunoprecipitated with phosphotyrosine antibody (PY20) from total cell lysates of spread, cyto D-, or PP2- treated cells (*P<0.01). (B) Tyrosine phosphorylated p190RhoGAP detected by phosphotyrosine antibody (4G10) in TrX-100-soluble and -insoluble fraction isolated from spread versus cyto D-treated cells. (C) Raft localization of p190RhoGAP and Src tyrosine kinase in spread versus PP2-treated cells, using methods shown in Fig. 2C.

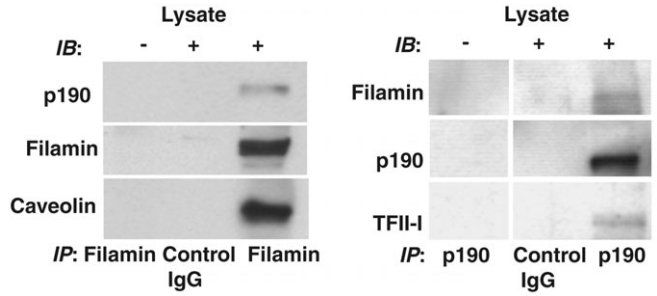

Fig. 4. Interactions between p190RhoGAP and filamin. Immunoblots of p190RhoGAP coimmunoprecipitated with anti-filamin antibody (mAb 1678) and vice versa. Mouse IgG was used as a control for immunoprecipitation. Caveolin-1 and TFII-I were used as a positive control for filamin and p190RhoGAP immunoprecipitation respectively.

and partners. Thus, we hypothesized that filamin may contribute to control of p190RhoGAP accumulation in lipid rafts. Immunoprecipitation experiments revealed that filamin co-precipitates with p190RhoGAP and vice versa (Fig. 4). In our positive control studies, we also confirmed that filamin binds caveolin-1, and that p190RhoGAP interacts with the transcription factor, TFII-I, as previously reported (Jiang et al., 2005a; Stahlhut and van Deurs, 2000).

The finding that filamin forms a complex with p190RhoGAP suggests that filamin could potentially mediate the effects of cytoskeletal distortion on p190RhoGAP shuttling to or from lipid rafts. To test this hypothesis, we compared the ability of p190RhoGAP to accumulate in lipid rafts in filamin-deficient human melanoma (M2) cells compared with M2 cells that were stably reconstituted with filamin (A7 cells) (Cunningham et al., 1992). The filamin-containing A7 cells spread on fibronectin and formed well-developed focal adhesions and stress fibers, whereas the filamin-deficient M2 cells attached but remained retracted with only a few small focal adhesions and rare short actin bundles (Fig. 5A). CytoD treatment of the filamin-containing A7 cells induced disassembly of focal adhesions and stress fibers, and prevented cell spreading (Fig. 5A), as observed in normal HMVE cells (Fig. 1B). Treatment of M2 cells with cytoD resulted in further disassembly of the rare focal adhesions and actin fibers that normally formed in these cells; however, it had a smaller effect on overall cell shape than it did in A7 cells (Fig. 5A).

When spread filamin-containing A7 cells were analyzed using the detergent-free sucrose gradient assay, p190RhoGAP was found in the caveolin-containing lipid raft fraction (fraction 4) in these melanoma cells (Fig. 5B). Disruption of cytoskeletal integrity by treatment with cytoD or placing cells in suspension resulted in its loss from the lipid raft fraction (Fig. 5B), as observed in HMVE cells (Fig. 2C). By contrast, treatment with cytoD or culturing the cells in suspension did not have any effect on the distribution of p190RhoGAP in filamin-deficient M2 cells, since it remained in fraction 4 in both control and cytoskeleton-disrupted cells (Fig. 5B). Quantification of the blots from multiple experiments revealed that the fraction of p190RhoGAP in the lipid raft in cytoD-treated and suspended A7 cells was reduced to a third or a tenth, respectively, of that of spread cells. In spread M2 cells, p190RhoGAP in the lipid raft fraction was slightly reduced compared with that in A7 cells, and cytoD-treatment or suspension culture did not cause any significant change in p190RhoGAP distribution in M2 cells (Fig. 5B). These results demonstrate that filamin is essential for cells to exclude p190RhoGAP from lipid rafts under conditions in which cell spreading is prevented or cytoskeletal integrity is compromised.

To determine if the filamin-dependent exclusion of p190RhoGAP from lipid rafts has functional consequences, we measured tyrosine phosphorylation of total cellular p190RhoGAP as a measure of its activation state. In spread filamin-containing A7 cells, p190RhoGAP was tyrosine phosphorylated, whereas disruption of the actin cytoskeleton using cytoD or suspension culture reduced p190RhoGAP phosphorylation by more than 50% (Fig. 5C). By contrast, cytoD and suspension culture had no effect on p190RhoGAP phosphorylation in filamin-deficient M2 cells (Fig. 5C),

suggesting that filamin is required for changes in cytoskeletal structure to influence p190RhoGAP's ability to accumulate in lipid rafts and become phosphorylated. Additional studies revealed that filamin-containing A7 cells that spread on fibronectin exhibited almost a two- to three-fold increase in Rho activity when treated with cytoD or placed in suspension (Fig. 5D), similar to the response displayed by normal HMVE cells (Fig. 1A). By contrast, Rho activity was not affected by cytoD or suspension culture in M2 cells that lack filamin (Fig. 5D).

To determine if filamin also plays a role in Rho signaling in the HMVE cells, we used siRNA to knock down filamin levels in these cells. Immunoblotting revealed that cells transfected with filamin siRNA displayed more than a 90% reduction of filamin protein expression (Fig. 6A), although it did not cause the morphological alterations displayed by M2 cells (not shown). When spread HMVE cells were transfected with control siRNA, p190RhoGAP was found in the lipid raft fractions when analyzed using the detergent-free sucrose gradient assay (Fig. 6B). Treatment with cytoD reduced this

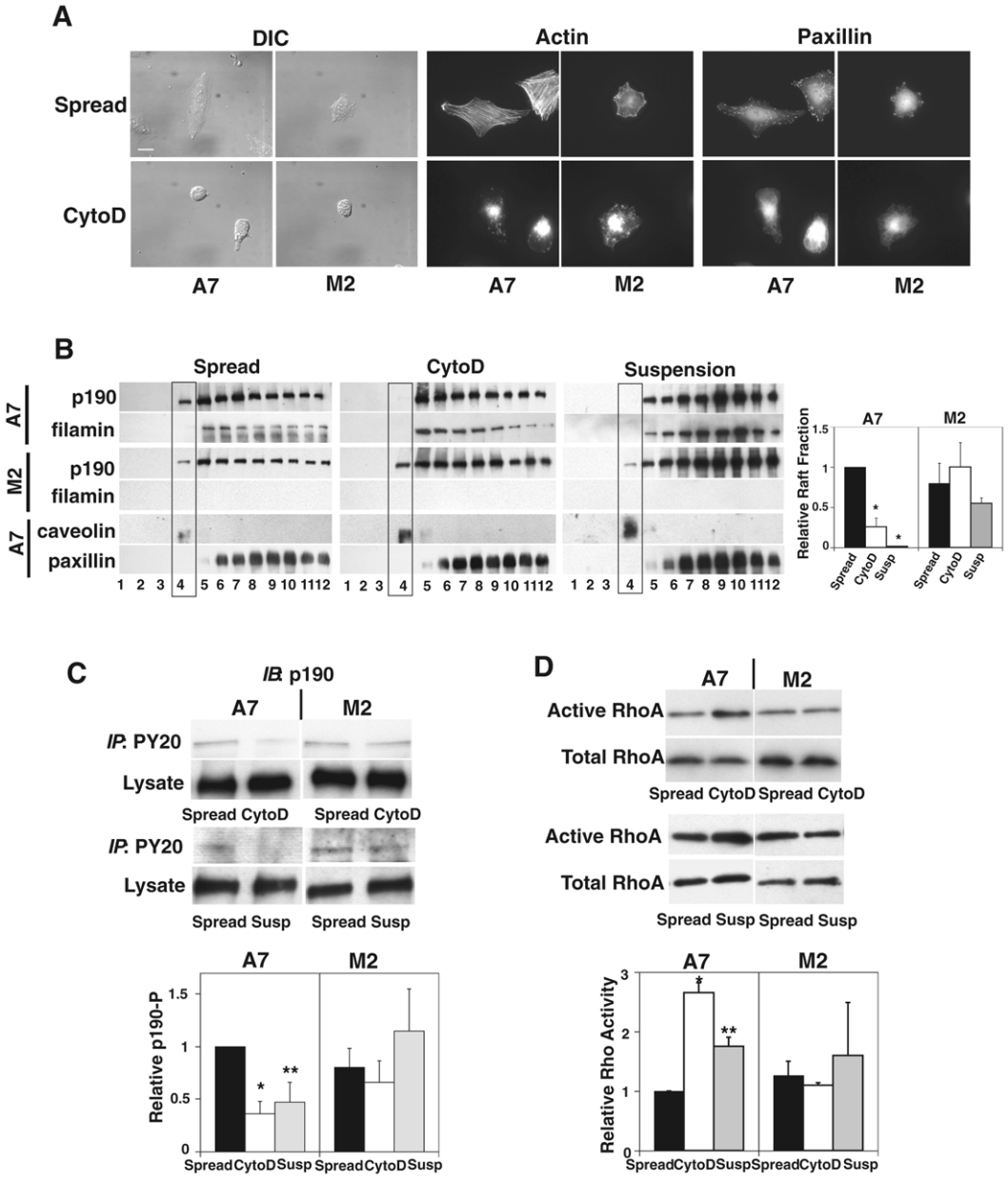

Fig. 5. p190RhoGAP localization in filamin null (M2) and repleted (A7) human melanoma cells. (A) DIC and fluorescence micrographs illustrating cell shape, stress fiber organization and focal adhesion formation in spread or cytoD-treated A7 and M2 cells. F-actin was visualized by staining with Alexa-488-phalloidin; focal adhesions were stained with an anti-paxillin antibody (bar, 5 μm). (B) Raft-localization of p190RhoGAP in spread, suspended, or cytoD-treated A7 and M2 cells. Quantitative results were normalized to spread A7 cells (*P<0.01). (C) Immunoblots showing phosphorylated p190RhoGAP as detected by immunoprecipitation with PY20 antibody in spread, suspended, or cytoD-treated A7 and M2 cells. Quantitative results were normalized to spread A7 cells (*P<0.03, **P<0.05). (D) Levels of active RhoA in spread, suspended, or cyto D-treated A7 and M2 cells 1 hour after replating. Quantitative results were normalized to spread A7 cells (*P<0.01, **P<0.03).

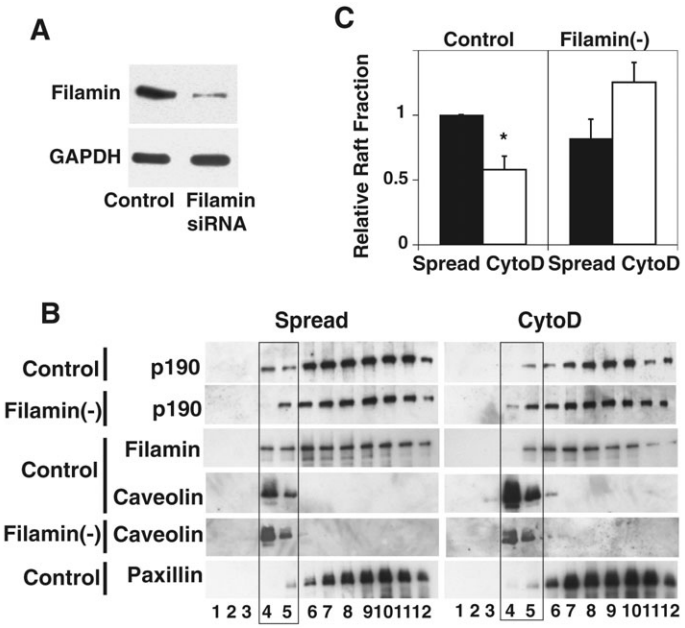

Fig. 6. p190RhoGAP localization in filamin knockdown HMVE cells. (A) Filamin expression in siRNA-mediated filamin knockdown HMVE cells. (B,C) Raft-localization of p190RhoGAP and filamin in spread versus cytoD-treated filamin-knockdown HMVE cells. Quantitative results were normalized to spread control cells without cyto D (*P<0.02).

level by approximately 50% while the distributions of caveolin and paxillin between the soluble and lipid raft fractions did not change (Fig. 6B,C). By contrast, when filamin was knocked-down using siRNA, baseline p190RhoGAP levels in the lipid raft fraction were slightly reduced compared with control cells, but cytoD did not further reduce p190RhoGAP levels in this fraction, and the distribution of caveolin remained unchanged under these conditions (Fig. 6B,C). CytoD also had less effect on Rho activity in filamin knockdown cells, much like we observed in M2 cells that lack filamin (not shown). Taken together, these results indicate that filamin is required to exclude p190RhoGAP from the lipid rafts and to prevent it from inactivating Rho in round cells or in cells in which the cytoskeletal restructuring that normally accompanies cell spreading is inhibited.

p190RhoGAP translocation to rafts is mediated by cleavage of filamin by calpain

When the distribution of filamin within the sucrose gradient fractions isolated from HMVE cells was analyzed by western blot analysis, we detected a progressive increase in the number of faster migrating (smaller molecular mass) fragments in the non-raft fractions (6-12), whereas these fragments were almost completely absent in cells cultured in suspension or in the presence of cytoD or latB (Fig. 7A). Filamin has been reported to be cleaved by the intracellular calcium-dependent protease, calpain (Gorlin et al., 1990), which also has been implicated in regulation of cytoskeletal structure, as well as Rho signaling (Bialkowska et al., 2000; Glading et al., 2002; Gorlin et al., 1990; Kulkarni et al., 2002; Perrin and Huttenlocher, 2002). When HMVE cells were treated with the calpain inhibitory peptide, ALLN (5 μg ml^{-1}), filamin fragments did not appear in the spread cells (Fig. 7A). Interestingly, treatment of spread cells with ALLN also inhibited accumulation of p190RhoGAP in the lipid raft fraction; however ALLN did not have any effect on p190RhoGAP accumulation in filamin knockdown cells (Fig. 7B). Thus, cleavage of filamin by calpain seems to play an important role in accumulation of p190RhoGAP in lipid rafts.

To directly determine whether intact filamin actively prevents p190RhoGAP accumulation in lipid rafts, we engineered a non-cleavable form of GFP-filamin in which its calpain targeting sequence (TYA: 1762-1764 aa) (Gorlin et al., 1990) was deleted (GFP-filamin-Δ). Although this mutation did not completely prevent filamin cleavage, the levels of smaller molecular weight degradation bands were significantly reduced (Fig. 7C). M2 cells transiently transfected with the GFP-filamin-Δ plasmid almost completely excluded p190RhoGAP (Fig. 7D) from the lipid raft fraction. These transfected M2 cells also exhibited a twofold increase in Rho activity (Fig. 7E), even though the cells were not treated with cytoD. CytoD treatment also did not further increase of Rho activity over the already high basal levels that were observed in these cells (not shown). Thus, the constitutive presence of intact (uncleaved) filamin protein in the non-raft fractions effectively excludes p190RhoGAP from lipid rafts, and thereby allows activated Rho to accumulate in the cell.

p190RhoGAP localization in FAK knockdown HMVE cells

Although treatment of cells with cytoD induce cytoskeletal disruption as its major effect, it also can cause disengagement of already bound integrins and inhibit certain signaling pathways, such as the focal adhesion kinase (FAK) pathway (Roovers and Assoian, 2003). Thus, to further dissect the relative roles of signals elicited by integrin ligation versus cell shape distortion in regulating Rho, we next asked whether filamin-mediated translocation of p190RhoGAP would be affected by the absence of the FAK since this kinase has been shown to mediate the effects of integrin ligation on Rho activity (Ren et al., 2000). When FAK was knocked down using siRNA (Fig. 8A), it did not affect the accumulation of p190RhoGAP in the lipid raft fraction of spread cells (Fig. 8B), even though it blocked p190RhoGAP phosphorylation (Fig. 8C) as previously demonstrated in other cell types (Arthur et al., 2000; Ren et al., 2000). Thus, FAK activation is necessary for the phosphorylation of p190RhoGAP, but not for its accumulation in lipid rafts induced by cell spreading, confirming that raft localization and p190RhoGAP phosphorylation represent independent mechanisms for regulating Rho activity.

Discussion

Mechanical distortion of cells and their internal cytoskeleton regulates a wide array of cellular processes, including growth, differentiation, motility, apoptosis, contractility and stem cell lineage switching (Chen et al., 1997; Huang et al., 1998; Jiang et al., 2005b; Mammoto et al., 2004; McBeath et al., 2004; Parker et al., 2002; Paszek et al., 2005; Polte et al., 2004; Tan et al., 2003). The small Rho GTPase has been shown to play a pivotal role in these forms of cell shape-dependent behavioral control (Mammoto et al., 2004; McBeath et al., 2004; Parker

et al., 2002; Tan et al., 2003). Rho exerts its effects on cell structure based on its ability to both promote changes in actin polymerization and control cytoskeletal tension generation (Burridge and Wennerberg, 2004; Etienne-Manneville and Hall, 2002; Hinz and Gabbiani, 2003). However, this is a bidirectional mechanism: changes in cytoskeletal organization and mechanical stresses also feed back to control Rho activity (Mammoto et al., 2004; Riveline et al., 2001; Tan et al., 2003). Although much is known about how Rho controls the cytoskeleton, little is known about how changes in cytoskeletal structure feed back to regulate Rho.

Our data show that accumulation of p190RhoGAP in lipid rafts is necessary for the early dip of Rho activity observed during initial cell spreading following plating on ECM. Previous studies showed that this early phase of Rho inactivation is mediated by p190RhoGAP phosphorylation, and this was interpreted as being triggered by integrin engagement (Arthur et al., 2001; Arthur et al., 2000). Although integrin binding and clustering directly activate these signaling responses, application of mechanical stresses to already ligated integrin receptors can elicit distinct signals (Matthews et al., 2006; Meyer et al., 2000; Wang et al., 2005). Importantly, the signals that are conveyed by this form of force application to integrins and are critical for cell cycle progression, apoptosis, differentiation, and motility are also distinct from those elicited by integrin engagement or clustering alone (Chen et al., 1997; Huang et al., 1998; Ingber, 1991; Ingber, 1990; Parker et al., 2002). Inhibition of integrin binding with soluble RGD

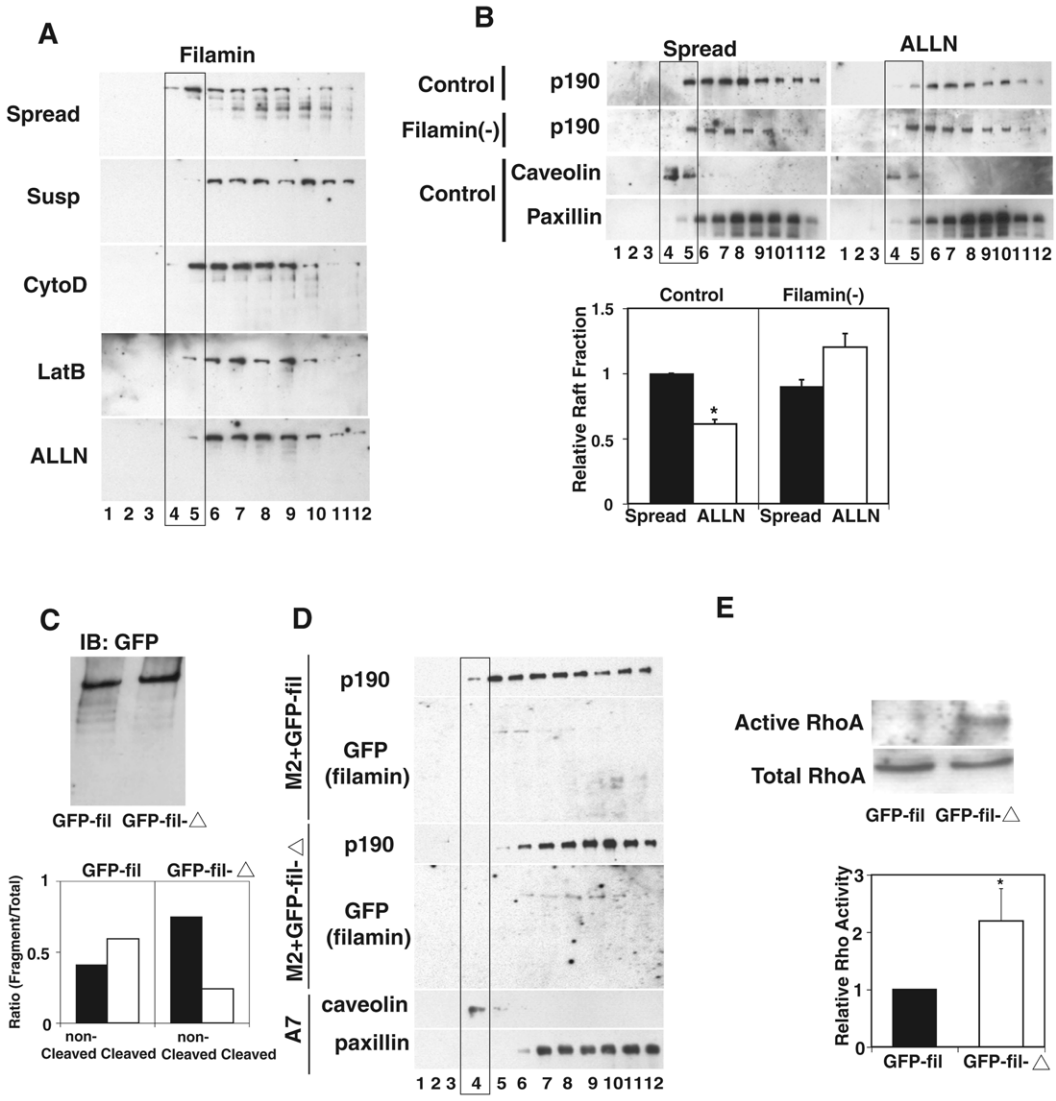

Fig. 7. Filamin cleavage controls the localization of p190RhoGAP. (A) Raft-localization and cleavage of filamin in HMVE cells that were cultured in suspension (Susp) or plated on fibronectin in the absence (Spread) or presence of cytochalasin D (CytoD), latrunculinB (LatB), or ALLN. (B) Raft-localization of p190RhoGAP in control versus ALLN-treated filamin knockdown cells. Quantitative results were normalized to spread control cells (*P<0.01). (C) Filamin protein in total cell lysate from M2 cells transfected with intact GFP-filamin (GFP-fil) or a GFP-filamin-Δ (GFP-fil-Δ) construct that lacks the calpain cleavage site. (D) Raft-localization of p190RhoGAP in total cell population of M2 cells transfected with GFP-fil or GFP-fil-Δ. (E) Levels of active RhoA in total cell population of M2 cells transfected with GFP-fil or GFP-fil-Δ (*P<0.02).

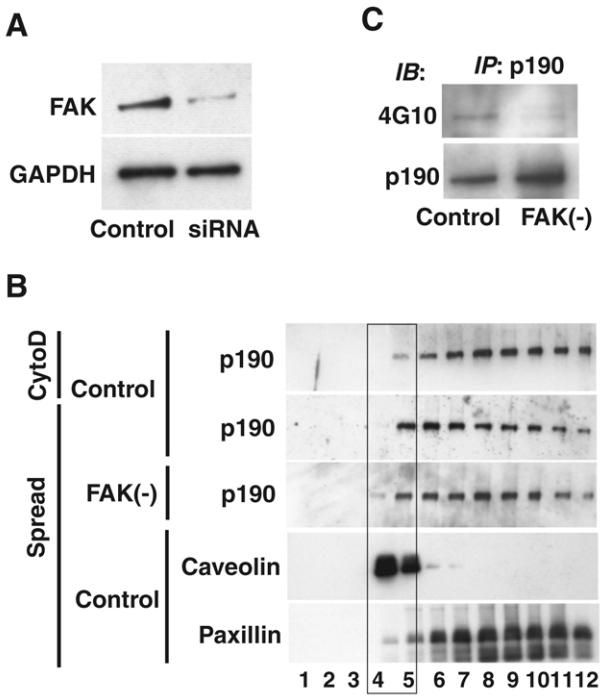

Fig. 8. p190RhoGAP localization in FAK knockdown HMVE cells. (A) FAK expression in HMVE cells transfected with FAK siRNA. (B) Raft-localization of p190RhoGAP in FAK-knockdown cells, as in Fig. 2C. (C) Tyrosine phosphorylation of p190RhoGAP in FAK-knockdown cells.

peptides can inhibit Rho in suspended cells (Arthur et al., 2000), so integrin binding alone clearly can have effects on this pathway. Some of the effects of cytoD we observed may therefore be due to its ability to promote disengagement of already bound integrins. However, our results demonstrate that the effects of cytoD on p190RhoGAP accumulation in lipid rafts is independent of FAK and src-dependent phosphorylation of p190RhoGAP, signaling events that were previously shown to mediate the effects of integrin ligation on Rho activity (Plopper et al., 1995). It is also important to note that while cytoD can alter some integrin signaling pathways (e.g. FAK), it does not interfere with others (e.g. cAMP) (Meyer et al., 2000). Furthermore, we found that cytoD treatment can increase Rho activity in suspended HMVE cells even though they lack ligated integrins (not shown), as described previously in other cell types (Ren et al., 1999). Thus, although cytoD and suspension have similar effects on Rho activity, disruption of the actin cytoskeleton apparently can activate Rho via a pathway that is distinct from that triggered by detachment from ECM and integrin disengagement alone, however both of which are necessary steps for Rho inhibition induced by initial cell adhesion and spreading on ECM.

Thus, in our HMVE cells, the accumulation of p190RhoGAP in lipid rafts appears to be independent of its phosphorylation state. But it contradicts a recent report which suggests that phosphorylation of p190RhoGAP-B on Tyr306 is necessary for raft localization in mouse embryo-derived fibroblasts (Sordella et al., 2003). This inconsistency may be due to the fact that the phosphorylation site of A isoform of p190RhoGAP we analyzed in this study that is regulated by Src is not homologous to the Tyr306 phosphorylation site in the B-isoform (Sordella et al., 2003). The use of constitutive phosphorylation target mutants in that study also may have resulted in generalized inhibition of protein translocation.

Our suggestion that filamin plays a pivotal role in cell shape-dependent control of Rho activity through modulation of p190RhoGAP accumulation in lipid rafts is based on the following findings: (1) p190RhoGAP physically interacts with filamin; (2) p190RhoGAP is not in the lipid raft fraction when wild-type cells are treated with cytoD, whereas it remains in the lipid raft fraction even after cytoskeletal disruption in filamin-deficient cells. In fact, in these cells p190RhoGAP appeared to be even more enriched in the lipid raft fraction after cytoD treatment. These data raise the possibility that filamin present in the cytoplasm or cytoskeleton interacts with p190RhoGAP and prevents its accumulation in the raft fraction so that it cannot inhibit Rho activity in round cells or in retracted cells that remain adherent to ECM but lack cytoskeletal integrity.

It remains to be elucidated how filamin senses cell shape changes and converts this cell architectural signal into changes of p190RhoGAP translocation. However, our results show that the cytoskeleton-associated protease, calpain, is central to this mechanism, and that cleavage of filamin at site (TYA: 1762-1764 aa) is able to inhibit its ability to sequester p190RhoGAP, thereby allowing it to accumulate in lipid rafts. We were able to identify direct binding between filamin and p190RhoGAP using recombinant proteins; however, the affinity was extremely low (not shown). Importantly, most filamin proteins are found outside of rafts, bound to the cytoskeleton or in multi-molecular complexes containing proteins other than caveolin-1. Apparently, changes in binding affinity alone are not sufficient to release p190RhoGAP from these complexes. Instead, cleavage of filamin by cytoskeleton-associated calpain molecules is required. Future studies will have to focus on how cell distortion and cytoskeletal restructuring modulate calpain activity. Other mechanisms, such as modulation of p190RhoGAP's affinity for filamin or for other cytoskeletal-associated proteins may be involved because cytoD did not completely block filamin degradation. Moreover, the filamin fragments themselves can still form complexes with other proteins and thereby exert different biological activities (Loy et al., 2003; Ohta et al., 1991; Stossel et al., 2001). In fact, the total amount of intact filamin did not significantly change between spread and round cells (not shown), therefore cleaved filamin fragments potentially could have functional activities. Further studies are necessary to analyze the kinetics of entry and exit from the lipid raft, as well as raft internalization and protein trafficking by direct microscopic visualization. Regardless of the mechanism, release from filamin and accumulation within lipid rafts of only a small subpopulation of p190RhoGAP protein is apparently sufficient to convey the cytoskeletal inhibitory signal to Rho.

Our results therefore suggest that changes in cell shape and associated deformation of the cytoskeleton govern these downstream responses by controlling the accessibility of p190RhoGAP to these critical sites of signal integration within lipid rafts. Interestingly, the organization of the lipid raft itself may be shape-dependent: they become internalized when spread fibroblasts are induced to round, and this influences

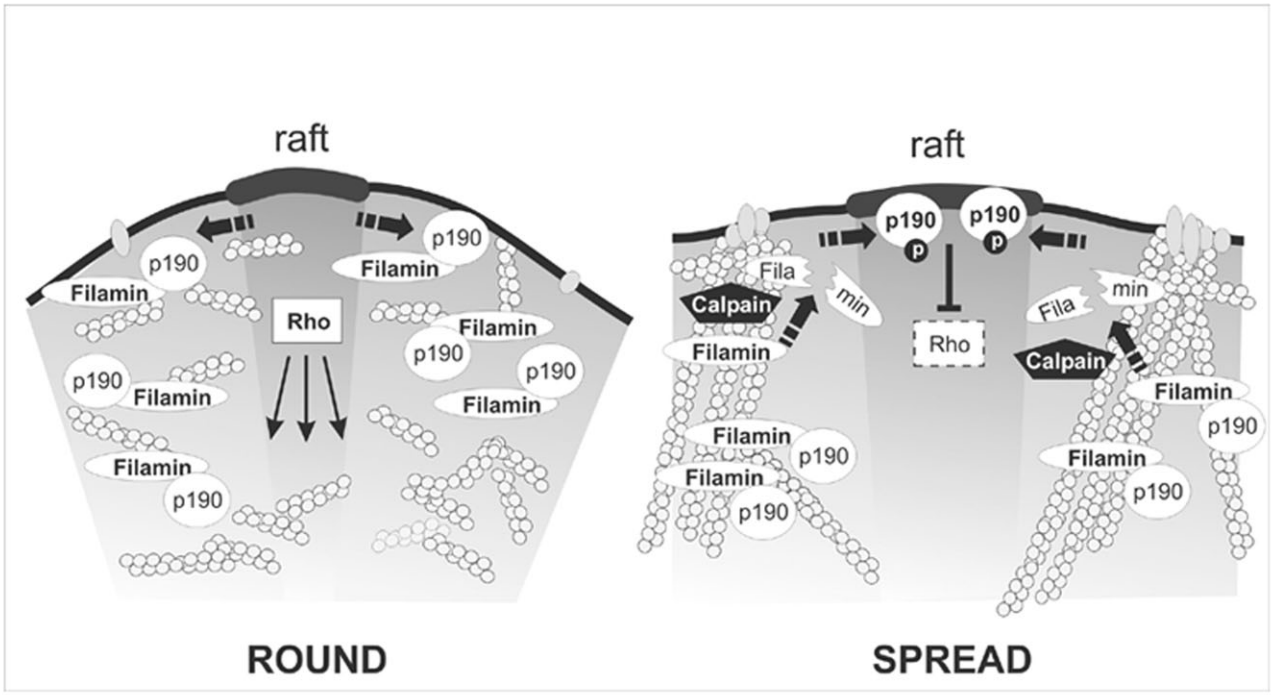

Fig. 9. Model for cell shape-dependent control of Rho activity. (A) Round cells: intact filamin protein binds p190RhoGAP and prevents its accumulation in lipid rafts. p190RhoGAP is inactive and hence Rho activity is high. (B) Spread cells: filamin is cleaved by calpain, thereby releasing p190RhoGAP and allowing it to accumulate in lipid rafts where it becomes activated and suppresses Rho activity.

downstream Rac signaling in these cells (del Pozo et al., 2004). Thus, raft internalization also could contribute in part to the inhibition of p190RhoGAP entry into lipid rafts that we observed in round HMVE cells. But some of our observations cannot be explained by raft internalization alone. For instance, p190RhoGAP was always present in the lipid raft fractions of filamin-deficient M2 cells and of siRNA-mediated filamin knockdown HMVE cells, whether treated with cytoD or cultured in suspension. Thus, regardless of the state of raft internalization, filamin protein appears to be necessary for sequestration of p190RhoGAP from lipid rafts in round cells.

In conclusion, these studies reveal a novel mechanism for the control of Rho activity by integrin-mediated changes in cytoskeletal organization that accompany cell spreading on ECM. This mechanism is mediated by the cytoskeletal protein, filamin, which must be cleaved by the cytoskeletal-associated protease, calpain, to manifest its effect on p190RhoGAP accumulation in lipid rafts, and hence, on Rho activity (Fig. 9). Interestingly, filamin also appears to influence the activity of a RacGAP (FilGAP) (Ohta et al., 2006), and thus, this may be part of a more general mechanism by which filamin links cytoskeletal structure to the activity of small GTPases. These findings also must be seen in the context of the p190RhoGAP phosphorylation mediated by Src and FAK, which has been reported to be induced by integrin ligation. We find that cytoD also influences Src-dependent phosphorylation of p190RhoGAP, and so this 'integrin signaling pathway' may have mechanical as well as chemical components. Nevertheless, our results reveal that there is yet another mechanism by which mechanical signals from ECM mediated by integrins and related changes in cytoskeletal structure may influence Rho activity: through filamin-dependent control of p190RhoGAP accumulation in lipid rafts. Future studies will be need to understand how these different integrin-related signaling mechanisms are integrated in living cells; however, they all impinge on the key protein p190RhoGAP to control Rho signaling within the cell.

Materials and Methods

Materials

Anti-RhoA polyclonal was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caveolin-1, anti-p190RhoGAP-A, anti-phospho-tyrosine (PY20), anti-FAK, anti-src, anti-TFII-I, and anti-paxillin antibodies were purchased from Transduction Laboratory (Lexington, KY). Anti-filamin (mab1680 and mab1678) and anti-GAPDH antibodies were from Chemicon (Temecula, CA). Anti-phospho-tyrosine (4G10) was from Upstate (Lake Placid, NY). Anti-GFP antibody was from Abcam (Cambridge, MA). Protein A-sepharose was purchased from Zymed (South San Francisco, CA). PP2 and ALLN were purchased from Calbiochem (SanDiego, CA); methyl-β-cyclodextrin was purchased from Sigma (St Louis, MI).

Cell culture

HMVE cells (Cambrex, Walkersville, MD) were cultured in EBM-2 (Cambrex), supplemented with 5% FBS and growth factors (bFGF, IGF, VEGF) according to the manufacturer's instructions. A human filamin-deficient malignant melanoma cell line (M2) and the same cells restored with full-length filamin (A7) were kindly provided by Dr Stossel and maintained as described (Cunningham et al., 1992).

Plasmid

pEGFP-filamin-full (21-2647 aa) was kindly provided by Dr Yong (National University of Singapore, Singapore). pEGFP-filamin-Δ, in which the calpain-target sequence (TYA: 1762-1764 aa) was deleted was constructed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Experimental system

Cells were serum-starved by incubation in 0.3% FBS/EBM-2 for 40-42 hours and then trypsinized and immediately replated with EBM-2 containing 1% FBS and growth factors. To control cell shape, cells were plated on bacteriological petri dishes (Petri dishes; Falcon, Lincoln Park, NJ) that were pre-coated with 666 ng cm^{-2} of fibronectin (BD Biosciences, SanDiego, CA) in carbonate buffer as previously described (Mammoto et al., 2004). 500 nM of Cytochalasin D (Sigma) or 3 μg ml^{-1} of latrunculin B (Sigma) was added to the cells after they were plated

on the fibronectin-coated dishes; cells were harvested for assays 1.0-1.5 hours after plating. For experiments in suspension, cells were detached with trypsin-EDTA and plated in EBM-2 containing 1% FBS and growth factors in 10 cm bacterial plastic dishes precoated with 1 mg ml^{-1} BSA to prevent cell adhesion to the plastic. Transient transfection was performed using the Superfect reagent (QIAGEN, Valencia, CA) according to the manufacturer's directions.

siRNA-mediated protein knockdown

Protein knockdown was performed using the RNA interference technique (Elbashir et al., 2001). siRNA duplexes used were as follows; p190RhoGAP, 5′-GGAUUGUGUGGAAUGUAAG-3′ and 5′-CUUACAUUCCACACAAUCC-3′; filamin, 5′-GGCAAAAGUGACCGCCAAU-3′ and 5′-AUUGGCGGUCACUUUUGCC-3′; FAK, 5′-GCGAUUAUAUGUUAGAGAUAGUU-3′ and 5′-CUAUCUCUAACAUAUAAUCGCUU-3′ (Yano et al., 2004). Cells were transfected with 30nM of siRNA duplexes using Silentfect (BioRad, Hercules, CA) according to the manufacturer's instructions and were used for each assay after incubation for 48 hours. As a control, siRNA duplex with irrelevant sequence (QIAGEN) was used. We checked the silencing efficiency by western blotting and RT-PCR. For p190RhoGAP and filamin, we used two different siRNA for each experiment and obtained the similar results.

Rho activity pull-down assay

Rho activity assay was performed and quantified using the Rho activation assay kit based on rhotekin pull-down assay according to the manufacturer's instruction (Cytoskeleton, Denver, CO). In brief, cells (1.5×10^6 cells) were washed with PBS, and extracted in 600 μl of cell lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100). Similar results were obtained when RIPA buffer was used as cell lysis buffer. Samples were centrifuged for 5 minutes at 10,000 ***g*** and the supernatant was incubated with rhotekin beads for 1.5 hours at 4°C. After washing the beads with buffer (25 mM Tris, pH 7.5, 40 mM NaCl, 15 mM $MgCl_2$), proteins were removed from the beads in Laemmli buffer, subjected to western blotting. The ratio of rhotekin-bound RhoA and RhoA in the total cell lysate (5% of total RhoA) was analyzed using NIH image software.

Purification of lipid-raft fractions

Caveolin rich lipid raft fractions were isolated either using the detergent-free sucrose gradient floating assay (Smart et al., 1995; Song et al., 1996) or by subcellular fractionation of Triton X-100 insoluble membrane fractions (Sordella et al., 2003) as described previously. In brief, for detergent-free floating assay, HMVE cells were washed with ice-cold PBS and lysed with 600 μl of 0.5 M Na_2CO_3 (pH 11). Cells were sonicated with minimal output in a Microson ultrasonic cell disrupter (Heat System Inc., Farmigdale, NY). 0.3 ml of lysates was adjusted to 45% sucrose by addition of 0.9 ml of 60% OptiPrep Density Gradient Medium (sigma) and placed at the bottom of the ultracentrifuge tube. This was overlaid with 0.8 ml of 35% sucrose and 0.5 ml of 5% sucrose in buffer A (25 mM MES, pH 6.5, 75 mM NaCl, 0.2 M Na_2CO_3). The samples were then ultracentrifuged at 150,000 ***g*** for 20 hours in an sw55Ti rotor (Beckman Coulter). Twelve 0.2 ml fractions were collected from the top of the gradient and analyzed by immunoblotting.

Immunoprecipitation assay

For immunoprecipitation of proteins from total cell lysates, we lysed cells with RIPA buffer and incubated 200 μg of total protein with antibodies for 3 hours, followed by incubation with protein A sepharose for 2 hours at 4°C.

Fluorescence microscopy

For morphological analysis, cell staining was performed as described before (Mammoto et al., 2004) and analyzed using fluorescence microscopy (Nikon Diaphot 300; Nikon, Japan) with IP lab software.

Immunoblot quantification and statistical analysis

Immunoblots were quantified by densitometric analysis of films using NIH image software. Error bars (s.e.m.) and *P*-values were determined from results from at least three or more experiments. The unpaired Student's *t*-test was used for analysis of statistical significance of replicate western blots.

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