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Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton

Xiang-Dong Ren, William B.Kiosses and Martin Alexander Schwartz[1]

Department of Vascular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

[1]Corresponding author
e-mail: schwartz@scripps.edu

Soluble factors from serum such as lysophosphatidic acid (LPA) are thought to activate the small GTP-binding protein Rho based on their ability to induce actin stress fibers and focal adhesions in a Rho-dependent manner. Cell adhesion to extracellular matrices (ECM) has also been proposed to activate Rho, but this point has been controversial due to the difficulty of distinguishing changes in Rho activity from the structural contributions of ECM to the formation of focal adhesions. To address these questions, we established an assay for GTP-bound cellular Rho. Plating Swiss 3T3 cells on fibronectin-coated dishes elicited a transient inhibition of Rho, followed by a phase of Rho activation. The activation phase was greatly enhanced by serum. In serum-starved adherent cells, LPA induced transient Rho activation, whereas in suspended cells Rho activation was sustained. Furthermore, suspended cells showed higher Rho activity than adherent cells in the presence of serum. These data indicate the existence of an adhesion-dependent negative-feedback loop. We also observed that both cytochalasin D and colchicine trigger Rho activation despite their opposite effects on stress fibers and focal adhesions. Our results show that ECM, cytoskeletal structures and soluble factors all contribute to regulation of Rho activity.

Keywords: cell adhesion/cytochalasin/fibronectin/microtubules/Rho

Introduction

Complex biological processes such as morphogenesis, wound healing and normal tissue homeostasis require precise control of cell behavior. This is achieved by collaborative effects of both soluble regulatory factors and extracellular matrices (ECM) to determine cell migration, differentiation, growth and survival (reviewed in Adams and Watt, 1993; Schwartz *et al*., 1995; Miyamoto *et al*., 1996; Frisch and Ruoslahti, 1997; Giancotti, 1997; Howe *et al*., 1998). Cell adhesion to ECM results in clustering of integrins in focal adhesions, structures that contain multiple signaling as well as cytoskeletal proteins. These structures are important sites of signal transduction and integration (Miyamoto *et al*., 1995). Formation of focal adhesions and the closely associated actin stress fibers requires activation of the small GTP-binding protein Rho (Ridley and Hall, 1992).

Rho is a member of the Ras superfamily of small GTP-binding proteins. Like Ras, Rho cycles between a GDP-bound inactive state and a GTP-bound active state. Rho-bound nucleotides are regulated primarily by two groups of proteins: guanine nucleotide exchange factors that catalyze exchange of GDP for GTP, and GTPase-activating proteins (GAPs) that stimulate hydrolysis of GTP to GDP. Upon binding to GTP, Rho interacts with and activates downstream effectors such as Rho kinase, protein kinase N and phosphatidylinositol 4-phosphate 5-kinase (reviewed in Machesky and Hall, 1996; Narumiya, 1996). Among these effectors, Rho kinase plays an important role in generating contractile force by increasing myosin light chain (MLC) phosphorylation (Amano *et al*., 1996; Kimura *et al*., 1996). Rho thereby regulates formation of actin stress fibers and focal adhesions, and cell migration (Chrzanowska-Wodnicka and Burridge, 1996; Amano *et al*., 1997; Burridge *et al*., 1997). Rho-induced synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2) is also required for the formation of focal adhesions and stress fibers, possibly by anchoring actin fibers to cytoskeletal proteins in focal adhesions (Gilmore and Burridge, 1996; reviewed in Ren and Schwartz, 1998). Rho has also been shown to stimulate DNA synthesis and participate in Ras-induced cell transformation (Yamamoto *et al*., 1993; Olson *et al*., 1995; Prendergast *et al*., 1995; Qiu *et al*., 1995), indicating an important role in cell growth. These effects of Rho can be blocked by treating cells with *botulinum* C3 exoenzyme (C3 treatment) which specifically ADP-ribosylates Rho at Asn41 to inhibit Rho function (Sekine *et al*., 1989; Paterson *et al*., 1990; Ridley and Hall, 1992).

There is strong experimental support for the idea that components of serum such as lysophosphatidic acid (LPA) or other soluble factors such as sphingosine-1-phosphate can activate Rho (Ridley and Hall, 1992; Seufferlein and Rozengurt, 1995), although it has not been confirmed by direct assays of Rho activity. Several lines of evidence suggest that Rho is also regulated by integrin-mediated cell adhesion to ECM. Effects of activating or inhibiting Rho on regulation of cell growth, MAP kinase and levels of inositol lipids by cell adhesion suggest that Rho is downstream of integrins (Chong *et al*., 1994; Renshaw *et al*., 1996; Schwartz *et al*., 1996). Integrin stimulation enhances stress fibers and focal adhesions in serum-starved Swiss 3T3 cells, indicating activation of Rho by integrins (Barry *et al*., 1997). Others, however, have argued that integrins are required in a structural sense for the formation of focal adhesions, but that cell adhesion does not modulate Rho activity (Hotchin and Hall, 1995). They have suggested instead that Rho is required for some integrin-dependent functions but is regulated only by soluble factors. In the absence of a biochemical assay for Rho

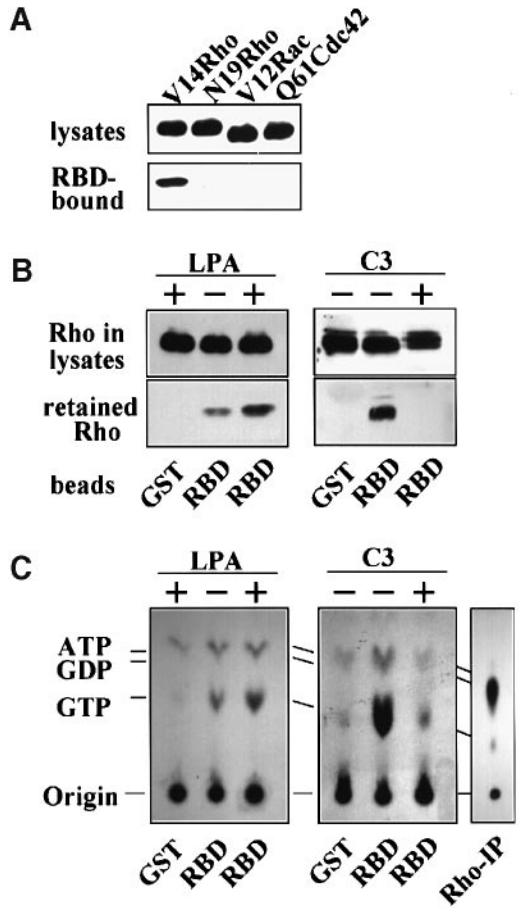

Fig. 1. Binding of GTP-Rho to GST–RBD. (**A**) Extracts of COS-7 cells transiently transfected with HA-tagged V14Rho, N19Rho, V12Rac or Q61Cdc42 were incubated with GST–RBD glutathione beads. After washing, the bound proteins were analyzed by Western blotting using anti-HA antibody (12CA5). Only V14Rho was retained by GST–RBD. (**B**) Lysates from untransfected Swiss 3T3 cells were incubated with GST–RBD beads, the beads washed and the bound protein analyzed by Western blotting with a monoclonal antibody against RhoA. The amount of Rho bound to RBD beads was increased by treating cells with 1 μg/ml LPA for 1 min (left panel), and abolished by C3 treatment of NIH 3T3 cells (right panel). NIH 3T3 cells were treated with 10% serum for 1 min before the assay (right panel). (**C**) Thin-layer chromatography of the bound nucleotides. Cell types and treatment were the same as in (B) except cells were labeled with [^{32}P]phosphate. Shown in the right panel are the nucleotides immunoprecipitated with HA-tagged wild-type Rho from radiolabeled cells.

activity, these issues have been difficult to resolve, as formation of cytoskeletal structures has been the only available indication of Rho activity.

To address these questions, it was necessary to directly assay Rho GTP-loading after stimulating cells with ECM and/or growth factors. The classical assay to detect Ras GTP-loading employs an antibody (Y13-259) that inhibits both the intrinsic and the GAP-enhanced GTPase activity of Ras (Downward *et al*., 1990). Detection of GTP-bound cellular Rho by immunoprecipitation is problematic because available Rho antibodies do not inhibit Rho GTPase activity, hence only GDP can be detected after immunoprecipitation (Laudanna *et al*., 1996; Figure 1C). An assay for Rho exchange activity based on the amount of Rho-associated GDP in radiolabeled lymphoid cell lines (Laudanna *et al*., 1996) is not applicable to adherent cell types such as fibroblasts due to much higher basal levels of nucleotide exchange and hydrolysis (data not shown).

In this report, we describe an assay to detect GTP-bound cellular Rho. Using this assay, we analyzed the contributions of cell adhesion, the cytoskeleton and soluble factors to Rho activation. The data reported here reveal several surprising aspects of Rho regulation.

Results

Detection of cellular GTP-Rho

To develop an assay for Rho activity, we took the advantage of the fact that Rho effectors interact only with GTP-bound Rho. Furthermore, binding of Rho to the Rho-binding domain (RBD) from the effector protein Rhotekin inhibited both the intrinsic and GAP-enhanced GTPase activity of Rho (Reid *et al*., 1996). Therefore, Rhotekin RBD was used to affinity-precipitate cellular GTP-Rho. This interaction withstood 0.5 M NaCl in RIPA buffer used to dissociate the GTP-binding protein from its effectors and GAPs, thus these conditions were used to prepare cell extracts (see Materials and methods). No residual Rho could be detected in the insoluble fraction from adherent or suspended cells using these extraction conditions (data not shown).

As an initial test, a fusion of the RBD with glutathione *S*-transferase (GST–RBD) was coupled to glutathione beads and incubated with lysate from COS cells expressing epitope-tagged mutants of Rho family GTP-binding proteins. The beads were rinsed and the retained proteins analyzed by Western blotting with an antibody to the epitope tag. GST–RBD beads retained recombinant V14Rho (GTP-bound) but no detectable N19Rho (GDP-bound), indicating high specificity for the GTP-bound state (Figure 1A). Neither GTP-bound Rac nor Cdc42 was retained by GST–RBD beads, again demonstrating high specificity, consistent with published data (Reid *et al*., 1996).

GST–RBD also bound endogenous Rho from Swiss and NIH 3T3 cell lysates, as detected by Western blotting with a monoclonal antibody against RhoA. Retention of Rho from Swiss 3T3 cells was increased 2- to 6-fold after 1 min stimulation of cells with LPA (Figure 1B, left panel) or serum, but not with PDGF (data not shown). Virtually no Rho was detected bound to agarose beads with GST alone. To further test the specificity of the interaction with endogenous Rho, NIH 3T3 cells were treated with *botulinum* C3 exoenzyme, which ADP ribosylates and inactivates Rho. This treatment completely blocked binding of Rho from serum-stimulated cells to GST–RBD beads (Figure 1B, right panel). Finally, to demonstrate that only GTP-bound cellular Rho was associated with RBD beads, cells were incubated with [^{32}P]phosphate to label the guanine nucleotide pool. Cell lysates were incubated with RBD beads, and the bound nucleotide eluted and analyzed by thin-layer chromatography. RBD beads bound only GTP, which increased in response to LPA (Figure 1C, left panel) and decreased after C3 treatment (Figure 1C, middle panel). By contrast, Rho that was immunoprecipitated with an antibody that does not block GAP activity yielded only GDP (Figure 1C, right panel). These results demonstrate that the GST–RBD

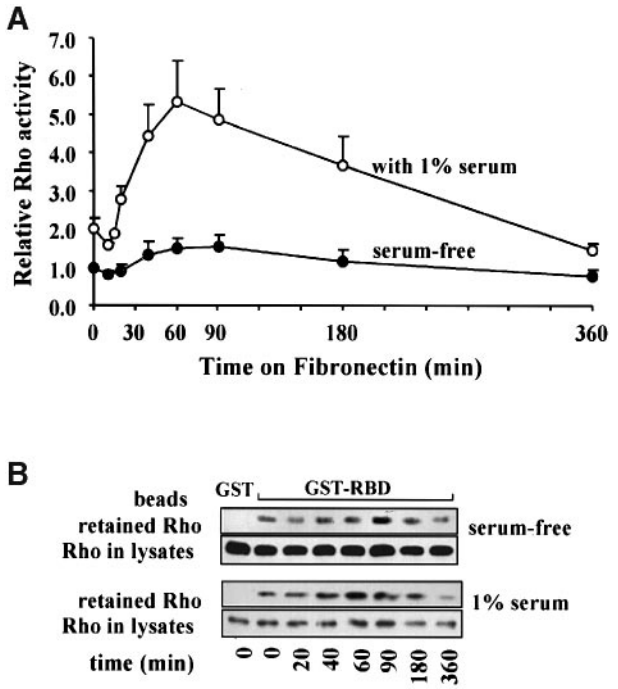

Fig. 2. Regulation of Rho activity by cell adhesion to fibronectin. (**A**) Swiss 3T3 cells, either serum-free or in 1% serum for 18 h, were detached and replated on fibronectin-coated dishes under the same conditions. Rho was assayed at indicated times. Rho activity is indicated by the amount of RBD-bound Rho normalized to the amount of Rho in whole cell lysates. Values represent Rho activity relative to serum-starved cells at time 0. Results are means ± SD from three or more experiments. (**B**) Western blots from one of the representative experiments showing activation of Rho.

beads exhibit high specificity for GTP-Rho and can detect activation of endogenous Rho by physiological stimuli.

Biphasic regulation of Rho during cell spreading on fibronectin

To investigate the role of an ECM protein in regulating Rho, suspended Swiss 3T3 cells were plated onto fibronectin-coated plastic dishes. Prior to and during replating, cells were maintained in either serum-free medium or 1% serum. In serum-starved cells, an initial period of low Rho activity was followed by a modest (~1.5-fold) but reproducible ($p < 0.025$) increase that peaked at 60–90 min then returned to basal levels (Figure 2). For cells in low (1%) serum, a period of low Rho activity was again observed, but occurred after a shorter lag and the transient increase that followed was greatly enhanced. Rho activation induced by plating cells on fibronectin was accompanied by an increase in phosphorylation of MLC (data not shown), an event that is downstream of Rho (Amano *et al*., 1996; Kimura *et al*., 1996).

To correlate these results with cytoskeletal dynamics, cells plated on fibronectin were fixed and stained with rhodamine–phalloidin to label actin stress fibers and with anti-vinculin to label focal adhesions (Figure 3). Staining of actin and vinculin in cells on fibronectin-coated coverslips showed that the initial phase of low Rho activity corresponded to the rapid spreading of cells on the substrate. During this period, cells had small focal complexes at the cell periphery but minimal stress fibers or focal adhesions. Stress fibers and focal adhesions formed only at later times, corresponding to the period of high Rho activity. In 1% serum, stress fibers and focal adhesions were dramatically enhanced compared with cells lacking serum, but followed a similar time course.

Cell adhesion to ECM downregulates growth factor-induced Rho activation

To further explore crosstalk between adhesion and soluble factors, we measured Rho activity in serum-starved suspended versus adherent cells after stimulation with LPA. These measurements showed that in both suspended and adherent Swiss 3T3 cells, Rho activation was initially of similar magnitude (Figure 4). Thus, LPA activation of Rho neither requires nor is enhanced by cell adhesion. At later times, however, Rho activity in adherent cells declined, whereas the Rho activation in suspended cells was sustained (Figure 4). This activation was also accompanied by equivalent increases in phosphorylation of MLC in adherent and suspended cells (data not shown). Even after 18 h, Rho activity and MLC phosphorylation in cells suspended in the presence of serum were ~50% higher than in stably adherent cells ($p < 0.005$) (Figure 5). In contrast, no increase of Rho activity was observed in suspended cells under serum-free condition (Figure 4, time 0). These data demonstrate that there is an adhesion-dependent downregulation of Rho following activation by LPA or serum.

Activation of Rho by colchicine and cytochalasin D

Microtubule-disrupting reagents have been shown to trigger formation of stress fibers and focal adhesions in starved Swiss 3T3 cells (Bershadsky *et al*., 1996), and this effect is Rho-dependent (Enomoto, 1996; Zhang *et al*., 1997; Liu *et al*., 1998). It has been suggested that microtubules provide compression-resistant struts against the tension generated by the contractile actin/myosin cytoskeleton (Ingber, 1993). This idea led one group to propose that loss of microtubules generates cortical tension which induces signaling events, leading to the formation of focal adhesions and stress fibers (Bershadsky *et al*., 1996). To test these models, serum-starved Swiss 3T3 cells were treated with colchicine, and Rho activity monitored. These assays showed that colchicine induced rapid Rho activation, similar to that triggered by LPA (Figure 6A). The vehicle (DMSO) caused a decrease in Rho activity, suggesting that the effect of colchicine may actually be higher than it appears in Figure 6A if one compares the colchicine effect to the zero time point. Taxol, a microtubule stabilizing drug, had no effect beyond that of the vehicle control. Activation of Rho by colchicine in this assay was followed by formation of actin stress fibers (not shown) as described previously (Enomoto, 1996; Zhang *et al*., 1997; Liu *et al*., 1998).

To explore the role of mechanical tension in this effect, two additional experiments were performed. First, cells in suspension were compared to adherent cells. Under these conditions, colchicine triggered equivalent induction of Rho activity (Figure 6B). Secondly, cells were treated with colchicine in the presence of cytochalasin D to disrupt actin filaments and block contractility. Remarkably, cytochalasin D alone induced activation of Rho (Figure 6B). Furthermore, cytochalasin and colchicine together resulted in greater activation than either one alone. No

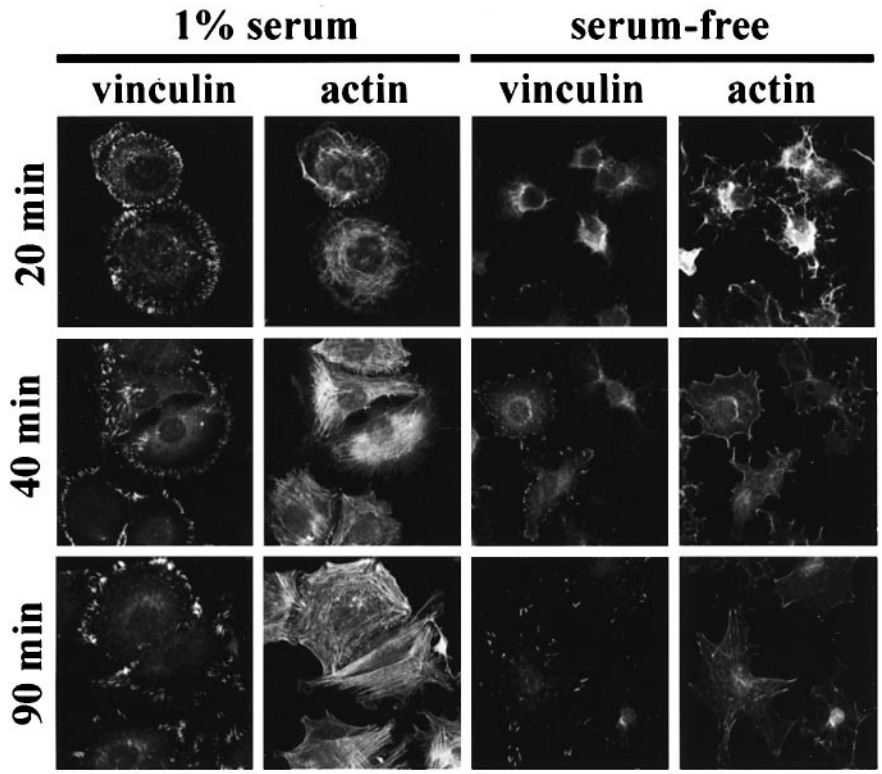

Fig. 3. Time course of focal adhesion and stress fiber formation. Cells were plated onto fibronectin-coated coverslips under the same conditions as described above. At the times indicated, cells were fixed and stained with rhodamine–phalloidin to detect actin stress fibers and anti-vinculin to detect focal adhesions.

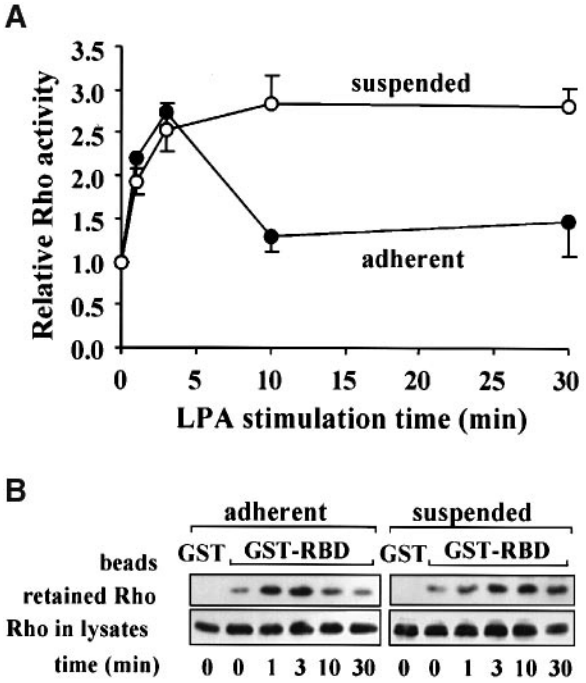

Fig. 4. Stimulation of Rho by LPA. (**A**) Serum-starved Swiss 3T3 cells were either suspended for 60 min in serum-free medium supplemented with BSA, or were adherent but transferred to the same medium. Rho activity was measured after stimulation with 1 μg/ml LPA. Results are means ± SD from three experiments. (**B**) Western blots from one experiment.

stress fibers were visible after cytochalasin treatment in the presence or absence of colchicine (data not shown). Moreover, the effects of these reagents were similar in attached or suspended cells (Figure 6B). These results strongly argue against models in which increased contractile force mediates the increase in Rho activity in response to microtubule disruption.

The activation of Rho by cytochalasin D was further characterized. Time course measurements showed that the effect was comparatively slow, requiring ~30–60 min to reach a maximum, and was sustained for >3 h before eventually returning to baseline (Figure 6C). Cytochalasin D stimulated Rho equally well in adherent or suspended cells (Figure 6B), indicating that the effect is not dependent on disruption of adhesions. When the effects of soluble factors were examined in the presence or absence of cytochalasin D, we observed that sphingosine-1-phosphate or serum induced similar increases in Rho activity under both conditions. The combined effects were approximately additive (Figure 6D). These results suggest that cytochalasin D activates Rho via a pathway that is distinct from that used by adhesion to ECM, serum or sphingosine-1-phosphate.

Discussion

In this report, we describe the development of an assay to detect GTP-Rho by affinity-precipitation. This assay revealed several surprising aspects of Rho regulation. First, it appears that Rho activation (and myosin phosphorylation) is triggered by LPA equally well in adherent and detached cells, despite the fact that typical Rho-dependent cytoskeletal structures cannot form in detached cells. Therefore, activation of Rho by LPA is independent of adhesion to ECM. Secondly, following the initial

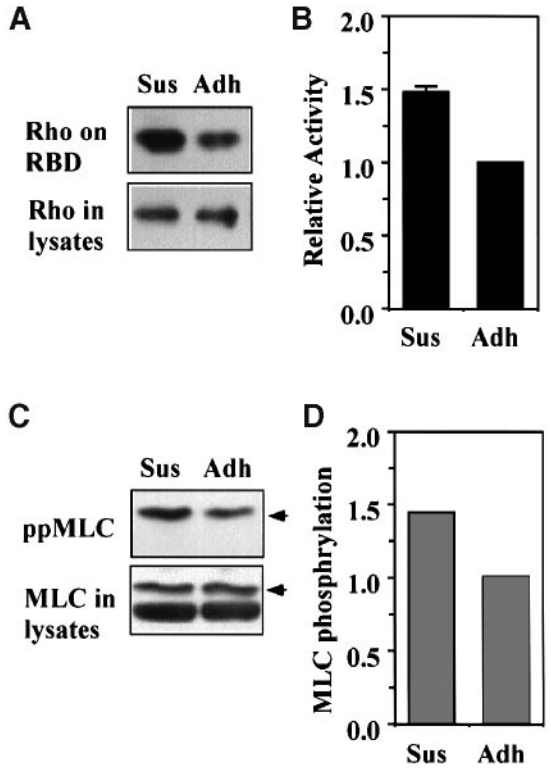

Fig. 5. Rho activity and MLC phosphorylation in suspended versus adherent cells. Swiss 3T3 cells were either maintained in suspension (Sus) for 18 h in the presence of 5% serum, or plated on tissue culture dishes under the same condition (Adh). (**A**) Western blots showing Rho activity in suspended and adherent cells. (**B**) Results from three experiments comparing Rho activity (means ± SD) between suspended and adherent cells. (**C**) Western blots showing MLC phosphorylation in suspended and adherent cells. (**D**) Densitometric analysis of the result in (C).

LPA-induced Rho activation, Rho is downregulated in adherent cells whereas it remains elevated in suspended cells. Furthermore, Rho activity and MLC phosphorylation remain higher in suspended cells in the presence of serum than in adherent cells. These results suggest the existence of a negative-feedback loop that occurs following Rho-dependent formation of focal adhesions. Such an effect would be predicted to lead to homeostasis of Rho activity. This regulatory circuit may serve to prevent excessive contraction under physiological conditions. The mechanism of this effect is at present unclear, but there are several candidates to mediate this negative feedback. One of the candidates is p190 RhoGAP, which has been shown to inhibit Rho-induced stress fiber formation (McGlade *et al*., 1993; Ridley *et al*., 1993) and can be recruited to focal adhesions (Burbelo *et al*., 1995). cAMP can also antagonize Rho function (Lang *et al*., 1996; Kreisberg *et al*., 1997; Laudanna *et al*., 1997) and is sometimes elevated following cell adhesion (Fong and Ingber, 1996). In addition, Cdc42 and Rac, two other members of the Rho family of GTP-binding proteins, have been shown to antagonize the effect of Rho (Kozma *et al*., 1997; van Leeuwen *et al*., 1997); they are transiently activated following adhesion to fibronectin, coincident with the period of low Rho activity (Price *et al*., 1998).

Our data also demonstrate that Rho can be activated after plating Swiss 3T3 cells on fibronectin, and that a low concentration of serum greatly enhances this activation.

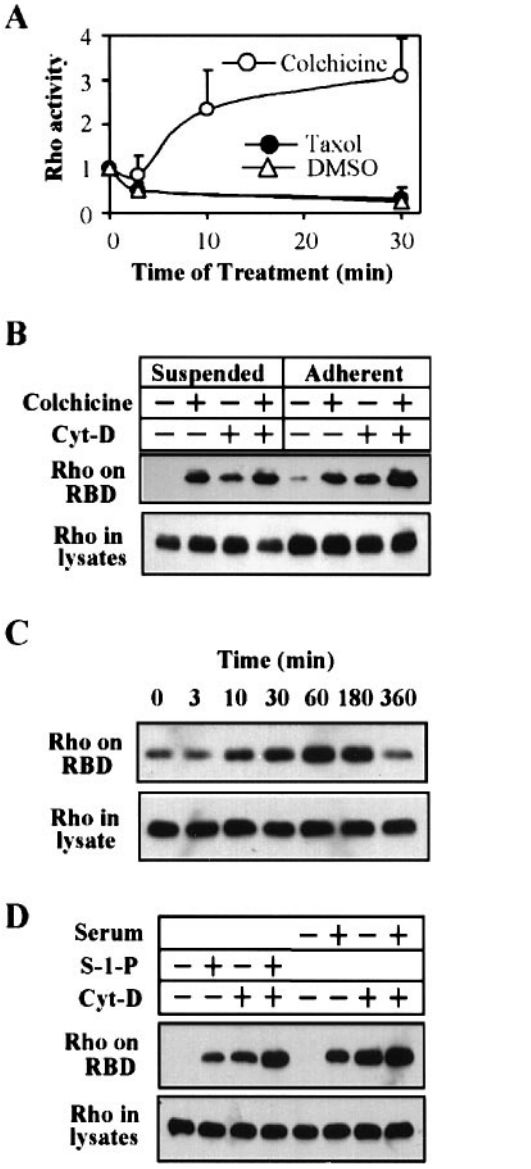

Fig. 6. Rho activation by cytochalasin D and colchicine. (**A**) Serum-starved Swiss 3T3 cells were treated with 10 μg/ml colchicine, 10 μg/ml taxol or 0.1% DMSO (to achieve the same concentration as in the colchicine and taxol samples). Rho activity was measured at the times indicated. (**B**) Serum-starved Swiss 3T3 cells, either detached and held in suspension for 60 min or left attached to the tissue culture dishes, were stimulated for 30 min with 10 μg/ml colchicine, 0.5 μg/ml cytochalasin D (Cyt-D) or both as indicated in the figure, prior to assaying Rho activity. Control cells were treated with DMSO for 30 min. (**C**) Cells were treated with 0.5 μg/ml cytochalasin D (Cyt-D) for the indicated times, then Rho activity was assayed. (**D**) Cells were treated with 0.5 μg/ml cytochalasin D or 0.05% DMSO for 30 min prior to stimulation with 1 μg/ml sphingosine-1-phosphate (S-1-P) for 2 min, or 5% serum for 5 min, then Rho activity assayed.

Therefore, ECM proteins not only make a structural contribution to the formation of focal adhesions but also directly transduce signals to regulate Rho. Rho activation during attachment and spreading on fibronectin occurred only after a significant (10–20 min) delay, however, and eventually (after 6 h) declined to a baseline level. Thus, increases in Rho activity are observed in freshly plated cells where new adhesions are being formed, but in stably adherent cells where adhesions are less dynamic the

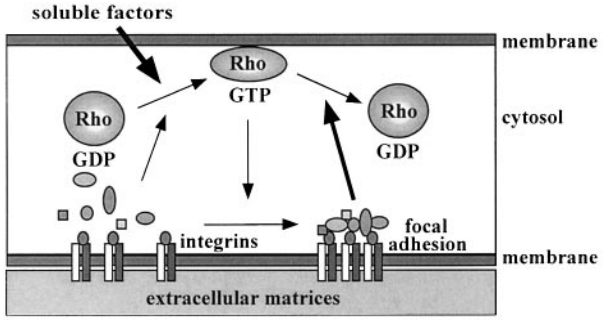

Fig. 7. Proposed model for the regulation of Rho by both soluble factors and ECM. Under serum-free condition, Rho activity is decreased and integrins are not significantly clustered therefore focal adhesions are not readily detectable. Adding LPA or serum activates Rho within minutes to generate contractile force, leading to clustering of integrins and formation of focal adhesions and stress fibers. Newly formed focal adhesions generate signals to downregulate Rho to prevent excessive formation of focal adhesions. Downregulation of Rho may also play a role in disassembly of focal adhesions required for cell migration. The actin cytoskeleton and microtubules suppress Rho activity possibly by controlling the localization of guanine nucleotide exchange factors or GAPs.

dominant effect is inhibitory. The regulation of Rho by adhesion and growth factors is summarized in Figure 7.

The biphasic regulation of Rho by adhesion and the crosstalk with soluble regulatory factors indicate that the pathways regulating Rho must exhibit considerable complexity. Elucidation of these pathways clearly poses a substantial challenge. While the physiological role for Rho regulation in these cells is poorly understood, we suggest that the observed regulatory effects closely match the requirements for cell migration. Either inhibiting or constitutively activating Rho decreases cell migration (Takaishi *et al*., 1993; Allen *et al*., 1998), indicating that dynamic Rho regulation is important for migration. Migrating cells lose adhesions at the trailing edge and form new adhesions at the leading edge. Thus, the biphasic regulation of Rho seen in freshly plated cells may parallel spatially regulated events in migrating cells where assembly and disassembly of adhesions occur in different regions.

Data from experiments with cytoskeletal drugs demonstrate activation of Rho by colchicine, consistent with the ability of colchicine to trigger formation of stress fibers and focal adhesions under these conditions (Enomoto, 1996; Zhang *et al*., 1997; Liu *et al*., 1998). Surprisingly, cytochalasin D, which disrupts stress fibers and focal adhesions, also induced activation of Rho. Furthermore, the activation of Rho by colchicine was independent of cell adhesion and was enhanced in the presence of cytochalasin. These results demonstrate plainly that the activation of Rho by colchicine is not mediated by an increase in cytoskeletal tension (Ingber, 1993; Bershadsky *et al*., 1996). They also underscore the fact that focal adhesion and stress fiber assembly does not necessarily provide an accurate indication of Rho activity. These results with cytochalasin D and colchicine may be of particular importance given their widespread use in studies of cellular regulation. There are instances where cytochalasins show stimulatory effects, which have been interpreted to demonstrate that the actin cytoskeleton serves an inhibitory function (Benya and Brown, 1988; Cantiello *et al*., 1991; Lub *et al*., 1997; Sheikh and Nash, 1998). The finding that Rho is activated under these conditions may necessitate re-evaluation of some of the previous studies.

In addition to its role in cytoskeletal organization, Rho has been implicated in cell cycle progression and cell transformation (Yamamoto *et al*., 1993; Olson *et al*., 1995; Prendergast *et al*., 1995; Qiu *et al*., 1995). Previous data suggested that Rho is implicated in integrin-mediated signals including PIP_2 synthesis (McNamee *et al*., 1993; Chong *et al*., 1994), MAP kinase activation (Renshaw *et al*., 1996) and FAK activation (Barry *et al*., 1997), and that low Rho activity might partially account for growth arrest in suspended cells (Schwartz *et al*., 1996). Our data, however, demonstrate that Rho activity is higher in suspended cells than in adherent cells in the presence of serum, and that the time course of Rho activation by adhesion does not correlate well with adhesion-induced PIP_2 synthesis (McNamee *et al*., 1993), activation of FAK (Hanks *et al*., 1992) or MAP kinase (Chen *et al*., 1994; Renshaw *et al*., 1996). These results do not exclude the possibility that Rho contributes to or is required for these events. They do not, however, support a model in which low Rho activity leads to growth arrest in suspended cells, or where Rho activity is the key point of regulation of these events by cell adhesion.

Materials and methods

DNA cloning and vector construction

The coding sequence for the Rho-binding domain of Rhotekin (amino acids 7–89) (Reid *et al*., 1996) was amplified from a mouse testicle cDNA preparation (Clontech) by PCR, cloned into pGEX-2T vector (Pharmacia), and confirmed by DNA sequencing. The pcDNA3 vector (Invitrogen) was modified to have a coding sequence of hemagglutinin (HA) immuno-tag followed by a *Bam*HI site with the same reading frame to the pGEX-2T vector. The GTP-binding proteins were subcloned into the pcDNA3-HA vector from the pGEX plasmids. LipofectAMINE reagents (Life Technologies) were used for transient transfections.

Affinity-precipitation of cellular GTP-Rho

Swiss 3T3 cells were washed with ice-cold Tris-buffered saline and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF). Cell lysates were clarified by centrifugation at 13 000 *g* at 4°C for 10 min, and equal volumes of lysates were incubated with GST–RBD (20 μg) beads at 4°C for 45 min. The beads were washed four times with buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 0.1 mM PMSF). Bound Rho proteins were detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology). Densitometry analysis was performed using AlphaImager™ system (Alpha Innotech). The amount of RBD-bound Rho was normalized to the total amount of Rho in cell lysates for the comparison of Rho activity (level of GTP-bound Rho) in different samples. Depending on cell conditions and types, and different batches of GST–RBD, the RBD-bound Rho accounts for ~0.5–5% of total Rho.

For metabolic labeling, Swiss 3T3 or NIH 3T3 cells were incubated with 50 μCi/ml [^{32}P]phosphate in phosphate-free medium overnight. After stimulation, cells were lysed in RIPA buffer supplemented with nucleotides (1 mM ATP, 200 μM each of GTP and GDP), and incubated with GST–RBD beads. After washing four times with the same buffer and four times with buffer B supplemented with the nucleotides, samples were analyzed by thin-layer chromatography followed by autoradiography (Renshaw *et al*., 1997). Aliquots of whole cell lysates were also analyzed and showed no changes in total nucleotide pools after treatment with LPA or C3. To inactivate endogenous Rho with C3 exoenzyme, NIH 3T3 cells were used as we have previously found that

LipofectAMINE reagent can introduce C3 into >90% of cells (Renshaw *et al*., 1996).

Suspension culture

Cells were detached with trypsin–EDTA and washed with DMEM containing 0.5 mg/ml soybean trypsin inhibitor. Cells were then resuspended in DMEM supplemented with 0.5 mg/ml lipid-free bovine serum albumin (for serum-free culture), incubated in 1% agarose-coated dishes for 60–90 min, and transferred to fibronectin-coated (25 μg/ml) dishes. For suspension culture overnight, 0.4% methylcellulose was included in the medium to prevent cells from clumping. Cells were handled very gently throughout, and were cooled to 4°C before collecting for lysis.

Immunofluorescence microscopy

Cells plated on glass coverslips were fixed in 2% formaldehyde–PBS for 30 min, permeabilized in 0.1% Triton X-100 at room temperature for 5 min, and stained with rhodamine–phalloidin (Molecular Probes) and fluorescein isothiocyanate (FITC)-anti-vinculin (Sigma). Images were acquired using Bio-Rad 1024 MRC laser scanning confocal imaging system.

Detection of phosphorylated myosin light chain

Phosphatase inhibitors (5 mM sodium vanadate, 20 mM NaF and 3 mM β-glycerophosphate) were added to the cell lysis buffer. Antibody pp2b (a generous gift from Dr Fumio Matsumura, Rutgers University, NJ) was used in Western blotting to detect the phosphorylated myosin light chain (Matsumura *et al*., 1998). A monoclonal IgM antibody (Sigma) against mouse myosin light chain was used to detect total myosin light chain (phosphorylated and non-phosphorylated) in whole cell lysates. Phosphorylated myosin was normalized to total myosin in each sample for comparison.

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