

The Rho kinases I and II regulate different aspects of myosin II activity

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The homologous mammalian rho kinases (ROCK I and II) are assumed to be functionally redundant, based largely on kinase construct overexpression. As downstream effectors of Rho GTPases, their major substrates are myosin light chain and myosin phosphatase. Both kinases are implicated in microfilament bundle assembly and smooth muscle contractility. Here, analysis of fibroblast adhesion to fibronectin revealed that although ROCK II was more abundant, its activity was always lower than ROCK I. Specific reduction of ROCK I by siRNA resulted in loss of stress fibers and focal adhesions, despite persistent ROCK II and guanine

triphosphate-bound RhoA. In contrast, the microfilament cytoskeleton was enhanced by ROCK II down-regulation. Phagocytic uptake of fibronectin-coated beads was strongly down-regulated in ROCK II-depleted cells but not those lacking ROCK I. These effects originated in part from distinct lipid-binding preferences of ROCK pleckstrin homology domains. ROCK II bound phosphatidylinositol 3,4,5P₃ and was sensitive to its levels, properties not shared by ROCK I. Therefore, endogenous ROCKs are distinctly regulated and in turn are involved with different myosin compartments.

Introduction

Small Rho GTPases are molecular switches that control actin cytoskeleton organization, cell adhesion and migration, gene expression, microtubule dynamics, and vesicle transport (Etienne-Manneville and Hall, 2002). Two homologous serine/threonine kinases (ROCK I and II, also known as ROK β and α , respectively) are major Rho effectors and are implicated in the formation of stress fibers and focal adhesions in nonmuscle cells, contractility of smooth muscle, and suppression of neurite outgrowth (Riento and Ridley, 2003). Both isoforms are composed of an NH₂-terminal kinase domain, coiled-coil domain, Rho-binding domain, and pleckstrin homology (PH) domain. Major ROCK substrates in the regulation of actomyosin contraction are myosin light chain 2 (MLC2; Kureishi et al., 1997) and the myosin-binding subunit of myosin phosphatase (MYPT; Kawano et al., 1999). Rho kinases can also phosphorylate and activate LIM kinases, which regulate an actin-depolymerizing protein, cofilin (Mae-kawa et al., 1999). The central role of ROCKs in microfilament architecture is underscored by the inhibitor Y-27632, which although not entirely specific for these kinases (Davies

et al., 2000; Narumiya et al., 2000) has been shown to block or reverse bundle formation and contraction.

ROCK I and II have been assumed to perform the same functions, but most studies have been accompanied by the overexpression of kinase dead, constitutively active mutant or wild-type forms of one isoform. Distinctions between the ROCKs have been only rarely drawn; ROCK I but not ROCK II, for instance, is sensitive to caspase 3-mediated cleavage in apoptotic cells (Coleman et al., 2001; Sebbagh et al., 2001). However, analysis of the ROCK II-deficient mouse suggested that there is no compensation for the loss of ROCK II by ROCK I (Thumkeo et al., 2003). Approximately 90% of null embryos died in utero, accompanied by placental defects, whereas those born were runted. Moreover, RhoE inhibits ROCK I, but not ROCK II, with a loss of stress fibers (Riento et al., 2003, 2005), indicating that ROCK II cannot compensate for the decreased ROCK I activity. However, although the importance of the rho kinases in many cellular processes is understood, no clear dissection of roles for ROCK I and II has been described. Here, we show distinct roles for ROCK I and II in the same cells. We show that although ROCK I rather than II is important for stress fiber formation, ROCK II acts as a counterbalance in regulating the microfilament bundle and focal adhesion site. ROCK II activity is involved in phagocytosis of matrix-coated beads, a function not sensitive to ROCK I depletion.

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Abbreviations used in this paper: FN, fibronectin; GRP1, general receptor for phosphoinositides-1; LPA, lysophosphatidic acid; MLC, myosin light chain; MYPT, myosin-binding subunit of myosin phosphatase; PH, pleckstrin homology; PI, phosphoinositide; PtdIns, phosphatidylinositol; REF, rat embryo fibroblast.

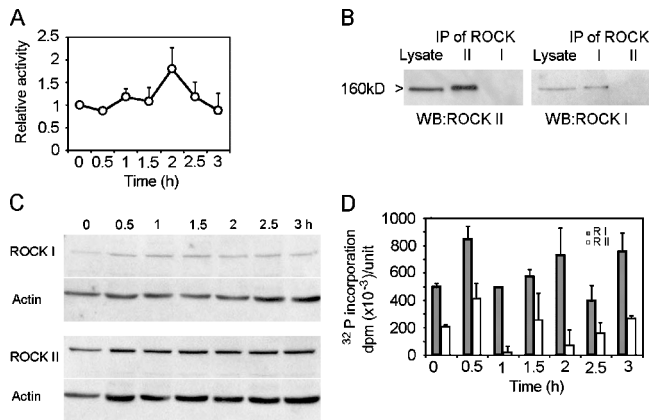


Figure 1. Activity profiles of ROCK I and II in fibroblast adhesion. (A) Serum-starved REF were plated on FN-coated plates and lysates were prepared at times up to 3 h. GTP-RhoA was pulled down with GST-Rhotekin-immobilized beads. RhoA protein levels in total cell lysate and pull down samples were calculated from Western blots and relative GTP-RhoA levels were normalized to time 0. Error bars show SD. (B) ROCK I and II immunoprecipitates and total cell lysates were subjected to SDS-PAGE and Western blotting with ROCK antibodies. Each antibody is monospecific and only recognizes one ROCK protein. (C) ROCK I and II and actin protein levels in cell lysate from REF plated on FN for the indicated times were analyzed by Western blotting. No changes through cell adhesion were seen. (D) ROCK I and II were specifically immunoprecipitated from lysates at the indicated times in fibroblast adhesion on FN. Kinase activities in immunoprecipitates were analyzed with GST-MLC as substrate. Background radiolabel levels from negative control precipitates were subtracted and the data was normalized to ROCK protein levels in the immunoprecipitates. Error bars show SD.

Results

ROCK I and II in fibroblast adhesion have different activity profiles

In response to seeding on fibronectin (FN) substrates, primary rat embryo fibroblasts (REF) attached, spread, and formed focal adhesions over a 2–3 h period. Using a pull-down assay, levels of GTP-RhoA peaked at around 2 h (Fig. 1 A), when large, stable focal adhesions formed in agreement with other studies (Ren et al., 1999). At all time points, GTP-RhoA could be detected, including in rounded cells at 0 h. Rho kinase activity was measured in immunoprecipitates at the same time points using antibodies monospecific for ROCK I or II (Fig. 1, B–D) and recombinant GST-MLC chimeric protein as substrate (Amano et al., 1996). There was no detectable alteration of either ROCK protein expression level during cell–matrix adhesion (Fig. 1 C). Three trends in activity could be observed. First, ROCK I activity levels, normalized to protein mass, were always higher than those of ROCK II (Fig. 1 D), although cells contained approximately twice the protein levels of ROCK II compared with ROCK I (unpublished data). Second, ROCK II activity was low throughout the adhesion process, except for a brief period ~30 min after seeding. Third, ROCK I activity, in particular, could be detected at all time points, and like GTP-RhoA levels, was quite substantial in rounded cells. Overall, the data indicated that the adhesion process had a particular requirement for ROCK I. As a further test, serum-starved REF were treated with lysophosphatidic acid (LPA), known to ele-

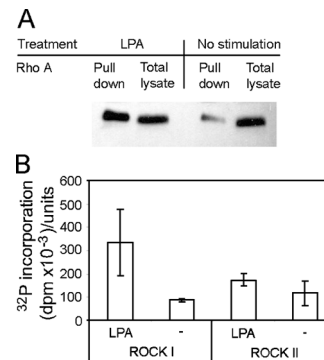


Figure 2. Rho kinase activity in relation to microfilament bundle formation. (A and B) Serum-starved REF were stimulated with 400 ng/ml LPA or control buffer for 5 min and lysed. (A) GTP-RhoA was pulled down from cell lysates and analyzed by Western blotting. One example from duplicate experiments is shown, with increased levels of GTP-RhoA after LPA treatment. (B) Each ROCK was specifically immunoprecipitated, and kinase activities in immunoprecipitates were analyzed as described in Materials and methods. Means of duplicates are shown. Whereas ROCK I activity was enhanced by LPA treatment, that of ROCK II was little changed.

vate GTP-RhoA levels in Swiss3T3 cells (Ridley and Hall, 1992). LPA treatment resulted in substantial elevation of GTP-RhoA levels as expected (Fig. 2 A). This was matched by a larger increase in ROCK I compared with ROCK II activities, correlating temporally with stress fiber and focal adhesion formation (Fig. 2 B).

The necessity for persistent ROCK activity throughout the adhesion process was examined by treating fibroblasts with the rho kinase inhibitor Y-27632 (Narumiya et al., 2000) at times from seeding to 3 h. In all cases, microfilament bundles dissipated, and any focal adhesions that formed at 1.5–3 h after seeding, disassembled. This confirmed that rho kinase activity was continuously required through the adhesion process and that addition of Y-27632 at any time would disrupt the microfilament architecture.

ROCK I but not ROCK II activity is essential for focal adhesion and stress fiber formation

To establish whether ROCK I and II were separable in their ability to regulate stress fibers and focal adhesions, siRNA techniques were used to reduce levels of each specifically. As an aid to identification of transfected cells, fluorochrome-conjugated nucleotides were used. These experiments showed a striking contrast in terms of cytoskeletal reorganization. Depletion of ROCK I in REF (Fig. 3 A) and Rat-1 cells (not depicted) led to almost complete loss of stress fibers and focal adhesions, as monitored by F-actin and paxillin localizations. Similar results were seen when distributions of vinculin, α -actinin, and phosphotyrosine were examined (unpublished data). Residual focal complexes at the cell margins were observed. Western blotting showed an ~70% reduction in ROCK I protein in the cell population as a whole, but normal levels of ROCK II (Fig. 3 B). Reduction in ROCK II had almost opposite effects, with stress fibers and focal adhesions becoming exaggerated beyond that normally seen in these fi-

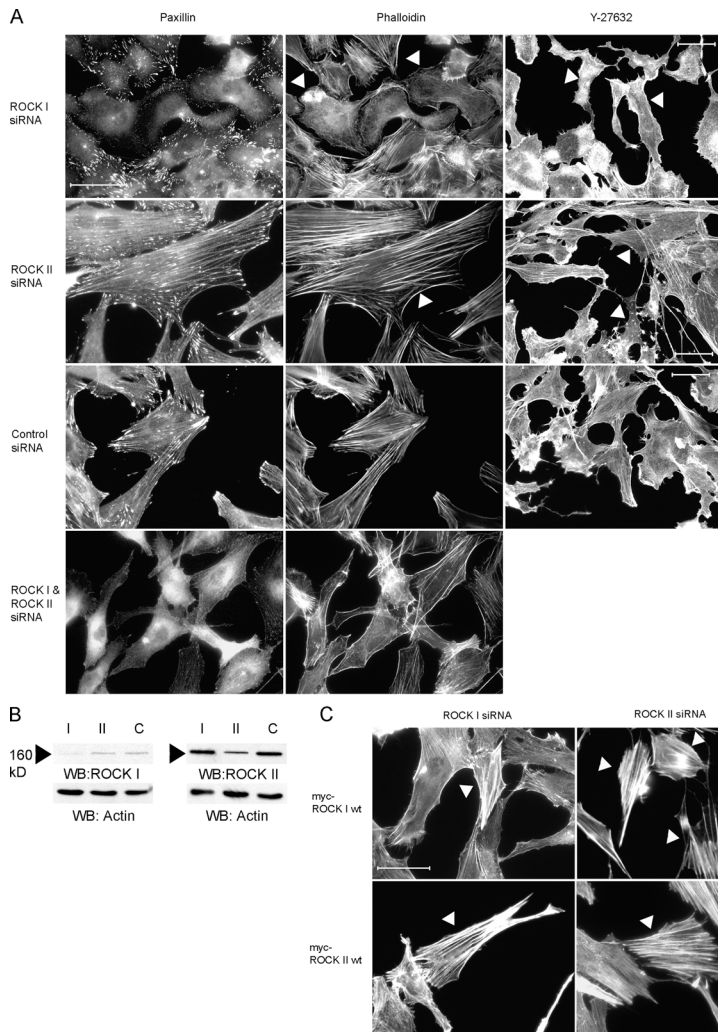


Figure 3. ROCK I but not ROCK II activity is essential for focal adhesion and stress fiber formation. (A) Fibroblast cultures were transfected with ROCK I, ROCK II, negative control, or a mixture of ROCK I and II siRNA duplexes. After maintenance culture for 2 d, cells were fixed and stained for paxillin and F-actin. Arrowheads indicate transfected cells. 2 d after transfection with siRNA duplexes, REF were incubated with Y-27632 (10 μ M) in the presence of FCS. After 30 min, cells were fixed and stained for F-actin. In all cases, stress fibers were sensitive to Y-27632 addition. (B) Total cell lysates of REF prepared 2 d after transfection with ROCK I (I), ROCK II (II), or control (C) siRNA duplexes were analyzed by Western blotting for ROCK I, ROCK II, and actin. In each case, total populations were analyzed, containing some nontransfected cells. Results from cultures where 80–90% of cells were transfected are shown. (C) REF were cotransfected with myc-tagged full-length human ROCK I or bovine ROCK II cDNA and cy3-labeled ROCK I or II siRNA duplexes. 2 d after transfection, cells were fixed and stained for myc and F-actin. Either ROCK cDNA can restore a normal phenotype. Arrowheads indicate cotransfected cells. Bars, 50 μ m.

broblasts (Fig. 3, A and B). Characteristically, ROCK II-depleted cells had an elongated and flattened morphology with long trailing extensions. Focal adhesion components could often be detected extending away from focal adhesions along the stress fibers. As before, Western blotting showed that protein levels of the alternate rho kinase (ROCK I in this case) were unchanged. ROCK II itself, however, was depleted by \sim 70% across the cell population. In ROCK II siRNA-treated cells, the prominent stress fibers and focal adhesions were completely susceptible to Y-27632 treatment (Fig. 3 A) or ROCK I siRNA oligonucleotides to accomplish double depletion of both rho kinases (Fig. 3 A). The morphological characteristics of double depleted cells were essentially additive. Stress fibers and focal adhesions were completely absent, but the extended flattened morphology of ROCK II-depleted cells was observed also, rather than the more convex, rounded cell margins of ROCK I-depleted cells. Phalloidin and vinculin fluorescence were quantitated in transfected fibroblasts. Both were significantly increased in ROCK II siRNA-transfected cells (1.6- and 1.4-fold, respectively, $P < 0.001$ for both F-actin and vinculin, $n = 27$) compared with mock-transfected controls ($n = 15$). Fluorescence levels in ROCK I siRNA-treated cells were not significantly different to con-

trols, despite the distinctly different organization of F-actin and vinculin.

Two conclusions can be drawn. First, the two rho kinases have separable roles, with ROCK I being largely responsible for stress fiber and focal adhesion formation. Second, ROCK II was incapable of substituting for ROCK I in the latter's absence. Control cells, transfected with an irrelevant siRNA oligonucleotide, were unaffected in cell morphology and actin cytoskeleton. Vector-based siRNA experiments to deplete ROCK II were not quite as successful (60% reduction in protein overall), but transfected cells acquired the same elongated phenotype with extensive microfilament bundles. In other control experiments, fibroblasts were cotransfected with siRNA together with full-length ROCK cDNA from a different mammalian species (and therefore not susceptible to siRNA down-regulation because of nucleotide differences) to show that the phenotypes could be corrected (Fig. 3 C). Cells in these experiments remained quite normal in morphology and actin cytoskeleton characteristics. However, it should be noted that in these experiments overexpression of either ROCK could restore a normal phenotype in cells treated with either siRNA oligonucleotide. Therefore, overexpression could obscure the specificity of the two rho kinases, but also indicated that when present in excess

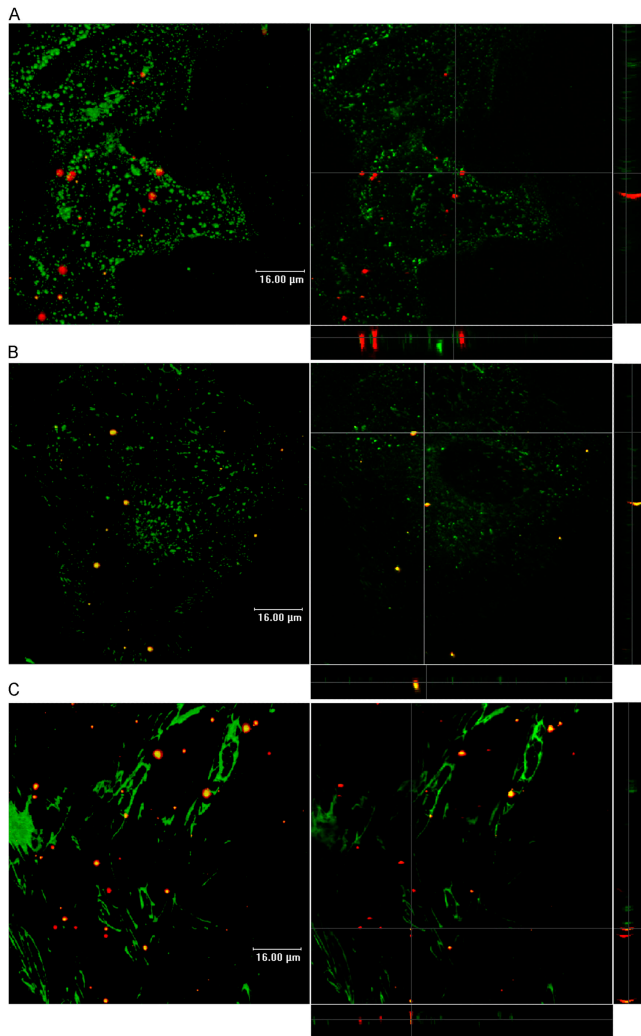


Figure 4. **ROCK II-deficient fibroblasts have low phagocytic activity.** REF transfected with ROCK I (A), ROCK II (B), and control (C) siRNA were analyzed for phagocytic activity with FN-coated latex red beads as described in Materials and methods. After a 1-h incubation with beads, the cells were fixed and stained for extracellular FN. Three-dimensional fluorescence images of FN (green) and latex beads (red) were recorded from laser scanning confocal microscopy. Intracellular, phagocytosed beads could be seen as red only, whereas beads remaining extracellularly could be detected in both red and green channels. Quantitation is shown in Table I.

ROCK II could promote stress fiber formation. REF were also seeded on serum, FN, or type I collagen-coated substrates, with near identical outcomes from siRNA treatments. Therefore, these effects were not related to a single integrin occupancy.

ROCK II activity facilitates phagocytosis

Another aspect of cell-matrix interactions was revealed by a study of phagocytic uptake of FN-coated latex beads. In these experiments, it was seen that ROCK II siRNA-treated cells were significantly impaired in comparison to control cultures or those depleted of ROCK I (Fig. 4 A and Table I). Laser scanning microscopy showed that fibroblasts could readily internalize FN-coated 1- μ m beads, but not if lacking ROCK II (Fig. 4 B). To facilitate quantitation, cells were exposed to the red fluorescing coated beads, fixed, and then treated with anti-

Table I. **Fibroblast phagocytosis of FN-coated beads**

| | <i>n</i> | Average of percentage of incorporated beads |
|---------------------------------|----------|---|
| ROCK I siRNA | 20 | 37.2 \pm 5.1 ^a |
| ROCK II siRNA | 22 | 18.3 \pm 3.6 |
| Control siRNA | 21 | 49.0 \pm 6.4 ^a |
| LY294002 | 15 | 18.9 \pm 3.3 ^b |
| DMSO (control for LY294002) | 15 | 34.5 \pm 5.0 |
| Blebbistatin | 21 | 14.0 \pm 2.2 ^b |
| DMSO (control for Blebbistatin) | 25 | 30.4 \pm 3.0 |
| Y27632 | 19 | 19.0 \pm 2.9 ^c |
| Control (control for Y27632) | 18 | 49.4 \pm 3.5 |

^a*P* < 0.002, ROCK I siRNA versus ROCK II siRNA and control siRNA versus ROCK II siRNA.

^b*P* < 0.02.

^c*P* < 0.001.

FN antibodies. Subsequent Alexa488 secondary antibody treatment therefore only detected externalized, but not internalized, beads. Dual fluorochrome imaging readily discriminated bead populations.

Rho kinase distributions and depletion by siRNA treatment

In further experiments, to show the differences between ROCK I and II, immunofluorescence microscopy revealed a distinct distribution of each protein (Fig. 5). Whereas ROCK I was mainly diffuse and perinuclear with some decoration of stress fibers, ROCK II was present at cell margins as well as in intensely staining of perinuclear locations. Specific siRNA treatment showed the concomitant down-regulation of each rho kinase as appropriate to the treatment. In many cases, staining was close to background, indicating that the overall 70% reduction in protein levels reflected incomplete transfection of the population (confirmed by microscopic analysis of cells for the fluorochrome-conjugated siRNA oligonucleotides). In many cases individual cells appeared to have almost total loss of the appropriate protein. Levels and distribution of staining were not markedly changed in the alternative kinase, consistent with siRNA specificity (Fig. 5). ROCK I distribution was similar to that reported previously (Riento et al., 2003).

Rho kinase-depleted cells are not deficient in GTP-bound RhoA

ROCK I-depleted cells that lacked stress fibers, as well as ROCK II-depleted cells that possessed them but had reduced phagocytic activity, contained similar levels of GTP-RhoA and Rac1, only slightly lower than equivalent negative control siRNA (Fig. 6 A). Moreover, ROCK I siRNA-treated fibroblasts could be cultured in the presence of serum, containing LPA, yet stress fiber formation was not restored. This result was consistent with the finding that ROCK I is substantially more responsive to LPA treatment.

Effects of ROCK depletion on growth

The very large spread areas of ROCK II and double-depleted cells suggested hypertrophy. This was confirmed in rounded

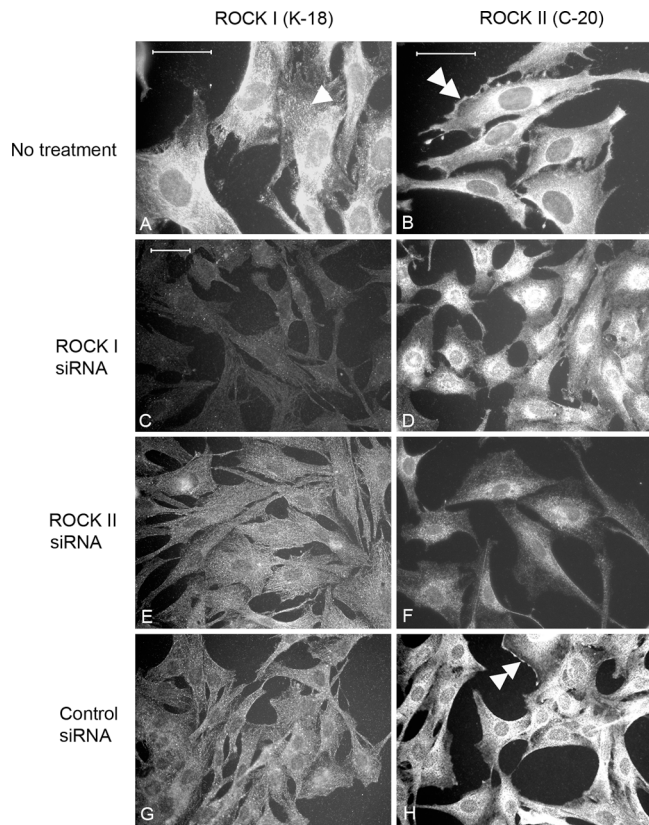


Figure 5. Endogenous ROCK I and II have distinct distributions. (A) ROCK I in REF cells is largely diffuse in the cytoplasm, more concentrated in perinuclear regions, and sometimes associated with stress fibers (arrowhead). It is not membrane associated unlike ROCK II (B; double arrowheads). ROCK II is also intensely concentrated in perinuclear regions (B, D, and H). After siRNA treatment to deplete ROCK I, its staining is correspondingly reduced (C), whereas that of ROCK II is unaltered (D). Reciprocal results are seen with ROCK II depletion (E and F). Control siRNA treatment was without effect on either rho kinase staining pattern (G and H). Bars, 50 μm .

cells by morphometry. Cells depleted of ROCK II were 38% larger in volume on average (Fig. 6 B). Cell cycle analysis showed a small population (2–3%) of apoptotic cells in both ROCK I- and II-depleted populations, against <1% in controls. However, growth was markedly retarded in treated cells (Fig. 6 C), with increasing numbers of binucleate cells after ROCK I siRNA treatment. This was consistent with a large pool of cells having a DNA content equivalent to G2/M phase. Extensive cell death from ROCK I siRNA treatment has been reported, but this was not seen here (Chevrier et al., 2002). Despite the clear changes in microfilament organization, both ROCK I- and II-depleted cells could be observed to round up and spread in the course of time-lapse video microscopy (unpublished data). Consistent with this data, treatment with Y-27632 also strongly retarded REF cell growth in a dose-dependent manner. Observations of treated cells indicated that they could enter mitosis but not successfully complete it.

Both ROCKs can phosphorylate the same substrates

Examination of the two major substrates for rho kinases, MLC2 (Kureishi et al., 1997) and MYPT (Kawano et al.,

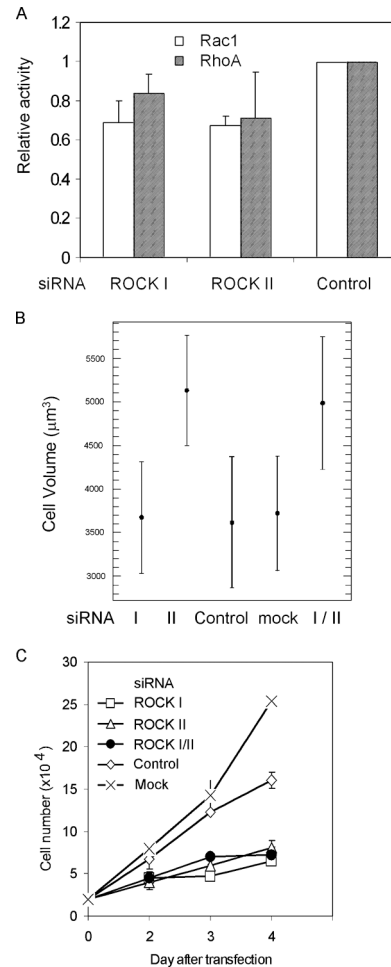
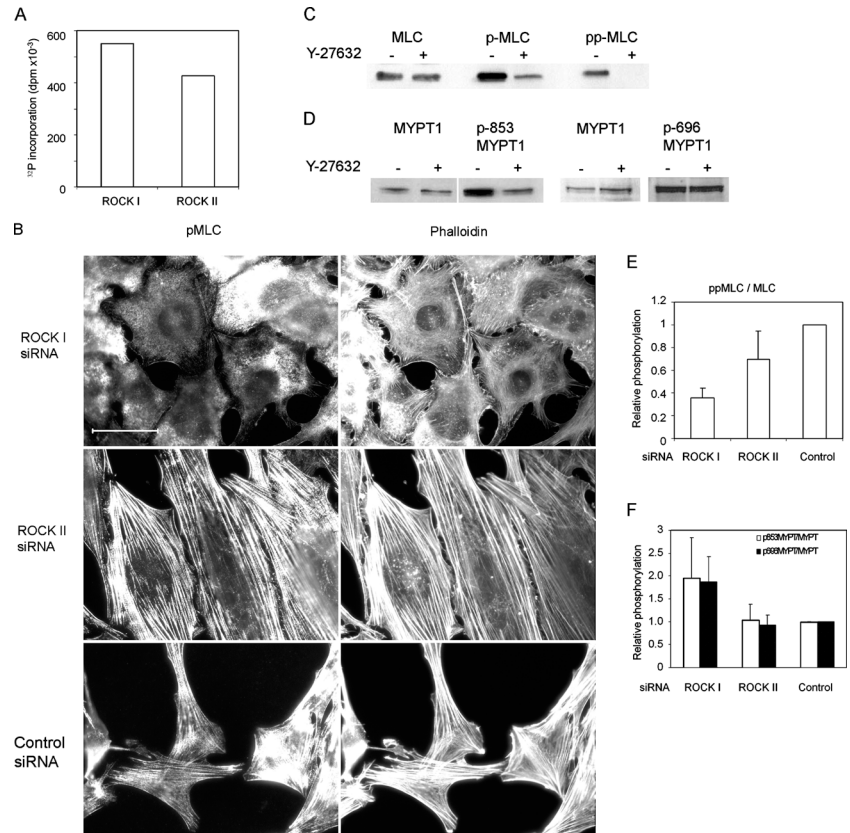


Figure 6. ROCK I- and II-deficient cells contain normal GTPase profiles but are growth retarded. (A) 2 d after siRNA duplex transfection, GTP-RhoA and GTP-Rac1 were analyzed by pull-down assays. Levels are normalized to negative control levels, and error bars indicate SD. In all cases, levels were close to those of control cells. (B) 2 d after transfection with siRNA duplexes, diameters of rounded cells were measured and volumes were calculated as described in Materials and methods. Data were analyzed by one-way ANOVA and showed that cells depleted of ROCK II were significantly larger than control, mock-transfected, or ROCK I-depleted cells ($P < 0.001$). Double depleted cells were similar to cells depleted of ROCK II alone. (C) At the indicated days after transfection, cell numbers were counted. Error bars show SD. In all cases, control transfections are luciferase DNA target duplexes, whereas mock cultures received oligofectamine only.

1999), showed that both could be phosphorylated by ROCK I and II in vitro (Fig. 1 D and Fig. 7 A). However, immunofluorescence microscopy with phosphospecific antibodies showed that phosphorylation of MLC2 on serine 19 was much reduced in ROCK I-depleted cells, but not in those lacking ROCK II. The large stress fibers in the latter cells were strongly decorated with anti-phosphoMLC2 antibodies (Fig. 7 B). Correlating with fluorescence microscopy, marked depletion of MLC2 phosphorylation was also seen in ROCK I siRNA-treated cells by Western blotting (Fig. 7 E). This was not seen in cells depleted of ROCK II, where MLC2 phosphorylation was not significantly different from mock-treated controls (Fig. 7 E). A further Western blotting control experiment showed a near to-

Figure 7. ROCKs can phosphorylate the same substrates. (A) ROCK I and II immunoprecipitates from REF were incubated with MBP-MYPT1 COOH-terminal fragment and γ -[32 P]ATP. Background radiolabel levels from control immunoprecipitates have been deducted. (B) 2 d after transfection with siRNA duplexes, cells were fixed and stained for pMLC2 (Ser19) and F-actin. ROCK I siRNA cells have depleted staining, whereas the abundant stress fibers of ROCK II siRNA stain strongly. Bar, 50 μ m. (C and D) Total cell lysates from REF treated with Y-27632 in the presence of serum were analyzed by Western blotting for MLC2 phosphorylation on Ser19 (p-MLC) or Thr18/Ser19 (pp-MLC) (C) and MYPT phosphorylation on Thr853 or Thr696 (D). Although MYPT phosphorylation on Thr853 was reduced, more marked reductions were seen in MLC2 phosphorylation after Rho kinase inhibition. (E and F) Total cell lysates from cells transfected with siRNA duplexes were analyzed by Western blotting for MLC2 phosphorylation on Thr18/Ser19 (E) and MYPT phosphorylation of Thr853 or Thr696 (F). Phosphorylation is normalized to the relevant protein level in the lysates. Phosphorylation levels of MYPT were increased in the absence of ROCK I, partly due to a decrease in myosin phosphatase protein levels in these cells. Error bars show SD.



tal ablation of MLC2 phosphorylation on threonine 18 and serine 19 after Y-27632 treatment (Fig. 7 C).

Two residues of MYPT, Thr696 and Thr853, are known substrates for rho kinases (Hartshorne et al., 2004). Western blotting with phosphospecific antibodies showed that both were increased after single siRNA depletion of ROCK I but not ROCK II (Fig. 7 F) when compared with controls. In part, this resulted from \sim 45% reduction in MYPT protein level in ROCK I-depleted cells. When both rho kinases were inhibited by Y-27632 treatment, phosphorylation levels of Thr853, but not Thr696, were reduced but not to background (Fig. 7 D). In total, the data suggest that both rho kinases contribute to MYPT phosphorylation in vivo, but the relationship is not simple because other kinases can phosphorylate these residues (Hartshorne et al., 2004; Wilkinson et al., 2005). The clearest correlation from these experiments was between MLC2 phosphorylation, ROCK I activity, and stress fiber formation.

Further analysis showed no changes in phosphorylation levels of the LIM kinase substrate, cofilin, in response to siRNA treatments. However, although LIM kinase 2 is suggested to be a substrate for rho kinases in vivo (Sumi et al., 2001a), the same residues in LIM kinase 2 are also phosphorylated by myotonic dystrophy kinase-related Cdc42-binding kinase α (Sumi et al., 2001b). LIM kinase 1 is downstream of p21-activated kinase (PAK1; Edwards et al., 1999) and is not phosphorylated by full-length ROCK I (Sumi et al., 2001a). Overall, because several GTPases may lead to LIM kinase activation and cofilin phosphorylation, these results are not unexpected.

ROCK II PH domain can bind to inositol phospholipids

A comparison of the homology between the two rho kinases shows that they have 92% identity in their kinase domains (Nakagawa et al., 1996), which is consistent with the ability to phosphorylate the same substrates. ROCK I and II also have closely similar rho-binding domains, although crystal structures of ROCK I with active RhoA and ROCK II alone are not completely similar (Shimizu et al., 2003; Dvorsky et al., 2004). There is no evidence currently that GTPases other than Rho can activate these kinases in vivo. The PH domains of ROCK I and II, on the other hand, have only 65–70% sequence identity (Nakagawa et al., 1996; Leung et al., 1996), suggesting that they may be a source of specificity. These were prepared as cDNAs encoding GFP-conjugated fusion proteins and transfected into cells to examine their subcellular localizations. Appropriately sized proteins were detected in cell lysates by Western blotting with ROCK antibodies (Fig. 8 A). At high expression levels in fibroblasts, both GFP-PH domain fusion proteins inhibited microfilament bundle formation. However, in low expressing cells, where such effects were not apparent, the ROCK II PH domain had a preferential distribution in ruffling membrane ($P < 0.04$), whereas that of ROCK I had a more general cell membrane distribution (Fig. 8 B) and both rho kinases PH domains could associate with microfilament bundles, especially when cells were cultured in the absence of serum (unpublished data). The ROCK II PH domain had a similar distribution to general receptor for phosphoinositides-1 (GRP1)-PH-GFP, which binds to phosphatidylinositol (PtdIns) 3,4,5P₃

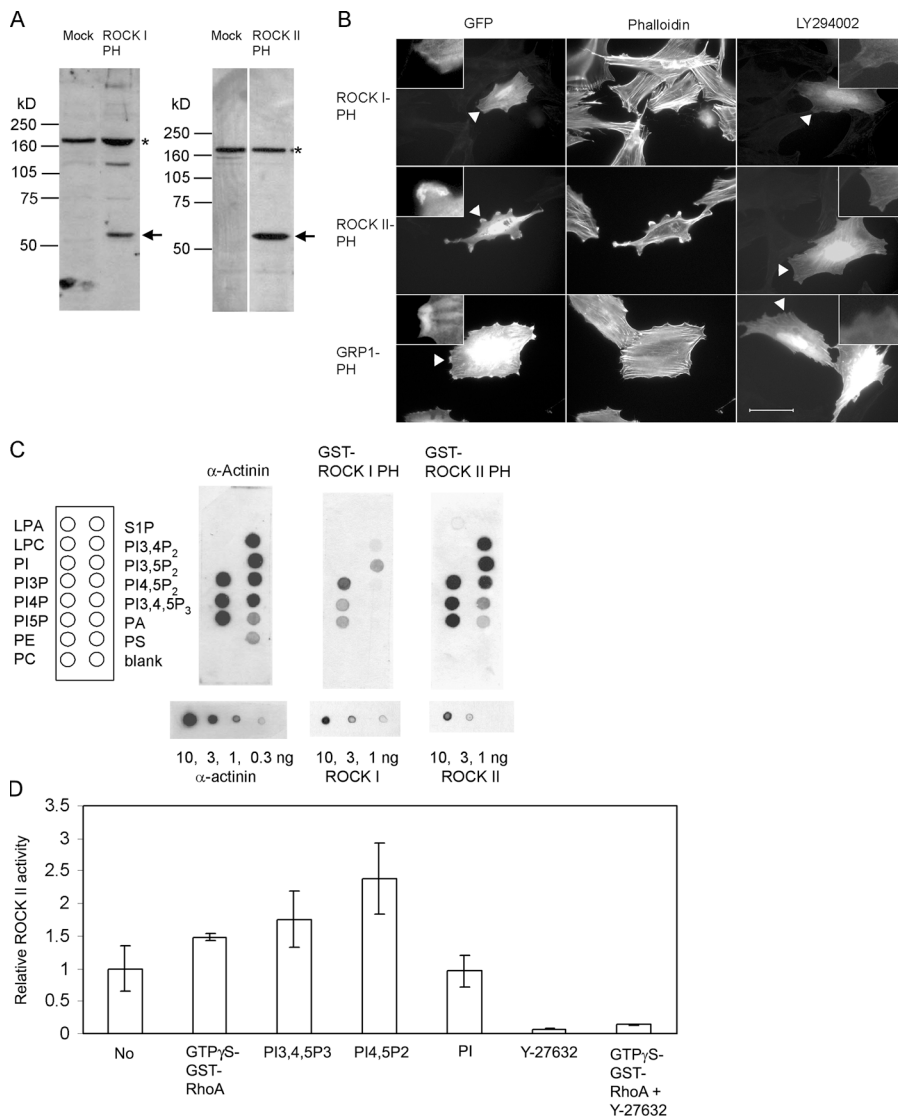


Figure 8. ROCK I and II PH domains do not bind the same PIs. (A) COS7 cells were transfected with ROCK I- or ROCK II-PH-GFP cDNA. After 24 h of transfection, total cell lysates were analyzed by Western blotting with ROCK antibodies. Asterisks show endogenous ROCK proteins and arrowheads indicate ROCK-PH-GFP proteins. (B) Fibroblasts were transfected with ROCK I- or ROCK II-PH-GFP or GRP1-PH-GFP cDNA. 1 d after transfection, cells were fixed and stained for F-actin. In other cases, transfectants were incubated with 30 μ M LY294002 for 60 min in the presence of serum and fixed. Arrowheads indicate cell margins with ruffles prominently containing ROCK II and GRP1-PH domains. These structures are absent after PI-3 kinase inhibition. Bar, 50 μ m. (C) GST-ROCK I-PH, GST-ROCK II-PH (3.3 nM), and α -actinin (10 nM) were incubated with phospholipid-loaded nitrocellulose membranes. Bound proteins were detected with ROCK or α -actinin antibodies as appropriate. Shown underneath each blot are calibrations where known amounts of each protein were spotted onto nitrocellulose membranes and probed similarly with antibodies. Different binding profiles of ROCK I and II are evident. (D) Recombinant myc-tagged ROCK II was purified from COS-7 cells (high salt method) and the kinase activity was analyzed in the presence of GTP γ S, GST-RhoA (1 μ M), PIs (100 μ M), or Y-27632 (1 μ M). Error bars show SD.

(Gray et al., 1999). Therefore, it seemed possible that the two PH domains had different preferences for membrane lipids. This has not been studied in any depth, though arachidonic acid was reported to activate ROCK II from muscle (Feng et al., 1999). Lipid binding assays revealed important differences between the two rho kinases (Fig. 8 C). ROCK I could not bind to PtdIns 3,4,5P₃, PtdIns3,4,P₂, or PtdIns 4, 5P₂. In contrast, ROCK II strongly bound to PtdIns3,4,5P₃ and PtdIns4,5P₂, consistent with its ruffling membrane distribution (Tall et al., 2000; Insall and Weiner, 2001). Both rho kinases could bind to PtdInsP isoforms. An identical assay with α -actinin was performed as a positive control (Fralely et al., 2003).

ROCK II could be expressed recombinantly and purified from COS-7 cells. When purified under high salt conditions (0.5 M), the kinase required the addition of RhoA-GTP γ S for activity, which could turn to be inhibited by Y-27632 (Fig. 8 D). Purification in physiological buffers resulted in constitutively active kinase that was, nevertheless, inhibited by Y-27632 (unpublished data). ROCK II purified under either condition had increased activity when PtdIns3,4,5P₃ or PtdIns4,5P₂

was added. No such increase was seen with PtdIns (Fig. 8 D). Therefore, ROCK II can at least be partially activated, even in the absence of RhoA-GTP, by two inositol phospholipids.

ROCK II activity is regulated by phosphoinositide (PI)-3 kinase activity

Confirmation of the relevance of this lipid binding was obtained by further experiments with fibroblasts. First, in the presence of the PI-3 kinase inhibitor LY294002 (Vlahos et al., 1994), membrane association of the GFP-PH domain of ROCK II was reduced, in contrast to the ROCK I equivalent that was still membrane associated (Fig. 8 B). PI-3 kinase inhibition also correlated with reduced ruffling membrane and was consistent with a loss of GRP1-PH from the membrane (Fig. 8 B). Second, the presence of LY294002 markedly depressed ROCK II activity, but had less effect on that of ROCK I in rounded cells (Fig. 9 A). Further evidence that ROCK II activity segregates with that of PI-3 kinase was obtained from phagocytic assays. Treatment of fibroblasts with LY294002 significantly reduced FN-coated bead uptake, as had ROCK II siRNA

treatment (Fig. 9 and Table I). The sensitivity of this process to rho kinase and myosin II inhibitors was confirmed by treatment with Y-27632 and blebbistatin, respectively (Table I). It was also seen that stress fibers dissipated as a result of blebbistatin treatment, confirmation that this process also required myosin II as previously reported (Limouze et al., 2004). In addition, transient transfection with cDNA encoding the p110 α subunit of PI-3 kinase led to corresponding protein expression and localization in membrane ruffles along with ROCK II but not ROCK I. ROCK II activity in these cells was increased, whereas that of ROCK I was unchanged (Fig. 9, B and C).

Discussion

Rho kinases are widespread and evolutionarily conserved downstream effectors of Rho GTPases: RhoA, B, and C are all potential activators whereas RhoE may be inhibitory (Riento et al., 2003). They are central regulators of the microfilament cytoskeleton, phosphorylating both MLC2 and MYPT. ROCK I and II are highly homologous in all domains except for their PH domains and phosphorylate the same substrates. Both are inhibited by Y-27632 (Narumiya et al., 2000), a compound that depletes microfilament bundles and focal adhesions in a range of cell types. However, the specificity of ROCK I and II in the regulation of cytoskeletal organization has not been previously recognized. This may be largely due to the fact that many experiments on ROCKs have used overexpression, either of wild-type, kinase dead, or constituent domains. Here, levels of one kinase have been reduced, whereas levels of the other remained unaltered. A key element of the current study is that the presence of GTP-RhoA and rho kinase does not directly correlate with kinase activity. The data is consistent with a further regulatory step involving PH domain interactions. ROCK I-depleted cells, for example, fail to assemble stress fibers and focal adhesions despite the continued availability of ROCK II and GTP-RhoA. The importance of ROCK PH domains is underscored by the observation that high transient expression of the ROCK I or II PH domains leads to disassembly of microfilament bundles. Others have similarly shown the dominant-negative properties of these domains (Chen et al., 2002). Previous work suggested PtdIns4,5P₂ as an activator of ROCK II (Feng et al., 1999), and it is possible that an unbound PH domain may engender kinase quiescence.

Several different experiments suggest that ROCK II may be regulated by PI-3 kinase activity. Moreover, purified recombinant ROCK II was partially activated by PtdIns3,4,5P₃ and PtdIns4,5P₂. Further work is required to fully understand these properties *in vivo*. For example, a recent paper suggests that ROCK PH domains may also bind myosin heavy chain directly (Kawabata et al., 2004). Once again, though, overexpression would likely mask specificity. The current experiments have revealed a major role for endogenous ROCK I in stress fiber and focal adhesion formation. ROCK II's role in these processes is dispensable, and it may even dampen this response because in its absence stress fiber formation is enhanced. This was confirmed by quantification of F-actin and vinculin. In contrast, phagocytosis of FN-coated beads, a process requiring

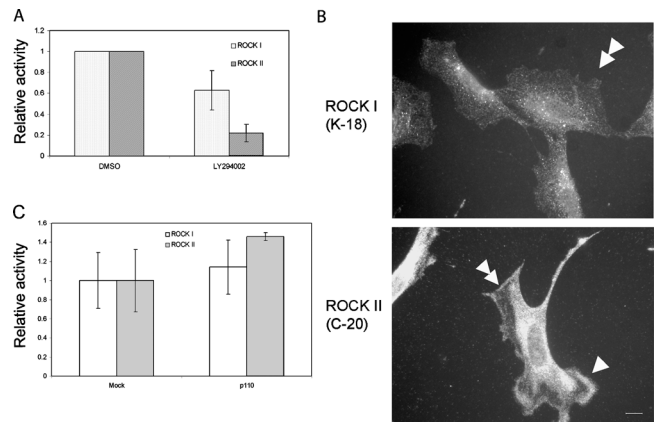


Figure 9. ROCK II activity is regulated by PI-3 kinase. (A) Serum-starved REF were trypsinized and incubated with 10 μ M LY294002 or DMSO for 30 min at 37°C. ROCK immunoprecipitates were incubated with MBP-MYPT1 COOH-terminal fragment and γ -[³²P]ATP. Background radiolabel levels from control immunoprecipitates have been deducted. ROCK activities from REF treated with DMSO was indicated as one. (B) REF was cotransfected with p110 α and pEGFP-N1. 1 d after transfection, cells were fixed and stained for ROCK I or II. Double arrowheads indicate transfected cells and the single arrowhead shows the localization of ROCK II in membrane ruffles. (C) ROCK immunoprecipitates from REF transfected with p110 α or control vector were incubated with GST-MLC and γ -[³²P]ATP, and the ROCK activities were measured. Activity of mock transfections is indicated as one. Bar, 10 μ m.

PI-3 kinase and myosin ATPase activities, is apparently dependent on ROCK II rather than ROCK I. It is interesting that they appear to regulate different aspects of cell-matrix interactions involving myosin II. PI-3 kinase activity has previously been implicated in phagocytosis and macropinocytosis (Cox et al., 2002; Araki et al., 2003; Swanson and Hoppe, 2004). Phagocytosis mediated by the complement receptor 3 pathway has been shown to require rho kinase activity and myosin II for the engulfment step (Olazabal et al., 2002). Phagocytosis may take several forms, but our evidence suggests that one ROCK isoform can regulate an integrin-mediated internalization of matrix-coated beads.

Although it may seem surprising that the two rho kinases have specific roles, our data is consistent with some recent findings. First, ROCK I is unable to compensate for the loss of ROCK II in knock-out mice. However, trophoblasts from such mice establish stress fibers in culture (Thumkeo et al., 2003). Second, RhoE can bind ROCK I, but not II, and inhibit its function, with a resultant loss of stress fibers (Riento et al., 2003, 2005). Last, in work reported since this study was completed, primary keratinocytes from ROCK I knock-out mice fail to establish stress fibers in response to epidermal growth factor (Shimizu et al., 2005), although ROCK II levels were unchanged. All these results are consistent with the hypothesis presented here that ROCK I has a major role in stress fiber formation and that the two rho kinases do not compensate for each other, except when overexpressed.

The two endogenous kinases also have distinct distributions in fibroblasts. ROCK I was diffusely, and widely distributed, sometimes associated with stress fibers, and more concentrated in perinuclear regions. This has been noted

previously (Riento et al., 2003). Cell membrane staining, particularly of ruffles was characteristic of ROCK II, but also accompanied by intense and more concentrated perinuclear distributions. These results are consistent with data showing a GTP-RhoA dependent membrane association of ROCK II (Sin et al., 1998).

Distinctions between ROCK I and II PH domains have not been studied previously. They have 65% identical sequences, but also have differences in proline-containing regions that may result in distinct tertiary structures. Chen et al. (2002) have suggested a regulatory role for ROCK II PH domain, based on injection of domain-specific antibodies into HeLa cells. It was noted, however, that such treatment had a mild effect on actin and focal adhesions. It is also interesting that neither PH domain binds LPA although a previous report showed that LPA, phosphatidylserine, and PI can activate ROCK II (Feng et al., 1999). Possibly, there are other binding sites in ROCK II for negatively charged lipids. One feature in common between ROCK I and II depletion was a lack of proliferation, yet little apoptosis. This and the appearance of binucleate cells are commensurate with previous papers suggesting that ROCK I is required for cytokinesis (Chevrier et al., 2002). However, a feature of ROCK II depletion was cellular hypertrophy. Moreover, it can be concluded that this was not due to ROCK I activity in these cells because hypertrophy was also seen in double-depleted cells. This recalls previous work showing that ROCK II can antagonize insulin signaling through an association with, and serine phosphorylation of, insulin receptor substrate-1 (Begum et al., 2002). Also, cells derived from p190-B Rho GTPase-activating protein-deficient mice had reduced size though insulin signaling was not inhibited (Sordella et al., 2002). Because ROCK activity was high in these cells, our results showing that deletion of ROCK II resulted in increased cell size are entirely consistent. Furthermore, our data also suggest that the intersection of ROCKs with insulin receptor signaling may be specific for ROCK II and not shared with ROCK I.

Materials and methods

Antibodies

Anti-Myosin (Light Chains 20K; MY-21), monoclonal anti-myc (9E10), and polyclonal α -actinin antibodies were purchased from Sigma-Aldrich. Polyclonal antibodies against ROCK I (K-18 and H-85), ROCK II (C-20 and H-85), RhoA (119), MYPT1 (E-19), pMYPT1 (Thr853), pMYPT1 (Thr696), and actin were obtained from Santa Cruz Biotechnology, Inc. Monoclonal anti-paxillin antibody (Z035) was purchased from Zymed Laboratories. Polyclonal antibodies against phospho-MLC2 (Thr18/Ser19) and phospho-MLC2 (Ser19) were purchased from Cell Signaling Technology.

Plasmids

pGEX-2T-rotectin RB and GST-PAK CRIB construct were gifts from A. Hall (University College London, London, UK), pCAG-myc-p160ROCK(WT) from S. Narumiya (Kyoto University, Kyoto, Japan), wild-type ROCK II (pEF-BOS-myc-Rho kinase (p164)) and pGEX-2T-MLC wild type from K. Kaibuchi (Nagoya University, Nagoya, Japan), and pcDNA3.1-p110 α from J. Domin (Imperial College London, London, UK). The pMAL-MYPT1 (aa 714–1004) construct was purchased from the University of Dundee (Velasco et al., 2002). Rat ROCK II cDNA was amplified by PCR from a REF cDNA library. Complementary DNAs coding for human ROCK I PH domain (aa 1113–1354) and rat ROCK II PH domain (aa 1145–1388) were amplified by PCR and subcloned into the EcoRI–BamHI site of pGEX-

3X (GE Healthcare) and the KpnI–SmaI or BamHI–EcoRI sites of pEGFP-N1 (BD Biosciences). Human GRP1-PH cDNA was obtained from A. Magee (Imperial College London, London, UK) and cDNA was subcloned into the BamHI–EcoRI site of pEGFP-N1. All constructs were confirmed by DNA sequencing.

Cell adhesion and pull-down assay

15-cm cell culture dishes (Corning Costar) were coated with 10 μ g/ml FN (Sigma-Aldrich) overnight at RT. Coated dishes were blocked with 1 mg/ml of heat-denatured BSA in PBS for 1 h at RT and washed three times with PBS. REF cells were maintained in α -MEM (Invitrogen) containing 5% FCS (Labtech International). 80% confluent REF cells were incubated overnight with serum-free α -MEM. REF were trypsinized, resuspended in α -MEM containing 0.25 mg/ml soybean trypsin inhibitor and 0.1 mg/ml BSA, and collected. After removal of trypsin inhibitor, REF were suspended in α -MEM containing 0.1 mg/ml BSA and kept in suspension for 30 min at 37°C. The cells were plated on FN-coated dishes and lysed at the various time points. Pull-down assays for GTP-loaded RhoA and Rac1 were performed according to Ren et al. (1999). The signals from Western blotting were analyzed by National Institutes of Health image version 1.61.

Rho kinase assay

REF cells were lysed in 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5% glycerol, 1% Triton X-100, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 25 mM NaF, 20 mM β -glycerophosphate, 1 mM Na₃VO₄, complete protease inhibitor cocktail (Roche), and 5 μ g/ml pepstatin (buffer A). The lysates were precleared by incubation with Ezview Red Protein G Affinity Gel (Sigma-Aldrich) for 15 min at 4°C. Each ROCK isoform was specifically immunoprecipitated from these lysates using goat polyclonal antibodies coupled to protein G beads for 30 min at 4°C. After washing with buffer A three times and with phosphorylation buffer (40 mM Tris-Cl, pH 7.4, 0.1% CHAPS, 10 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, phosphatase inhibitor cocktail [Sigma-Aldrich], 10 mM NaF, 10 mM β -glycerophosphate, 1 mM Na₃VO₄, EDTA free protease inhibitor cocktail [Roche], and 5 μ g/ml pepstatin) once, immunoprecipitates in the same buffer were incubated with 4 μ g GST-MLC or maltose binding protein (MBP)-MYPT1 (714–1004) and γ -[³²P]ATP (3 μ Ci, 0.11 TBq/mmol, 74 MBq/ml, 20 μ M final concentration; GE Healthcare) at 30°C for 10 min. The supernatants from the reactions were adsorbed onto P81 phosphocellulose (Whatman). The membranes were washed with 1% H₃PO₄ four times for 10 min, and membrane-bound radiolabel was measured by scintillation counting. In a preliminary series of experiments, rho kinase activity was measured in lysates that had been allowed to stand at 4°C for times up to 60 min. These experiments showed that activity persisted through the period of preclearing and immunoprecipitation. A slow decline in activity was noted so that the method adopted here was commensurate with the time required for specific purification of each kinase, with minimal loss of activity. In some experiments, trypsinized REF were suspended in α -MEM containing 0.1 mg/ml BSA and 10 μ M LY294002 (Merck Biosciences) or DMSO for 30 min at 37°C and then plated on FN-coated plates. In other cases, serum-starved REF in a 15-cm dish were stimulated with 400 ng/ml LPA (Sigma-Aldrich) for 5 min and then lysed for kinase analysis.

To examine the ability of phospholipid to activate rho kinases, recombinant myc-ROCK I and ROCK II were transiently expressed in COS7 cells. 1 d after transfection, cells were lysed in buffer A lacking Triton X-100 and lysates were cleared by centrifugation. Recombinant proteins were immunoprecipitated with anti-c-Myc-Agarose Affinity Gel (Sigma-Aldrich) for 1 h at 4°C, and eluted with 100 μ g/ml of c-Myc peptide (Sigma-Aldrich) in phosphorylation buffer containing 0.15 M or 0.5 M NaCl. Within 3 h of purification, kinase assays were performed as described in the previous paragraph with 3 ng of rho kinases, GST-MLC (4 μ g) in the presence of 1 μ M GTP γ S-GST-RhoA and/or 100 μ M phospholipid liposome, or 1 μ M Y-27632. Liposomes composed of PtdIns, PtdIns4,5P₂, or PtdIns3,4,5P₃ (BIOMOL Research Laboratories, Inc.) were prepared as described previously (Couchman et al., 2002).

To calculate the ratio of ROCK I and II protein levels in REF, Western blots and silver staining of immunoprecipitated proteins were quantitated. Two different sets of ROCK I- and II-specific antibodies were used, recognizing distinct domains of the kinases, with similar results. By calibrating the immunoprecipitates (using excess antibody) and relating the same antibodies to Western blots, the protein levels could be compared.

Transfection and immunofluorescence microscopy

REF (10⁴ cells) were cultured in 24-well plates on 13-mm-diam coverslips overnight and transfected with ROCK PH-GFP or GRP1-PH-GFP plasmids

using Oligofectamine Transfection Reagent (Invitrogen) as described by the manufacturer. 24 h after transfection, cells were fixed with 4% PFA in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Double fluorescence microscopy was performed using the described antibodies, followed by incubation with appropriate fluorochrome-conjugated secondary antibodies and phalloidin (Alexa Fluor 488/568/647; Molecular Probes). For localization of ROCK I and II proteins, cells were fixed with methanol/acetone (50:50) at -20°C for 5 min. Controls for nonspecific cross-reacting of secondary antibodies were included and gave no staining above background. Samples were analyzed on a Provis AX module fluorescence microscope (Olympus; objectives: UPlanApo 40 \times 1.0 oil iris, UPlanApo 60 \times 1.4 oil; images were collected using a SPOT Insight Mono digital camera) or on a confocal microscope (model TCS NT; Leica; objective Fluorotar 100 \times 1.4 oil PH3), and images were processed using Adobe Photoshop 7.0. 24 h after transfection, 30 μM LY294002 or DMSO was added into transfectants in growth medium for 1 h. Cells were fixed and stained for F-actin. In some experiments, 10 μM Y-27632 (Merck Biosciences) was added to fibroblast culture seeded on FN-coated plates for 30 min before the indicated times, and then cells were fixed. Cells were stained for focal adhesion components, including paxillin and F-actin. COS7 cells (3×10^4 cells) in 12-well plates were cultured overnight in DME (Invitrogen) containing 10% FCS and transfected with ROCK PH-GFP plasmids using Lipofectamine Transfection Reagent (Invitrogen) as described by the manufacturer. At 24 h after transfection, cells were lysed with 1 \times Laemmli sample buffer and denatured. Samples were subjected to SDS-PAGE and Western blotting with ROCK antibodies.

Silencing of ROCKs

Sense and antisense siRNA oligos corresponding to the target sequence for rat ROCK I (AATCGGCAGAGGTGCATTGG), for rat ROCK II (AACGTG-GAAAGCCTGCTGGAT), and for luciferase as negative control (AACGTACGCGGAATACTTCGA) were synthesized by Ambion and annealed. Uniqueness of individual target sequences was confirmed by a BLAST search of rat and mouse databases. Duplex RNAs were labeled by Cy3 or FAM used by Silencer siRNA Labeling Kit (Ambion). Transfection of siRNA (200 nM) was performed using Oligofectamine Reagent. Effective gene silencing was observed at the 50-nM level. 24–72 h after transfection, cells were analyzed. Cotransfection of Cy3-labeled siRNA duplexes with myc full-length ROCK I or II plasmid were performed in control experiments and cells were stained for myc-epitope and F-actin. Sense and anti-sense DNA encoding a short hairpin RNA corresponding to the target sequence for rat ROCK II, AAGTCGTGCTGCACTGGATG, was cloned into the site of BamHI and HindIII of pRNA-U6.1/Hygro vector (GenScript Corp.). REF cells transfected stably with this construct or empty vector were selected in growth medium containing hygromycin B (50 $\mu\text{g}/\text{ml}$) for 2 wk. Single clones were isolated and grown for cytoskeletal analysis.

Cell proliferation, cell cycle, and diameter analysis

REF cells were trypsinized at the indicated days after transfection with siRNA duplex and were analyzed as described in the following sentences. For cell proliferation assay, cell numbers were counted with a hemocytometer. Cell cycle characteristics were analyzed with FACS (BD FACSCalibur; Cell Quest software) after staining with 25 $\mu\text{g}/\text{ml}$ propidium iodide in PBS containing 0.1% Triton X-100 and 0.5 mg/ml RNase A. To analyze cell diameter, trypsinized cells were suspended in growth medium and plated onto plastic dishes. At 2 min after plating, cells were fixed with 4% PFA and images were taken. Diameters of cells were measured with Adobe Photoshop and cell volumes were calculated.

Protein lipid overlay assay

Human ROCK I PH domain and rat ROCK II PH domain were expressed in *Escherichia coli* BL21 as GST fusion proteins and purified using glutathione-agarose (Sigma-Aldrich) as described in Dowler et al. (2002) but in the absence of detergent. Protein lipid overlay assay was performed on PIP Strips (Echelon Biosciences) according to the methods of Dowler et al. (2002). Binding was visualized with anti-ROCK I and II antibodies that had been characterized and shown to react with the respective PH domains. α -Actinin from chicken gizzard (Sigma-Aldrich) was used as a positive control.

Phagocytosis assays

Sulfate-modified latex red beads (average bead size 1.0 μm ; Sigma-Aldrich) in 1 ml of a solution containing of 35 mM NaHCO_3 and 15 mM Na_2CO_3 , pH 9.6, were coated with 30 $\mu\text{g}/\text{ml}$ of bovine FN (Sigma-Aldrich) overnight at RT. After washing with PBS twice, coated beads were blocked with 1% BSA in PBS for 2 h at RT. After further washing, FN-

coated beads were suspended in 1 ml PBS. Control beads were coated with BSA alone. Fibroblast cultures were untreated or exposed to one of 30 μM LY294002, 15 μM (–)-blebbistatin (Merck Biosciences), 30 μM Y-27632, or DMSO as reference for LY294002 and blebbistatin in α -MEM containing 5% FCS for 2 h (LY294002 and Y-27632 treatments) or 0.5 h (blebbistatin treatment). Control or siRNA-transfected cells were incubated at 4°C for 10 min and then $\sim 9 \times 10^9$ FN-coated beads were added. Cells were further incubated at 4°C for 30 min to allow beads to interact with the cell surface, and then incubated at 37°C for 1 h before fixation with 4% PFA. Fixed cells were stained with anti-FN polyclonal antibody followed by Alexa488 goat anti-rabbit IgG to detect FN-coated beads remaining extracellularly. Three-dimensional fluorescence images of FN (green) and latex beads (red) were recorded from laser scanning confocal microscopy. Numbers of double-stained and total numbers of latex beads were counted and percentages of phagocytosed beads were compared by statistical analysis.

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