P21-activated kinase 1: convergence point in PDGF- and LPA-stimulated collagen matrix contraction by human fibroblasts

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Fibroblast three-dimensional collagen matrix culture provides a tissue-like model that can be used to analyze cell form and function. The physiological agonists platelet-derived growth factor (PDGF) and lysophosphatidic acid (LPA) both stimulate human fibroblasts to contract floating collagen matrices. In this study, we show that the PDGF and LPA signaling pathways required for matrix contraction converge on p21-activated kinase 1 (PAK1) and its downstream effector cofilin1 and that contraction depends on cellular ruffling activity, rather than on the protrusion and retraction of cellular dendritic extensions. We also show that, depending on the agonist, different Rho effectors cooperate with PAK1 to regulate matrix contraction, Rho kinase in the case of PDGF and mDia1 in the case of LPA. These findings establish a unified framework for understanding the cell signaling pathways involved in fibroblast contraction of floating collagen matrices.

Introduction

Fibroblasts synthesize, organize, and maintain connective tissues during development and in response to injury and fibrotic disease (Trinkaus, 1984; Tomasek et al., 2002; Desmouliere et al., 2004). Cells cultured in three-dimensional (3D) collagen matrices have been used to study fibroblast–matrix interactions in a tissue-like environment. Fibroblast morphology in the 3D environment ranges from dendritic to stellate to bipolar, depending on matrix stiffness and tension (Cukierman et al., 2002; Grinnell, 2003), which is similar to cells in tissues (Goldsmith et al., 2004; Langevin et al., 2005) and quite distinct from the flattened morphology of fibroblasts on two-dimensional (2D) tissue culture surfaces.

Cells can exert mechanical force on their surroundings (Bershadsky et al., 2003; Ingber, 2003; Katsumi et al., 2004; Meshel et al., 2005), and fibroblasts in 3D collagen matrices use this force to contract the matrix (Brown et al., 1998; Tomasek et al., 2002; Grinnell, 2003; Petroll and Ma, 2003; Vanni et al., 2003; Wakatsuki and Elson, 2003). The mechanism by which fibroblasts regulate the contraction of 3D collagen matrices has been shown to vary according to growth factor stimulus, mechanical environment, and the differentiation state of the cells.

The physiological agonists PDGF and lysophosphatidic acid (LPA) both stimulate floating matrix contraction, even though these agonists have opposite effects on the movement of cellular dendritic extensions within the matrices. PDGF increases their protrusion; LPA causes their retraction (Grinnell et al., 2003).

Studies with C3 exotransferase showed that the small G protein Rho is required for floating matrix contraction by either PDGF or LPA (Grinnell et al., 1999), but only PDGF-stimulated, and not LPA-stimulated, contraction was inhibited by blocking the Rho effector Rho kinase (Abe et al., 2003; Lee et al., 2003). Therefore, PDGF and LPA regulate floating collagen matrix contraction, in part, by different signaling mechanisms, and it has remained an open question as to whether there is a point of convergence.

p21-activated kinases (PAKs) were first identified as Rac- and Cdc42-interacting proteins (Manser et al., 1994) and are now known to be important in the regulation of cytoskeletal organization and cell migration (Bokoch, 2003; Zhao and Manser, 2005). Early on, PAK1 was recognized as a downstream effector for PDGF (Bokoch et al., 1996; Dharmawardhane et al., 1997), but more recently was shown to also be important for LPA-mediated signaling (Menard and Mattingly, 2003; Jung et al., 2004). In this study, we show that the PDGF and LPA signaling pathways that regulate matrix contraction converge on PAK1 and its downstream effector cofilin and that contraction depends on cellular ruffling activity, rather than on

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Abbreviations used in this paper: 2D, two-dimensional; 3D, three-dimensional; ADF, actin-depolymerizing factor; LPA, lysophosphatidic acid; PAK1, p21-activated kinase; PI3, phosphoinositide 3; SH3, Src homology domain; siRNA, small interfering RNA.

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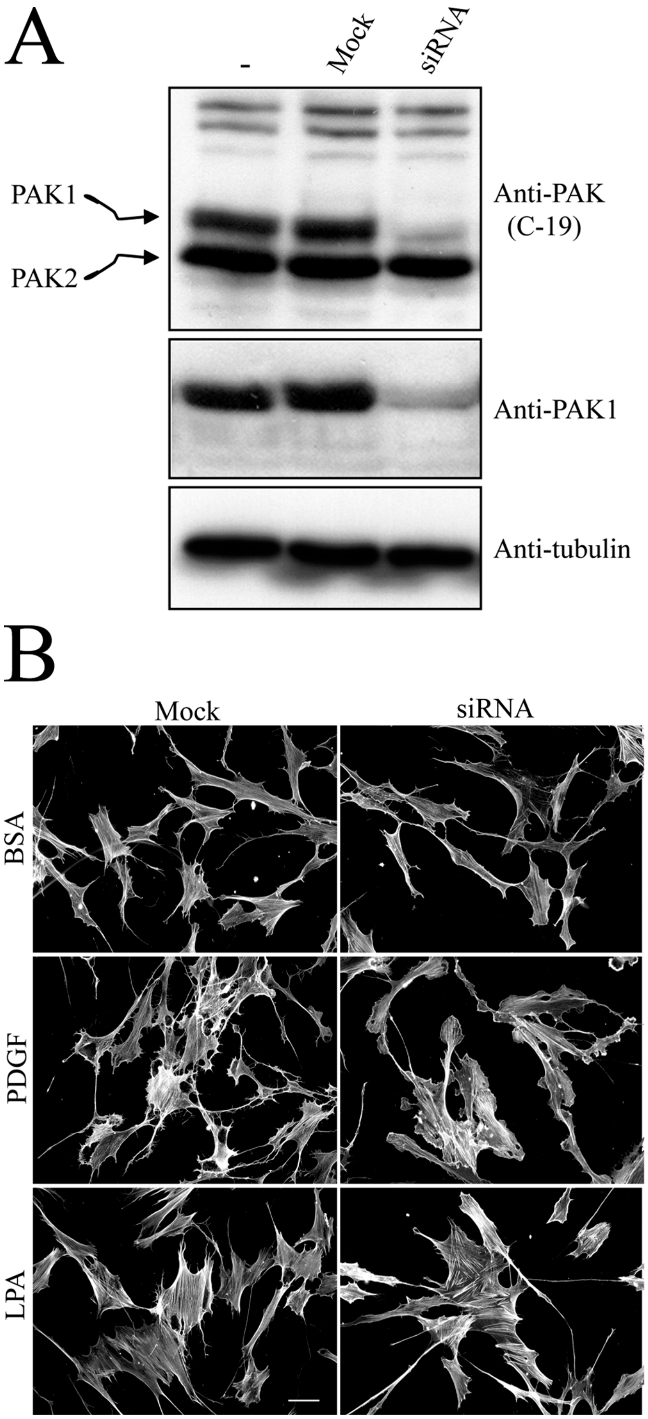

Figure 1. **PAK1 silencing in human fibroblasts and cell morphology.** (A) Cells were transfected for 12 h with 700 nM siRNA or sense RNA only (Mock) and cultured for an additional 24 h in growth medium without siRNA. Extracts were prepared and subjected to SDS-PAGE and immunoblotted to analyze levels of PAK1, PAK2, and tubulin. (B) PAK1-silenced and mock-transfected cells were harvested and incubated for 1 h on collagencoated glass coverslips in DME containing 5 mg/ml BSA and 10 µM LPA or 50 ng/ml PDGF as indicated. At the end of the incubation, samples were fixed and stained for actin. Bar, 50 µm.

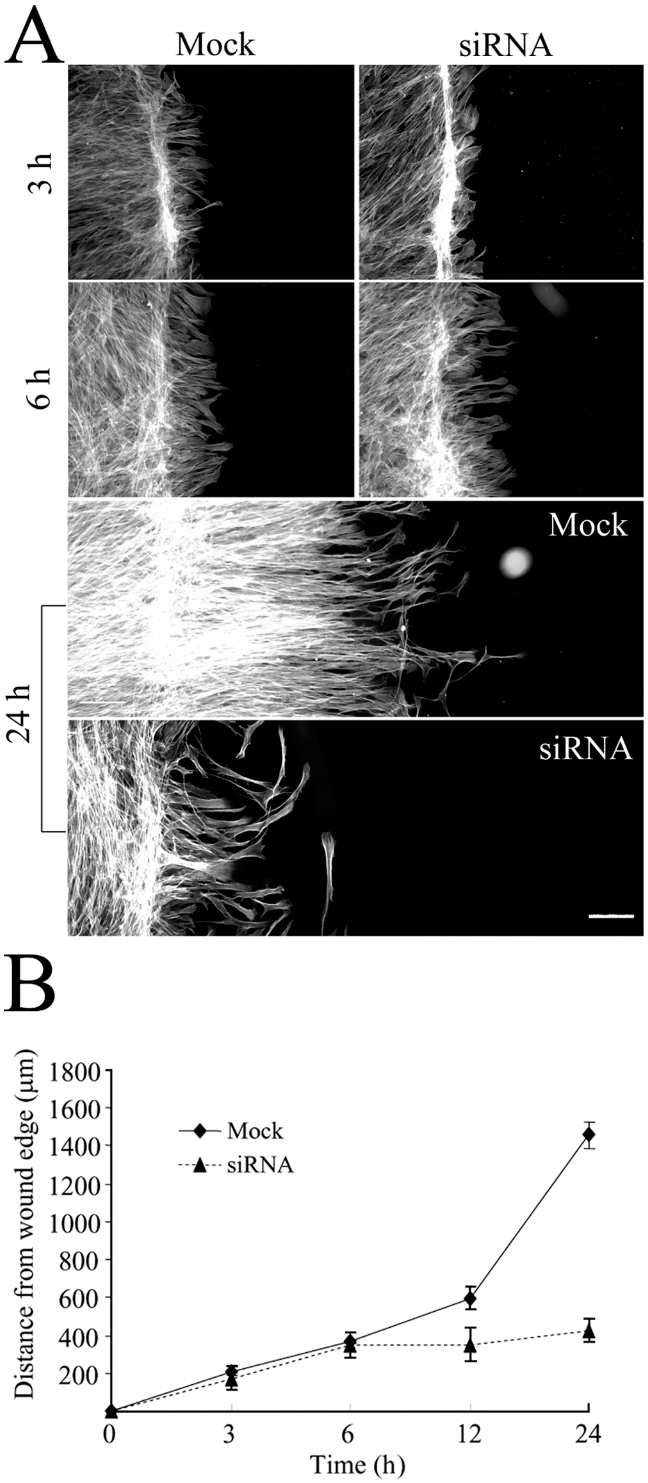

Figure 2. **Silencing PAK1 inhibits cell migration.** (A) PAK1-silenced and mock-transfected cells were harvested and cultured overnight on collagen-coated coverslips. After scrape wounding, the cultures were incubated in DME containing 5 mg/ml BSA and 50 ng/ml PDGF. At the end of the incubations, samples were fixed and stained for actin. (B) Migration was quantified by determining the average distance of cell migration from the wound edge based on measurement of 10 separate microscopic fields and 5 cells within each field. Bar, 150 µm.

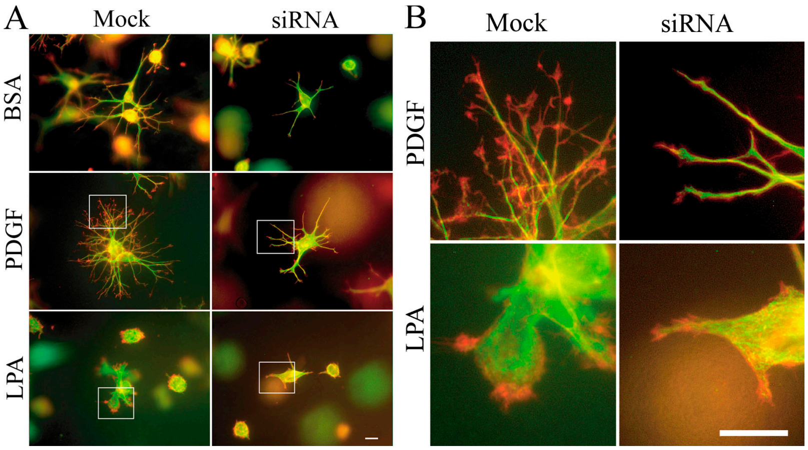

Figure 3. **Dendritic extensions and ruffling by PAK1-silenced human fibroblasts incubated for 1 h in collagen matrices.** (A) PAK1-silenced and mock-transfected cells were harvested and used to prepare floating collagen matrices. Samples were incubated for 1 h in DME containing 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA, as indicated. At the end of the incubations, samples were fixed and stained with anti-tubulin antibody for microtubules and rhodamine-phalloidin for actin. (B) Enlarged view of dendritic extension tips in the boxed areas. Green, microtubules; red, actin. Bar, 20 μm.

protrusion and retraction of cellular dendritic extensions. We also show that, depending on the agonist, different Rho effectors are required to cooperate with PAK1 to regulate matrix contraction, Rho kinase in the case of PDGF and mDia1 in the case of LPA.

Results

Effects of PAK1 silencing on fibroblast morphology and of migration on collagen-coated coverslips

We used small interfering RNA (siRNA) to knock down PAK1 expression in human fibroblasts. Fig. 1 A shows an example of immunoblot analysis performed on cell lysates prepared from cells after a 36-h transfection with PAK1-specific double-stranded siRNA. Levels of PAK1 in the PAK1 siRNA, but not in mock-transfected cells, were reduced by almost 95% without affecting levels of PAK2.

Fig. 1 B shows the morphology of PAK1-silenced versus mock-transfected cells by fluorescence visualization of actin. Compared with control cells, knocking down PAK1 had no detectable effect on cell spreading or response to PDGF and LPA in 2D culture. Treatment with PDGF caused the appearance of small lamellipodia along the cell margins, and treatment with LPA increased formation of actin stress fibers.

Transfection studies with modified PAK1 constructs have demonstrated a role for PAK1 in cell motility (Sells et al., 1999). Consistent with this finding, human fibroblast migration was decreased by knocking down PAK1. Fig. 2 A shows the typical appearance of cultures that were scrape wounded and incubated in medium containing PDGF. During the initial 3–6 h of culture, both mock-transfected and PAK1 knockdown cells extended lamellipodia into the scrape region. By 24 h of incubation, however, PAK1 knockdown cells had migrated substantially further into the wound region compared with the controls. Fig. 2 B shows the results quantitatively with the difference in cell migration evident by 12 h.

PAK1 silencing inhibits cell ruffling and matrix contraction by fibroblasts in 3D collagen matrices

Fibroblasts within 3D collagen matrices protrude a dendritic network of extensions that expands in response to PDGF stimulation and retracts in response to LPA stimulation (Abe et al., 2003). Fig. 3 (1 h) and Fig. 4 (4 h) show representative photomicrographs of the network under basal (BSA), expanded (PDGF), and retracted (LPA) conditions. Fibroblast dendritic extensions have microtubule cores (Figs. 3 and 4, green) with ruffling, actin-rich tips (red). Retraction of the extensions in response to LPA stimulation occurred within 1 h in PAK1-silenced

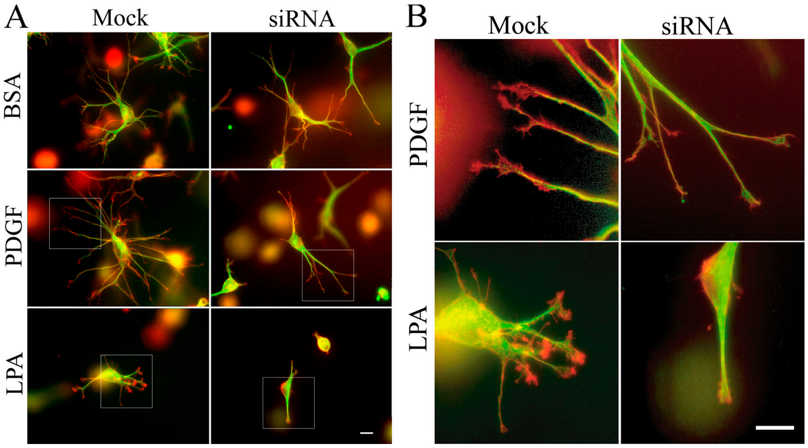

Figure 4. **Dendritic extensions and ruffling by PAK1-silenced fibroblasts incubated for 4 h in collagen matrices.** (A) PAK1-silenced and mock-transfected cells were harvested and used to prepare floating collagen matrices. Samples were incubated for 4 h in DME containing 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA, as indicated. At the end of the incubations, samples were fixed and stained with anti-tubulin antibody for microtubules and rhodamine-phalloidin for actin. (B) Enlarged view of dendritic extension tips in the boxed areas. Green, microtubules; red, actin. Bar, 20 μm.

Table I. **Morphometric analysis of cell extensions in 3D collagen matrices**

Cell sample	Growth factor	Projected cell area	Branch length	Number of branches
		μm^2	*μm*	
Mock	none	1,583 ± 422	57 ± 20	9.8 ± 3.3
siRNA	none	941 ± 359	45 ± 19	6.3 ± 2.5
	P value	0.048	0.043	0.14
Mock	PDGF	2,547 ± 773	74 ± 23	12.7 ± 3.4
siRNA	PDGF	1,006 ± 279	44 ± 15	8.0 ± 2.5
	P value	0.003	0.0003	0.03

PAK1-silenced and mock-transfected cells were harvested and used to prepare floating collagen matrices. Samples were incubated for 4 h in DME containing 5 mg/ml BSA and 50 ng/ml PDGF, as indicated. At the end of the incubations, samples were fixed and stained with rhodamine-phalloidin for actin. For each value, measurements were made on 50 cells that were photographed at random.

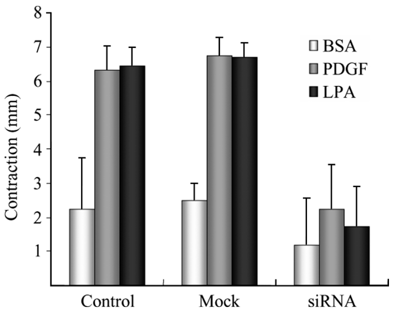

Figure 5. **Inhibition of collagen matrix contraction in PAK1-silenced cells.** Nontransfected (Control), PAK1-silenced, and mock-transfected cells were harvested and used to prepare floating collagen matrices. Samples were incubated for 4 h in DME with 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA added as shown. At the end of the incubations samples were fixed and the extent of matrix contraction was measured as the decrease in matrix diameter. Data shown are arithmetic mean ± SD for three separate experiments.

or mock-transfected cells, after which most fibroblasts either were round or had short extensions. Expansion of the dendritic network in response to PDGF was reduced in PAK1 knockdown cells. Table I shows morphometric analysis of a representative experiment. After 4 h in basal- and PDGF-containing medium, the projected cell area, branch length, and number of branches were all lower in PAK1-silenced cells. Therefore, PAK1 played a role in regulation of fibroblast dendritic extensions, but formation of the extensions was PAK1 independent.

The actin-rich tips of fibroblast extensions (protruded or retracted) showed prominent ruffling activity. In general, ruffling was more evident in PDGF- and LPA-stimulated cells than in basal (BSA) medium, and Figs. 3 B and 4 B show that cell ruffling induced by PDGF or LPA was markedly reduced in PAK1-silenced cells, compared with mock-transfected cells. Therefore, PAK1 appeared to be necessary for cell ruffling, regardless of whether the cells were stimulated by PDGF or LPA.

Collagen matrix contraction experiments also were performed using PAK1-silenced and mock-transfected cells. Contraction was quantified by measuring the diameter of collagen matrices before (∼12 mm) and after contraction and then calculating the difference. Fig. 5 shows that after 4 h in the presence of LPA or PDGF, contraction was ∼6–7 mm for control cells and ∼2 mm for PAK1 knockdown cells. The latter value is comparable to the level of basal contraction without agonist stimulation. These findings demonstrated that PAK1 was required for both PDGF- and LPA-stimulated matrix contraction.

Inhibiting phosphoinositide 3 (PI3) kinase selectively blocks PDGF-stimulated collagen matrix contraction and cell ruffling

Current studies suggested PAK1 as a potential point of convergence in PDGF and LPA regulation of cell ruffling and collagen matrix contraction. Activation of cell ruffling and PAK in response to PDGF stimulation depends on PI3 kinase (Hooshmand-Rad et al., 1997; Sells et al., 2000). In previous papers, the role of PI3 kinase in contraction of floating collagen matrices was tested, but the findings were inconsistent (Ahlen et al., 1998; Skuta et al., 1999; Han et al., 2002). In preliminary experiments, we established that 20 μM LY294002 inhibited PDGF-stimulated PI3 kinase activity based on measurement of Akt phosphorylation (unpublished data). Fig. 6 A demonstrates that this concentration of the PI3 kinase inhibitor (LY) blocked PDGF-stimulated, but not LPA-stimulated, matrix contraction. In addition, Fig. 6 B shows that blocking PI3 kinase inhibited PDGF-stimulated, but not LPA-stimulated, cell ruffling. Therefore, PI3 kinase was required for both cell ruffling and contraction stimulated by PDGF, whereas the link between LPA and PAK1 appeared to be PI3 kinase independent, as has been reported (Menard and Mattingly, 2003).

Pertussis toxin treatment selectively blocks LPA-stimulated matrix contraction and cell ruffling

LPA receptors couple to multiple G protein signaling pathways (Anliker and Chun, 2004; Moolenaar et al., 2004), and Gα_i has been implicated in floating collagen matrix contraction (Skuta et al., 1999). Fig. 7 A shows that overnight treatment with pertussis toxin inhibited LPA-stimulated, but not PDGF-stimulated, matrix contraction. In addition, Fig. 7 B shows that pertussis toxin treatment inhibited cell ruffling stimulated by LPA. These findings provided evidence for a link between PAK1-dependent cell ruffling and contraction stimulated by LPA, which was distinct from PDGF.

Downstream role of cofilin1 in PAK1-dependent collagen matrix contraction

The actin dynamics required for fibroblast ruffling can be controlled at the level of actin-depolymerizing factor (ADF)/cofilin by the PAK1 effector LIM kinase (Arber et al., 1998; Yang et al., 1998; Bamburg, 1999; Edwards et al., 1999). Fig. 8 A shows that with fibroblasts in 3D collagen matrices, PDGF and LPA stimulated cofilin1 phosphorylation and that stimulation was inhibited in PAK1-silenced cells. Contraction experiments were performed after knocking down cofilin1 using siRNA, which, as shown in Fig. 8 B, could be reduced by >70%. Fig. 8 C demonstrates that in cofilin1-silenced cells, collagen matrix contraction was inhibited. Also, cells ceased their ruffling

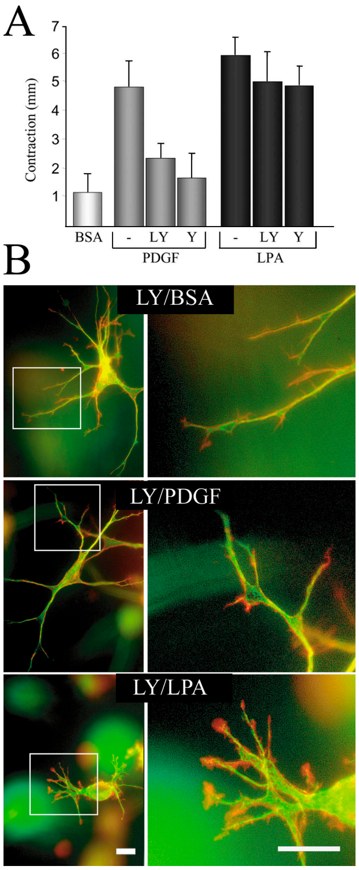

Figure 6. **PI3 kinase and Rho kinase are required for PDGF-stimulated, but not LPA-stimulated, collagen matrix contraction.** (A) Fibroblasts were harvested and used to prepare floating collagen matrices. Samples were incubated for 4 h in DME with 5 mg/ml BSA and 50 ng/ml PDGF, 10 μM LPA, 20 μM LY294002 (LY; PI3 kinase inhibitor), and 10 μM Y27634 (Y; Rho kinase inhibitor) added where indicated. At the end the incubations, samples were fixed and the extent of matrix contraction was measured as the decrease in matrix diameter. Data shown are arithmetic means ± SD for three separate experiments. (B) Selected samples from A were fixed and stained with anti-tubulin antibody for microtubules (green) and rhodaminephalloidin for actin (red). Enlarged view of dendritic extension tips in the boxed areas. Bar, 20 μm.

activity (unpublished data). Therefore, it could be concluded that cofilin1 was a downstream effector for PAK1 in LPA- and PDGF-stimulated collagen matrix contraction.

Different Rho effectors cooperate with PAK1 to regulate LPA- and PDGF-stimulated matrix contraction

Together, the experiments identified PAK1 as a downstream convergence point for the regulation of both cell ruffling and collagen matrix contraction stimulated by PDGF and LPA. As already mentioned, PDGF- and LPA-dependent floating collagen matrix contraction requires activity of the small G protein Rho (Grinnell et al., 1999), but only PDGF-stimulated contraction was dependent on the Rho effector Rho kinase (Abe et al., 2003; Lee et al., 2003). Consistent with this observation, Fig. 6 A shows that blocking Rho kinase (Y) inhibited PDGF-dependent, but not LPA-dependent, contraction of floating collagen matrices. Moreover, blocking Rho kinase did not cause a decrease in cell ruffling (unpublished data). Therefore, rather than functioning in the same signaling pathway as PAK1, it seemed likely that PAK1 and Rho acted in parallel cooperative fashion.

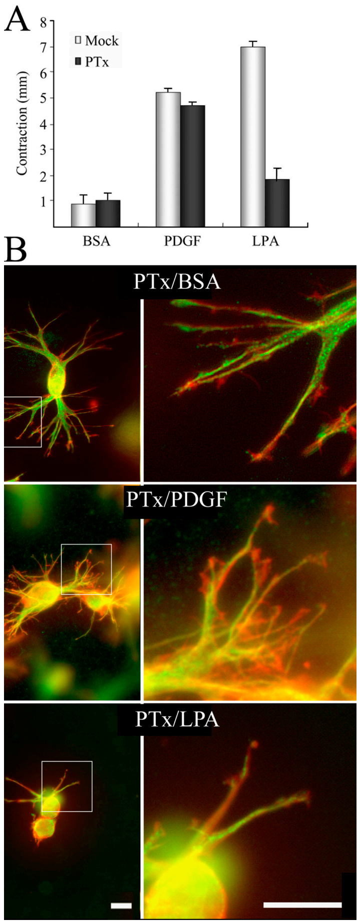

Figure 7. **Pertussis toxin inhibits LPA-stimulated, but not PDGF-stimulated, collagen matrix contraction.** (A) Fibroblasts in monolayer culture were incubated overnight with 25 ng/ml pertussis toxin. Subsequently, the cells were harvested and used to prepare floating collagen matrices. Samples were incubated for 4 h in DME with 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA added where indicated. At the end the incubations, samples were fixed and the extent of matrix contraction was measured as the decrease in matrix diameter. Data shown are arithmetic means ± SD for three separate experiments. (B) Selected samples from A were fixed and stained with anti-tubulin antibody for microtubules (green) and rhodamine-phalloidin for actin (red). (right) Enlarged view of dendritic extension tips in the boxed areas. Bars, 20 μm.

Along with Rho kinase, mDia1 has been implicated as a Rho effector for regulation of actin cytoskeletal dynamics and force generation (Watanabe et al., 1999; Geiger and Bershadsky, 2001). Therefore, we tested the possibility that mDia1 might be the Rho effector required for LPA-stimulated matrix contraction. This was accomplished by knocking down mDia1expression.

Fig. 9 A shows that levels of mDia1 in mDia1-specific siRNA, but not in mock-transfected cells, were reduced by >95%. mDia1-silenced cells were able to spread on collagen-coated coverslips and form vinculin-containing focal adhesions, although the cells were rounder and had reduced actin stress fibers compared with controls (unpublished data), as has been reported (Watanabe and Higashida, 2004). Fig. 9 B shows the

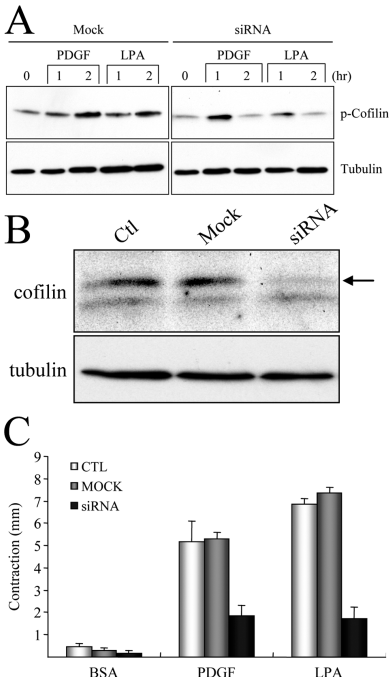

Figure 8. **Cofilin1 is downstream of PAK1 in LPA and PDGF regulation of collagen matrix contraction.** (A) PAK1-silenced and mock-transfected cells were harvested and used to prepare floating collagen matrices. Samples were incubated for the times shown in DME containing 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA, as indicated. At the end of the incubations, extracts of the samples were prepared and subjected to immunoblotting with antibodies directed against phospho-cofilin1 and tubulin. (B) Cells were transfected for 36 h with 500 nM of cofilin1 siRNA or sense RNA only (Mock) and then cultured an additional 24 h in growth medium without siRNA. Extracts were prepared and subjected to SDS-PAGE and immunoblotted to analyze levels of cofilin1 (arrow) and tubulin. (C) Nontransfected (CTL), cofilin1-silenced, and mock-transfected cells were harvested and used to prepare floating collagen matrices. Samples were incubated for 4 h in DME with 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA added as shown. At the end of the incuba-tions, samples were fixed and the extent of matrix contraction was meas-ured as the decrease in matrix diameter. Data shown are arithmetic means ± SD for three separate experiments.

results of collagen matrix contraction studies with mDia1-silenced and mock-transfected cells. Silencing mDia1 selectively inhibited LPA-stimulated contraction without affecting PDGF-stimulated contraction. However, knocking down mDia1 had no effect on cell ruffling (unpublished data). Therefore, different Rho effectors cooperated with PAK1 in fibroblast contraction of floating collagen matrices depending on the agonist used to stimulate contraction, which was Rho kinase in the case of PDGF and mDia1 in the case of LPA.

Control experiments were also performed to confirm activation of mDia1 by LPA. This was accomplished by taking advantage of the observation that LPA-stimulated formation of stable (nocodazole-resistant) microtubules depends on mDia1 (Cook et al., 1998; Palazzo et al., 2001). Serum-starved mDia1-silenced and mock-transfected cells were agonist stimulated and tested for the development of nocodazole- resistant microtubules (Gundersen et al., 1994; Cook et al., 1998). Fig. 9 C shows that after agonist stimulation by PDGF or LPA, a subpopulation of microtubules in mock-transfected cells became nocodazole resistant, but that in mDia1-silenced cells LPA was no longer able to stimulate formation of stable microtubules.

Discussion

Fig. 10 summarizes the results of our studies. We found that PDGF and LPA regulate floating collagen matrix contraction through signaling pathways that converge on PAK1 and its downstream effector, cofilin, and that contraction depends on cellular ruffling activity. Moreover, different Rho effectors were observed to cooperate with PAK1 in regulating contraction, Rho kinase in the case of PDGF and mDia1 in the case of LPA.

Previous work (for review see Grinnell, 2003) had demonstrated that the physiological agonists LPA and PDGF stimulated fibroblast contraction of floating collagen matrices, but the relationship between the signaling mechanisms involved had not been elucidated. These agonists have opposite effects on the overall movement of fibroblast dendritic extensions: retraction in response to LPA and protrusion in response to PDGF (Grinnell et al., 2003). Moreover, blocking Rho kinase or myosin II activity was shown to inhibit PDGF-dependent, but not LPA-dependent, matrix contraction (Abe et al., 2003; Lee et al., 2003).

PAK1 is a downstream effector for both PDGF- (Bokoch et al., 1996; Dharmawardhane et al., 1997) and LPA-mediated signaling (Menard and Mattingly, 2003; Jung et al., 2004). Therefore, we analyzed the possible role of PAK1 in matrix contraction, using siRNA to silence PAK1 expression, and learned that knocking down PAK1 resulted in inhibition of contraction stimulated by either agonist.

Microinjection and expression studies had implicated PAK1 in ruffling and motility of cells on 2D surfaces (Dharmawardhane et al., 1997; Sells et al., 1997, 1999, 2000). Although PDGF and LPA have opposite effects on the overall movement of fibroblast dendritic extensions, both agonists were found to stimulate membrane ruffling activity. However, ruffling by cells in 3D matrices was inhibited in PAK1-silenced cells. In addition, blocking PI3 kinase selectively inhibited PDGF-stimulated cell ruffling and matrix contraction, whereas blocking Gα_i selectively inhibited LPA-stimulated ruffling and contraction.

As previously stated, PAK1-regulation of the actin dynamics required for fibroblast ruffling can be controlled at the level of ADF/cofilin phosphorylation by the PAK1 effector LIM kinase (Arber et al., 1998; Yang et al., 1998; Bamburg, 1999; Edwards et al., 1999). We found that LPA and PDGF both stimulated cofilin1 phosphorylation, and that stimulation was blocked in PAK1-silenced cells. Moreover, silencing cofilin1 with siRNA blocked LPA- and PDGF-dependent matrix contraction, as well as membrane ruffling. Therefore, we suggest that matrix contraction requires cellular ruffling activity stimulated by PAK1 and cofilin1. Although LIM kinase is the likely intermediate between PAK1 and cofilin1, we have not succeeded in developing conditions using siRNA to effectively silence LIM kinase expression and directly test its role.

On collagen-coated coverslips, PAK1-silenced fibroblasts showed markedly decreased migration, but cells spread normally and increased formation of lamellipodia after PDGF stimulation. That PAK1-silenced cells formed normal lamellipodia in 2D culture, but lacked ruffles in 3D matrices, suggests

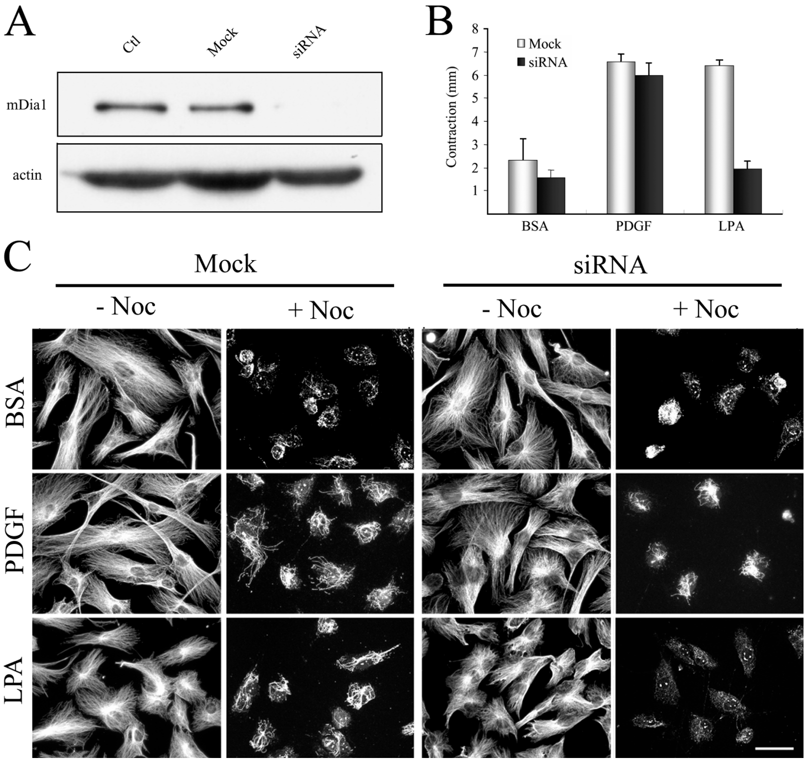

Figure 9. **mDia1 cooperates with PAK1 in LPA regulation of collagen matrix contraction silencing in human fibroblasts.** (A) Cells were transfected for 12 h with 700 nM siRNA or sense RNA only (Mock) and cultured an additional 24 h in growth medium without siRNA. Extracts were prepared and subjected to SDS-PAGE and immunoblotted to analyze levels of mDia1 and actin. (B) mDia1-silenced and mock-transfected cells were harvested and used to prepare floating collagen matrices. Samples were incubated for 4 h in DME with 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA added as shown. At the end of the incubations, samples were fixed, and the extent of matrix contraction was measured as the decrease in matrix diameter. Data shown are arithmetic means ± SD for three separate experiments. (C) At the end of the transfection period, mock- and siRNA-transfected cells were incubated in serum-free medium for 36 h, followed by 4 h in DME with 5 mg/ml BSA and 50 ng/ml PDGF or 10 μM LPA added as shown. Stable microtubules were detected as previously described (Gundersen et al., 1994; Cook et al., 1998). Subsequently, the indicated samples were treated with 2 μM nocodazole for 2 h . After two rinses with microtubule-stabilizing buffer (MSB; 85 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 2 M glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride), samples were treated with 1 ml of MSB containing 200 μg/ml saponin for 5 min at 37°C to extract tubulin monomer, rinsed with MSB, and fixed with methanol (−20°C) for 10 min, and then stained with anti-tubulin antibody. Bar, 50 μm.

that lamellipodia and ruffles may be regulated independently; PAK-independent mechanisms of cell ruffling and lamellipodia formation have been previously described (Joneson et al., 1996; Westwick et al., 1997). Differences in fibroblast adhesion to 3D matrices versus 2D coverslips may also be important. Besides LIM kinase, regulation of ADF/cofilin and fibroblast ruffling can be controlled by integrin interactions and testicular protein kinase 1 (Toshima et al., 2001; LaLonde et al., 2005). Cells interacting with 3D matrices have fewer stress fibers, smaller focal adhesions, and decreased activation of focal adhesion kinase compared with cells in 2D culture (Cukierman et al., 2002; Grinnell, 2003; Wozniak et al., 2004). It is possible, therefore, that in 3D matrices fibroblasts become completely dependent on the PAK1 pathway, whereas human fibroblasts on 2D collagen-coated surfaces can regulate cell ruffling and lamellipodia formation by multiple mechanisms.

Also noteworthy is the discovery that the dendritic extensions of PAK1-silenced fibroblasts in 3D matrices showed decreased expansion in response to PDGF. This decrease may have resulted from an increase in microtubule catastrophe in the absence of PAK1 (Wittmann et al., 2003, 2004) because microtubules were shown to be required for formation of the fibroblast dendritic network (Grinnell et al., 2003).

Based on molecular architecture, PAKs can be categorized into two subgroups with three members each (Jaffer and Chernoff, 2002; Bokoch, 2003; Zhao and Manser, 2005). The group I PAKs, including PAK1, share a high degree of amino acid homology and influence diverse cellular processes, such as cellular morphology, migration, and gene regulation. Our results identify PAK1 as the major isoform involved in regulation of fibroblast ruffling in 3D collagen matrices and collagen matrix contraction, although expression of PAK2 is at least five times higher than PAK1 in human fibroblast based on Western blotting results.

Structurally, the major differences between PAK1 and -2 are in the NH_2-terminal regulatory domain in which PAK1 has five proline-rich Src homology 3 (SH3)–binding motifs, compared with two SH motifs in PAK2 (Bokoch, 2003). It has been reported that phosphorylation of the threonine 212 residue in one of PAK1's unique SH3 motifs is important for regulation of neuronal growth cone dynamics (Rashid et al., 2001). Whether phosphorylation at this site is also important in regulation of fibroblast contraction of collagen matrices has yet to be determined.

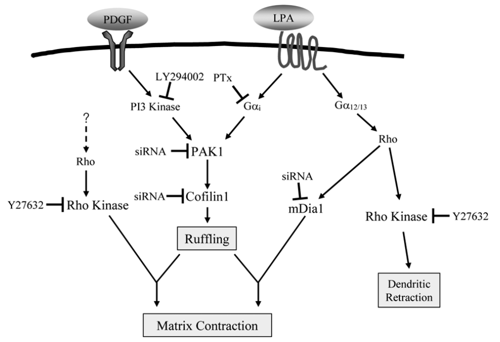

Figure 10. **Signaling pathways in floating collagen matrix contraction.** Model showing convergence of PDGF and LPA signaling on PAK1 and cofilin1, cell ruffling, and collagen matrix contraction. Rho kinase cooperates with PAK1 for PDGF-stimulated contraction, whereas mDia1 cooperates with PAK1 for LPA-stimulated contraction. Rho kinase also is required for LPA-stimulated retraction of dendritic extensions.

Although Fig. 10 shows PDGF and LPA converging separately on PAK1, Rac is likely to be immediately upstream of PAK1. Preliminary studies showed that dominant-negative expression of N17Rac1, but not of N17Cdc42, completely abolished the formation of dendritic extensions (unpublished data), which was a phenotype more pronounced than observed by knocking down PAK1. Given the potential for indirect effects by overexpression of dominant-negative Ras family mutants (Feig, 1999), coupled with the difficulty of transfecting genes into early passage human fibroblasts, our initial results cannot yet be clearly interpreted.

During collagen matrix contraction, individual collagen fibrils are translocated toward the cell surface (Grinnell and Lamke, 1984; Yamato et al., 1995; Tamariz and Grinnell, 2002). Such translocation of collagen fibrils, when it involves fibroblasts on 2D coverslips, requires a mechanism of contractile force generation (Meshel et al., 2005). In the case of cell migration, PAK1-dependent cell ruffling is usually thought to be important for cells to reach forward (Jaffer and Chernoff, 2002; Bokoch, 2003), whereas the small G protein Rho has been implicated in generation of contractile force required for cell translocation (Webb et al., 2002; Ridley et al., 2003).

This study builds on our previous finding that Rho was required for floating matrix contraction (Grinnell et al., 1999) to show that different Rho effectors are involved in contraction dependent on the agonist (i.e., Rho kinase in the case of PDGF and mDia1 in the case of LPA). Because blocking Rho kinase or silencing mDia1 using siRNA inhibited contraction selectively, and neither treatment blocked cell ruffling, we propose that the Rho effectors act in parallel to and cooperatively with the PAK1 signaling pathway and play a role in the force generation required for matrix contraction.

At this time we can only speculate as to why LPA requires mDia1 rather than Rho kinase for floating matrix contraction. Certainly, LPA activates Rho kinase in these cells because blocking Rho kinase has been shown to inhibit retraction of dendritic extensions without preventing matrix contraction (Abe et al., 2003; Lee et al., 2003). Rho and Rho kinase have been implicated in myosin II–dependent force generation (Etienne-Manneville and Hall, 2002; Riento and Ridley, 2003). The role of mDia1 in force generation is less clear, however (Watanabe et al., 1999; Geiger and Bershadsky, 2001). Significantly, mDia1 has been implicated in the stabilization of both microtubules (Palazzo et al., 2004) and actin filaments (Watanabe and Higashida, 2004), and microtubule dynamics (Dogterom et al., 2005; Grishchuk et al., 2005) as well as microfilament depolymerization (Mogilner and Oster, 2003) have the potential to generate force independently of actomyosin.

Materials and methods

Materials

DME and trypsin/EDTA solution were obtained from Invitrogen. BSA (fatty acid free) and LPA were obtained from Sigma-Aldrich. Vitrogen 100 collagen was obtained from Cohesion Technologies, Inc. PDGF (BB isotype) was obtained from Upstate Biotechnology. LY294002, pertussis toxin, and Y27632 were obtained from Calbiochem-Novabiochem. Total PAK- (C-19), PAK1- (N-20), cofilin1-, phospho-cofilin1– (mSer 3), and mDia1-specific antibodies were obtained from Santa Cruz Biotechnology, Inc. rhodamine-B-isothiocyanate–conjugated phalloidin, oligofectamine, and Opti-MEM I were obtained from Invitrogen.

Cell culture

Human foreskins were obtained from anonymous donors and provided by the University of Texas Southwestern Medical Center. Fibroblasts from human foreskin specimens (<10th passage) were maintained in 75-cm^2 tissue culture flasks (Falcon) in DME supplemented with 10% FBS. Fibroblasts were harvested from monolayer culture with 0.25% trypsin/EDTA for 4 min at 37°C, followed by 10% FBS in DME. All incubations with cells were performed at 37°C in a humidified incubator with 5% CO_2.

For experiments with collagen matrices, cells in neutralized solutions of 1.5 mg/ml of collagen were prewarmed to 37°C for 3–4 min, and 0.2-ml aliquots were placed in 24-well culture plates (Corning). Unless otherwise specified, cell density was 2×10^5 cells/matrix for matrix contraction and immunoblotting experiments and 2×10^4 cells/matrix for observing cell morphology. Each aliquot of collagen matrix occupied an area outlined by a 12-mm diam circular score within a well. After polymerization for 60 min, matrices were gently released from the underlying culture dishes with a spatula and allowed to float in 0.5 ml of basal medium (DME containing 5 ml/ml BSA). Growth factors and inhibitors were added at the times indicated in the figure legend.

For experiments with collagen-coated surfaces, harvested cells (2×10^4) were incubated for the times indicated in the figure legend on 12-mm^2 glass coverslips. The coverslips were coated for 20 min with 50 µg/ml collagen and then rinsed with Dulbecco's PBS (1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 150 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, and 6 mM Na_2HPO_4, pH 7.2). Subsequently, the cultures were incubated with 1 ml DME containing 5 mg/ml BSA and growth factors or inhibitors as indicated in the figure legend.

PAK1, cofilin1, and mDia1 gene silencing

To knock down PAK1, cofilin1, and mDia1 expression in human fibroblasts, the following primer pairs for siRNA were designed and obtained from the University of Texas Southwestern Medical Center siRNA core facility. PAK1 siRNA: 5′-AACACACAAUUCAUGUCGGTT-3′ and 5′-AACCGACAUGAAUUGUGUGTT-3′. Cofilin1 siRNA: 5′-GCGGUGCUCUUCUGCCUGAUU-3′and 5′-UCAGGCAGAAGAGCACCGCUU-3′. mDia1 siRNA: 5′-AUUCUUCUGCAUCAUAUGGTT-3′ and 5′-CCAUAUGAUGCAGAAGAAUTT-3′. For annealing, 20 µM of each single-strand 21-nt RNA was incubated in annealing buffer (100 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, and 2 mM magnesium acetate) for 2 min at 95°C, followed by 2 h at 37°C. To accomplish high efficiency transfection, fibroblast cultures (60–70% confluent) were rinsed with antibiotic-free DME and treated with trypsin-EDTA for 1 min to elicit cell rounding, but not detachment. Subsequently, antibiotic-free 10% FBS/DME was added at a ratio of 4:1 to quench the trypsinization. After cells were rinsed with antibiotic-free DME, they were incubated with Opti-MEM I containing 700 nM siRNA-(PAK1 and mDia1) or 500nM siRNA-(cofilin1) annealed oligonucleotides. After 12 (PAK1 and mDia1) or 36 h (cofilin1), the transfection medium was removed and replaced with 10% FBS/DME containing antibiotics for an additional 24 h, at which time cells were subcultured. Mock-transfected cells were treated with only the sense direction oligonucleotide at double the concentration.

Cell migration assay

To measure 2D migration, mock- and PAK1-silenced fibroblasts were incubated overnight on collagen-coated coverslips with DME containing 5 mg/ml BSA and 0.1% FBS. The cell cultures were scrape wounded with a pipette tip and then incubated in DME containing 5 mg/ml BSA and 50 ng/ml PDGF.

Fluorescence microscopy

For immunostaining, collagen matrix samples were fixed for 10 min with 3% paraformaldehyde in PBS (3 mM KCl, 1 mM KH_2PO_4, 150 mM NaCl, and 6 mM Na_2HPO_4, pH 7.2.) at room temperature, blocked with 2% BSA/1% glycine in PBS for 30 min, and permeabilized for 15 min with 0.5% Triton X-100 in PBS. Samples were then incubated for 1 h at 37°C with mouse anti–β-tubulin (1:100 dilution in 1% BSA/PBS) followed by 45 min at 37°C with FITC-conjugated goat anti–mouse IgG. For actin staining, samples were incubated with Alexa Fluor 594–conjugated phalloidin (1:200 dilution in 1% BSA/PBS) for 30 min at 37°C. Samples were mounted on glass slides with Fluoromount G. (Southern Biotechnology Associates, Inc.) Images were collected using a fluorescent microscope (Eclipse 400; Nikon) using 10×/0.45, 20×/0.75, and 40×/0.75 infinity corrected objectives (Plan Apo; Nikon). Images were collected at room temperature using a camera (SenSys; Photometrics) and MetaView acquisition software

(Universal Imaging Corp.). Subsequent image processing was performed using Photoshop 5.5 or 7.0 (Adobe) in accordance with the *Journal of Cell Biology* image acquisition and manipulation instructions.

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