Different Molecular Motors Mediate Platelet-derived Growth Factor and Lysophosphatidic Acid-stimulated Floating Collagen Matrix Contraction*

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Fibroblast-collagen matrix contraction has been used as a model system to study how cells organize connective tissue. Previous work showed that lysophosphatidic acid (LPA)-stimulated floating collagen matrix contraction is independent of Rho kinase, whereas plateleterived growth factor (PDGF)-stimulated contraction is Rho kinase-dependent. The current studies were carried out to learn more about the molecular motors responsible for LPA- and PDGF-stimulated contraction. We found that neither PDGF nor LPA-dependent contractile mechanisms require myosin II regulatory light chain kinase or increased phosphorylation of myosin II regulatory light chain (measured as diphosphorylation). Low concentrations of the specific myosin II inhibitor blebbistatin blocked PDGF-stimulated matrix contraction and LPA-stimulated retraction of fibroblast dendritic extensions but not LPA-stimulated matrix contraction. These data suggest that PDGF- and LPA-stimulated floating matrix contraction utilize myosin II-dependent and -independent mechanisms, respectively. LPA-dependent, Rho kinase-independent force generation also was detected during fibroblast spreading on collagen-coated coverslips.

Form and function of multicellular organisms depend on tissue-specific programs of cell motility. Fibroblasts synthesize, organize, and maintain connective tissues during development and in response to injury and fibrotic disease. The motile mechanisms that fibroblasts use to remodel the extracellular matrix during these morphogenetic processes have been studied by using cells cultured in three-dimensional collagen matrices (1, 2).

As fibroblasts exert force on and move collagen fibrils of the matrix, collagen concentration around the cells increases, and the corresponding decrease in matrix volume (typically referred to as contraction) can be measured as a decrease in the diameter of free matrices or a decrease in height of restrained matrices. During contraction of restrained matrices, collagen fibrils become oriented in the same plane as restraint, and mechanical loading develops. In floating matrices, on the other hand, contraction occurs without collagen fibrils developing a particular orientation, and the matrix remains mechanically unloaded (1–5).

The signaling mechanisms used by fibroblasts to regulate collagen matrix contraction depend on whether the cells are mechanically loaded or unloaded at the time that contraction is initiated as well as on the growth factor used to initiate contraction. For instance, stimulation of fibroblasts by lysophosphatidic acid (LPA)1 but not by platelet-derived growth factor (PDGF) causes robust force generation in restrained matrices (6), whereas LPA and PDGF stimulate floating matrix contraction equally well (7, 8).

Floating matrix contraction has presented something of an enigma because LPA stimulation of fibroblasts in floating matrices causes activation of the small G protein Rho (GTP loading) (9), but blocking Rho kinase with the pharmacological reagent Y27632 does not inhibit contraction (10). Conversely, PDGF stimulation of cells in matrices causes activation of Rac not Rho (9), but blocking Rho kinase inhibits PDGF-stimulated contraction (10).

For fibroblasts migrating on two-dimensional substrata, the ability of cells to exert tractional force on the substratum depends on activation of Rho and Rho kinase (11, 12), which is believed to increase cell contraction through increased phosphorylation of myosin II regulatory light chain (MLC) (13, 14). In addition to Rho kinase, the spatial and temporal pattern of MLC phosphorylation in fibroblasts is controlled by MLC kinase (15–17). Other mechanisms of force generation independent of MLC phosphorylation also have been described (18, 19), even in smooth muscle cells (20, 21) where MLC phosphorylation generally is thought to be the key regulator of contractile activity (22).

The current studies were carried out to learn more about the molecular motors responsible for LPA- and PDGF-stimulated fibroblast-collagen matrix contraction. We found that neither PDGF- nor LPA-dependent contractile mechanisms require MLC kinase or increased phosphorylation of MLC (measured as diphosphorylation). Low concentrations of the specific myosin II inhibitor blebbistatin blocked PDGF-stimulated matrix contraction and LPA-stimulated retraction of fibroblast dendritic extensions but not LPA-stimulated matrix contraction. These data suggest that PDGF- and LPA-stimulated floating matrix contraction utilize myosin II-dependent and -independent mechanisms, respectively. LPA-dependent and Rho kinase-independent force generation also was detected during fibroblast spreading on collagen-coated coverslips.

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1 The abbreviations used are: LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; MLC, myosin II regulatory light chain; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PIPES, 1,4-piperazinediethanesulfonic acid; siRNA, small interfering RNA.
**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle's medium (DMEM) and trypsin/EDTA solution were obtained from Invitrogen. Bovine serum albumin (fatty acid-free), t-arachidonic acid, and lysophosphatidic acid (LPA) were obtained from Sigma. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Necross, CA). Goat serum was obtained from Zymbled Laboratories Inc. Vitrogen “100” collagen was obtained from Cohesion (Palo Alto, CA). Platelet-derived growth factor (PDGF) (BB isotype) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Myosin light chain was obtained from Calbiochem-Novabiochem. BSA and ammonium molybdate tetrahydrate, pH 7.0) (100 mM NaH2PO4, 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40, 0.5% Triton X-100) were obtained from Invitrogen. Fluoro-AMINE and Opti-MEM I were obtained from Invitrogen. Fluoromount G was obtained from Southern Biotechnology Associates (Birmingham, AL).

**Collagen and Collagen Matrix Culture**—Fibroblasts were from human foreskin specimens and cultured up to 10 passages in Falcon 75-mm tissue culture flasks in DMEM supplemented with 10% (v/v) FBS (DMEM, 10% FBS) at 37°C in a 5% CO2 humidified incubator. The culture medium was changed every 3–4 days. Cells were harvested by 0.25% trypsin/EDTA for 3 min at 37°C followed by DMEM, 10% FBS. For monolayer culture experiments, harvested cells were seeded at a density of 4 × 104 cells on 22-mm square glass coverslips (Fisher) that previously had been collagen-coated (50 μg/ml for 30 min) and then cultured in DMEM containing 5 mg/ml BSA, growth factors, and inhibitors as indicated. Collagen matrix cultures were prepared using Vitrogen 100 collagen as described previously (8, 23). Briefly, neutralized collagen solution (1.5 mg/ml) containing harvested cells (105 cells/ml unless indicated otherwise) was prefemined to 37°C for 4 min, and then aliquots (200 μl) were placed on an area outlined by a 12-mm-diameter circular score within a well of 24-well culture plates (Greiner Biotechnology, Santa Cruz, CA). Rhodamine-conjugated phallolidin and FITC-conjugated goat anti-mouse IgG H+L were obtained from Molecular Probes, Inc. (Eugene, OR). Horseradish peroxi-
dase-conjugated goat anti-mouse IgG H+L and goat anti-rabbit IgG H+L were obtained from ICN Biomedicals, Inc. (Aurora, OH). Polyvi-
ylidene difluoride membranes were obtained from Millipore Corp. (Bedford, MA). β-Nicotinamide adenine dinucleotide (reduced form) was obtained from Sigma. Enhanced chemiluminescence (ECL) Western blotting reagent was obtained from Amersham Biosciences. Oligo-
fecDNA and Opti-MEM I were obtained from Invitrogen. Fluoro-
mount G was obtained from Southern Biotechnology Associates (Birmingham, AL).

**RESULTS**

**Rho kinase and Collagen Matrix Contraction**—Fig. 1 shows typical results of experiments measuring the dependence of collagen matrix contraction on Rho kinase activity. Consistent with previous studies (10), addition of the Rho kinase inhibitor Y27632 had little effect on LPA-stimulated contraction of floating matrices although PDGF-stimulated contraction was re-
duced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. If, however, matrices were restrained and PDGF-stimulated contraction was reduced markedly. 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nase, MLC kinase has been reported to play a role in fibroblast-collagen matrix contraction (24, 26, 27). To test whether MLC kinase was important for LPA-stimulated collagen matrix contraction, experiments were carried out using fibroblasts in which MLC kinase levels were knocked down using the siRNA method.

Fig. 2A shows by immunoblotting with anti-MLC kinase antibodies that cell transfection with MLC kinase-specific oligonucleotides but not mock transfection markedly reduced the levels of the two MLC kinase isotypes in human fibroblasts. Actin was used as a loading control. In corresponding fashion, Fig. 2B shows by immunofluorescence that the cortical staining intensity of MLC kinase was reduced in MLC kinase-silenced cells, although background staining of the cell nucleus was similar in specific and mock transformants as well as controls. Fibroblasts in which MLC kinase was determined as above to be reduced were used to measure matrix contraction in the presence of LPA or PDGF with or without Rho kinase inhibitor. Fig. 3 shows results from a typical experiment. The extent of contraction of MLC kinase-silenced cells was not detectably different from control cells. Moreover, LPA-stimulated floating matrix contraction occurred in MLC kinase-silenced cells even if Rho kinase also was blocked.

MLC Phosphorylation and Collagen Matrix Contraction—Other experiments were carried out to test the effects of LPA and PDGF on MLC phosphorylation with and without Rho kinase inhibitor. Previously, we showed by using the urea-glycerol method that LPA but not PDGF stimulated both monophosphorylation and diphosphorylation of fibroblasts on collagen-coated coverslips, but for technical reasons the urea-glycerol method could not be used for fibroblasts in collagen matrices (23). Therefore, MLC phosphorylation in fibroblasts in collagen matrices was assessed using antibodies specific to (di)phosphorylated MLC with total MLC as a loading control. Diphosphorylated MLC as well as the monophosphorylated form has been implicated in cell contractility (28, 29).

Fig. 4 shows a representative blot and quantification of results from several experiments. After 15 min of growth factor stimulation, levels of diphosphorylated MLC were highest in cells in LPA-containing medium and lowest in PDGF-containing medium. Prior addition of Rho kinase inhibitor markedly reduced phosphorylation in every case. These observations suggested that stimulation of floating collagen matrix contraction did not require growth factor stimulation of MLC phosphorylation (measured as diphosphorylation).

Myosin II and Collagen Matrix Contraction—The foregoing observations suggested that neither MLC kinase nor stimulation of MLC phosphorylation was necessary for collagen matrix

**Fig. 1.** Rho kinase dependence of collagen matrix contraction. Collagen matrices containing fibroblasts were released from culture dishes to initiate contraction either immediately after polymerization (floating matrices) or after preincubation overnight in DMEM (DME), 10% FBS (restrained matrices). Contraction medium was DMEM/BSA and 10 μM LPA or 50 ng/ml PDGF as shown. Where indicated, Rho kinase inhibitor Y27632 (10 μM) was added to the incubations 15 min before the growth factors. At the end of the incubations, 4 h for floating matrices and 1 h for restrained matrices, samples were fixed, and the extent of matrix contraction was measured as decrease in matrix diameter.

**Fig. 2.** MLC kinase silencing with siRNA. Fibroblasts were nontransfected or transfected for 48 h in DMEM with 3.5 μM siRNA or without siRNA (mock) after which the medium was switched to DMEM, 10% FBS for 96 h. A, cell extracts were prepared, subjected to SDS-PAGE, and immunoblotted to determine levels of MLC kinase and actin. B, harvested cells were incubated 4 h on collagen-coated coverslips and then fixed and stained to determine the distribution of MLC kinase. Bar, 50 μm.

**Fig. 3.** MLC kinase dependence and Rho kinase dependence of collagen matrix contraction. Details are the same as in Fig. 1 except cells used were either nontransfected (no treatment), mock-transfected, or MLC kinase-silenced (siRNA+) fibroblasts as indicated. DME, DMEM.
contraction. We could not exclude, however, the possibility that residual MLC kinase or constitutive levels of MLC phosphorylation were sufficient for contraction to occur. Additional experiments were carried out to test more directly the role of myosin II in contraction using the specific myosin II inhibitor blebbistatin (30).

Fig. 5 shows a dose-response curve for the effect of blebbistatin on floating matrix contraction. Concentrations of the drug in the range 5–20 μM markedly inhibited PDGF-dependent contraction, which is the same concentration range that blocks myosin II ATPase activity (30). In marked contrast, LPA-dependent contraction was only inhibited by blebbistatin at higher drug concentrations.

We also tested the effect of blebbistatin on LPA-stimulated retraction of fibroblast dendritic extensions. As described previously, fibroblasts in collagen matrices project and retract a dendritic network of extensions. PDGF stimulates the size and complexity of this network, whereas LPA stimulates its retraction in a Rho kinase-dependent manner (9). Retraction occurs independently of and is not required for matrix contraction (10). Fig. 6 shows the morphology of fibroblasts with retracted (LPA) and projected (PDGF) extensions. As described previously, addition of the Rho kinase inhibitor Y27632 blocked retraction stimulated by LPA (Fig. 6, LPA+Y27632). Treatment with blebbistatin at 5 (LPA+Bbst5) or 20 μM (Fig. 6, LPA+Bbst20) also blocked LPA-stimulated retraction but had no effect on the extensions formed in the presence of PDGF (PDGF+Bbst20). These findings show that LPA-dependent retraction of fibroblast dendritic extensions requires myosin II. Taken together, the foregoing results indicated that PDGF and LPA stimulated floating collagen matrix contraction through different molecular motors and that LPA was able to exert force by a Rho kinase-independent, myosin II-independent mechanism.

Rho Kinase and Cell Spreading on Collagen-coated Cover-slips—Finally, experiments were carried out to learn if the LPA-stimulated, Rho kinase-independent mechanism of force generation also could be demonstrated during cell spreading on collagen-coated coverslips. Fig. 7 shows that in PDGF-containing medium, fibroblasts spread with prominent stress fibers...
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Fig. 7. Rho kinase dependence of fibroblast spreading on collagen-coated coverslips. Cells were incubated on collagen-coated coverslips in DMEM and 5 mg/ml BSA for 15 min with or without Rho kinase inhibitor Y27632 (10 μM) as indicated after which 10 μM LPA or 50 ng/ml PDGF was added as shown. The incubations were continued for 4 h at which time cell extracts were prepared and subjected to SDS-PAGE and immunoblotted with antibodies against monophosphorylated (M) or diphosphorylated (Y) MLC as indicated. Bar, 50 μm.

Fig. 8. Myosin light chain phosphorylation by fibroblasts on collagen-coated coverslips. Cells were incubated on collagen-coated coverslips in DMEM and 5 mg/ml BSA for 15 min with or without 10 μM Y27632 as indicated after which 10 μM LPA or 50 ng/ml PDGF was added as shown. The incubations were continued for 4 h at which time cell extracts were prepared and subjected to SDS-PAGE and immunoblotted with antibodies against diphosphorylated (dip MLC) and total (t) MLC as indicated.

DISCUSSION

The current studies were carried out to learn more about the molecular motors responsible for LPA- and PDGF-stimulated fibroblast-collagen matrix contraction. Our findings suggest that PDGF- and LPA-stimulated floating matrix contractions utilize myosin II-dependent and -independent mechanisms, respectively.

Previous studies (10) suggested that LPA-stimulated force generation by fibroblasts in floating collagen matrices does not require Rho kinase activity. In the current work, we found that fibroblasts contracted the matrices even when MLC kinase was knocked down using siRNA, and LPA was added. Moreover, in the presence of Rho kinase inhibitor, MLC diphosphorylation was substantially reduced. Although these findings suggested that contraction was independent of MLC phosphorylation, we could not exclude the possibility that residual MLC kinase or constitutive levels of MLC phosphorylation were sufficient for contraction to occur. Monophosphorylated MLC might have been sufficient (28, 29).

Additional experiments were carried out, therefore, using the specific myosin II inhibitor blebbistatin. At concentrations sufficient to inhibit myosin II ATPase activity (30), blebbistatin blocked not only PDGF-dependent floating matrix contraction but also LPA-dependent retraction of the dendritic network of cellular extensions. LPA-stimulated retraction of fibroblast dendritic extensions is a Rho kinase-dependent activity independent from and not required for floating matrix contraction (10). Although blebbistatin also blocked LPA-dependent floating matrix contraction, inhibition only occurred at concentrations above those shown to be required to inhibit myosin II.

Taken together, the foregoing results suggested that LPA can stimulate floating matrix contraction through a molecular motor other than myosin II. Myosin Ic recently was shown to play a role in retraction of the neuronal growth cone lamellipodium (31), but preliminary studies using siRNA specific for myosin Ic demonstrated that myosin Ic knockout did not affect LPA-dependent floating matrix contraction.²

Also, it has been hypothesized that microtubule dynamics might play a role in exerting force on the cell plasma membrane (32). Nevertheless, because addition of the microtubule disrupting reagents nocodazole or vinblastine or the microtubule-

² M. Abe, C.-H. Ho, K. E. Kamm, and F. Grinnell, unpublished observations.
stabilizing drug taxol at concentrations expected to interfere with microtubule dynamics but not myosin II required for cell motility. The mechanism of force generation by fibroblasts in collagen matrices involves Rho kinase (24, 25) and phosphorylation, and is different from LPA-stimulated matrix contraction. Because collagen in these matrices is required for initial wound contraction (1, 46, 47), are not required for initial wound remodeling (48, 49). How fibroblasts initially begin to move into and remodel the wound matrix is not well understood. The present research implicates PDGF/Rho kinase-dependent and LPA/Rho kinase-independent mechanisms as potential candidates to explain this phenomenon.

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