Stable Transfectants of Smooth Muscle Cell Line Lacking the Expression of Myosin Light Chain Kinase and Their Characterization with Respect to the Actomyosin System*

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We constructed a plasmid vector having a 1.4-kilobase pair insert of myosin light chain kinase (MLCK) cDNA in an antisense direction to express antisense mRNA. The construct was then transfected to SM3, a cell line from vascular smooth muscle cells, producing a few stable transfectants. The down-regulation of MLCK expression in the transfectants was confirmed by both Northern and Western blots. The control SM3 showed chemotactic motility to platelet-derived growth factor-BB, which was supported by lamellipodia. However, the transfectants showed neither chemotactic motility nor developed lamellipodia, indicating the essential role of MLCK in the motility. The specificity for the targeting was assessed by a few tests including the rescue experiment. Despite this importance of MLCK, platelet-derived growth factor-BB failed to induce MLC20 phosphorylation in not only the transfectants but also in SM3. The mode in which MLCK was involved in the development of membrane ruffling is discussed with special reference to the novel property of MLCK that stimulates the ATPase activity of smooth muscle myosin without phosphorylating its light chain (Ye, L.-H., Kishi, H., Nakamura, A., Okagaki, T., Tanaka, T., Oiwa, K., and Kohama, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6666–6671).

The formation of acute atherosclerotic plaques results largely from a stimulated invasion of vascular smooth muscle cells (VSMCs) from the tunica media into the tunica intima. Future therapeutics to correct atherogenesis will benefit from the development of procedures to control these regulatory steps. A number of cytokines, such as platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor, transforming growth factor-β, angiotensin II, bradykinin, thrombin, and catecholamines, appear to play significant roles in the control of VSMC replication (reviewed in Ref. 1). It is currently difficult, if not impossible, to simultaneously target the many molecules involved in these complex signal pathways. In contrast to the complexity of upstream signaling, all VSMC types utilize common contractile machinery for driving their cell division and movement, i.e. the actomyosin contractile system. Thus, a protocol to control actomyosin activities would be a powerful tool for controlling VSMC proliferation and migration, regardless of which pathways are activated by growth factors in the VSMCs of atherosclerotic plaques.

The actomyosin system functions both in VSMC contraction and at the leading edge of migratory VSMCs. Several proteins are known to regulate actomyosin activities in VSMCs by modulating either the actin or myosin function (reviewed in Ref. 2). Myosin light chain kinase (MLCK) is one such regulatory protein. Upon the binding of Ca2+/calmodulin, MLCK phosphorylates a myosin subunit, known as the 20-kDa regulatory light chain (MLC20). In all smooth muscle cells, including VSMCs, the phosphorylation of MLC20 by MLCK serves to trigger myosin interaction with actin (reviewed in Ref. 3). Although most MLCK studies have focused on its regulatory roles in smooth muscle contraction, MLCK also functions in the regulation of cell migration (4, 5). For example, upon activation by PDGF-BB, VSMCs become highly motile and develop membrane ruffling (6–9), in which actin filaments (6–9) and phosphorylated MLC20 (10) accumulate at the inner surface of the cell membrane.

MLCK is composed of several functional domains: actin-binding domain at the N terminus (11–18), kinase domain in the center, and the myosin-binding domain at the C terminus (19). In addition to the regulatory role of the kinase domain, we recently found that actin- and myosin-binding domains regulate the actin-myosin interaction; the former inhibits the interaction (18), and the latter activates the interaction (20). How are these regulatory modes of MLCK involved in the actual motility of VSMCs? As the first step to approach this question, we are interested in down-regulating MLCK in VSMCs.

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The abbreviations used are: VSMC, vascular smooth muscle cell; PDGF, platelet-derived growth factor; MLCK, myosin light chain kinase; MEM, minimal essential medium; PGE2, prostaglandin E2; bp, base pair(s); PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; CAPS, 3-(cyclohexylamino)propane-sulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
Targeting of MLCK in Smooth Muscle Cell

![Diagram of Domain Structure](image)

**Domain Structure of Rabbit Smooth Muscle MLCK Molecule.** Localization of 1366-bp MLCK cDNA was obtained by the PCR method shown, with special reference to the domain structure of rabbit smooth muscle MLCK (22). The 1366-bp MLCK cDNA was subcloned into the pCXLp vector in an antisense direction so that it expressed antisense mRNA in the transfected cells. The actin-binding and telokin domains were assigned from Ye et al. (18) and Gallagher and Herrington (43), respectively.

In the present study, we inhibited the expression of MLCK in SM3 cells, a cell line established from rabbit VSMC (21), by overexpressing the antisense RNA of MLCK in SM3. The data indicate that the down-regulation of MLCK gives rise to significant loss of both lamellipodia formation and cell migration of SM3 when stimulated by PDGF-BB. These results suggest that MLCK is involved in inducing lamellipodia to induce VSMC migration. Inhibition of MLCK may be a useful future approach to regulate VSMC migration in vivo.

**Experimental Procedures**

**Cell Cultures—**SM3 is a multipassagable smooth muscle cell line established from rabbit aorta smooth muscle (21). SM3 cells remain in a proliferative form in the presence of fetal bovine serum (21), while they differentiate into a contractile form upon removal of fetal bovine serum. Throughout the present study, SM3 cells were maintained in minimum essential medium (MEM; Life Technologies, Inc.), supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) at 37 °C in 5% CO₂.

Reagents—PDGF-BB of human recombinant as expressed in PDGF-BB of rabbit smooth muscle MLCK, in order to detect only the sense mRNA of MLCK. The probe for β-actin was a 32P-labeled, random-primed cDNA probe of full-length chicken β-actin, the template for which was excised from SP64-actin construct by Mikawa’s laboratory. The conditions for the hybridization were as follows: 10% dextran sulfate, 1% SDS, 50% deionized formamide, 2% sodium citrate/sodium chloride (SSC), 5× Denhardt’s solution, and 100 μg/ml denatured salmon sperm DNA at 42 °C. The final washing was carried out in 0.1× SSC and 0.1% SDS at 42 °C. The nitrocellulose membrane thus treated was exposed to x-ray film at –80 °C.

**Western and Dot Blot Analyses—**SM3, A3, A8, and C1 cells were washed with ice-cold saline and fixed with 10% trichloroacetic acid for 15 min on ice, followed by scraping with a rubber policeman and centrifugation at 10,000 × g. The precipitate was suspended in 10 μl of SDS-sample buffer consisting of 40 mM Tris-HCl (pH 6.8), 50 mM dithiothreitol, 1% SDS, 7.5% glycerol, 0.002% bromphenol blue, and 1.4 μl 2-mercaptoethanol and was sonicated briefly. An aliquot of 2 μl Tris was added to adjust the pH to 6.8. The sample was boiled for 5 min and stored at −80 °C until electrophoresis. The proteins were separated by SDS-PAGE with 8% acrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon™ PVDF Transfer Membrane; Millipore, Bedford, MA) at 40 V for 3 h in the presence of 0.1× CAPS at pH 11.0 and 10% methanol.

5 μg of protein isolated from SM3, A3, A8, and C1 cells as described above was spotted onto a nitrocellulose membrane (Protran; Schleicher & Schuell), dried, treated with 5% nonfat dry milk in PBST (0.05% Tween 20, 50 mM Tris, 150 mM NaCl, 0.1% SDS, pH 7.4) for 1 h, then reacted with a polyclonal antibody raised against chicken gizzard actin (Mikawa’s laboratory). The conditions for the incubation were as follows: 10% nonfat dry milk in PBST at room temperature for 1 h, followed by incubation with goat anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Following the wash in PBST, the immunoreactive proteins were visualized on x-ray film with an enhanced chemiluminescence detection system (ECL Western blotting detection system; Amersham Pharmacia Biotech). We subjected the 152-kDa major band to densitometry (see below) by excluding fuzzy staining.

**Cell Migratory Assay—**A cell migratory assay was performed using the modified Boyden chamber method (28). We used a 96-well microplate chamber with 3.2-mm diameter wells containing polycarbonate filters with 8-μm pores (Neuro probe, Inc., Gaithersburg, MD). The cells were trypsinized and suspended in MEM. 2 × 10⁵ cells suspended in 100 μl of MEM were applied per well in the upper chamber. After a wash in PBST, the blots were reacted with goat anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Following the wash in PBST, the immunoreactive proteins were visualized on x-ray film with an enhanced chemiluminescence detection system (ECL Western blotting detection system; Amersham Pharmacia Biotech). We subjected the 152-kDa major band to densitometry (see below) by excluding fuzzy staining.
incubated in MEM with and without 10 ng/ml PDGF-BB so that they produced lamellipodia. The cells were fixed with 3.5% formaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 2 min. After two washes with PBS for 10 min each, the cells were stained with rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR) that was dissolved in methanol at a concentration of 0.2 unit/ml and diluted to 1:100 in PBS. After the excess phalloidin was removed by washing with PBS for 10 min three times, the cells on the coverslips were mounted with PermaFluor (Immunon, Pittsburgh, PA) and observed with a fluorescence microscope. We considered cells that developed a broad accumulation of F-actin on the cell edge (more than 5 μm in width and 30% in the length of the whole periphery of the cell) as lamellipodia-positive. The number of scored cells was about 130 on average in the 10 fields.

Rescue Experiment—The Xba1–Pst1 fragment containing MLCK cDNA was isolated from the pBst/S3M3-FL (see “Plasmid Construction”) and ligated with a sense orientation into an expression vector, pME18S, which has 5′RFl promoter (28). We named the resulting vector MLCKpME18S. The sequence of the insert in MLCKpME18S was confirmed by an Applied Biosystems model 377 DNA sequencer (Applied Biosystems, Foster City, CA). For DNA transfection, A3, A8, and C1 cells were plated on 120-mm coverslips on the day before the transfection. They were co-transfected by 1 μg of MLCKpME18S and 1 μg of pEGFP-C1 (CLONTECH Laboratories, Palo Alto, CA) using FuGENE-6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. In brief, 6 μl of FuGENE-6 was mixed with 94 μl of serum-free Opti-MEM (Life Technologies, Inc.). After a 5-min incubation at room temperature, the mixture was added slowly to the rescue mixture of MLCKpME18S plus pEGFP-C1 (a total of 2 μg of DNA in 2 μl) or the control mixture of pME18S plus pEGFP-C1 (a total of 2 μg of DNA in 2 μl). After a 15-min incubation at room temperature, the FuGENE-6/DNA mixture was added to the cells. Transfected cells were incubated for 48 h and subjected to the staining of lamellipodia described as above.

MLC20 Phosphorylation Assay—We performed a MLC20 phosphorylation assay using glycerol-PAGE and Western blot essentially as described elsewhere (30). Briefly, cells cultured on a 100-mm dish were stimulated with 10 ng/ml PDGF-BB for 5 min, and then 20 μg/ml actinomycin D was added to inhibit the transcription. After 10 min, the cells were harvested, and 10% SDS-PAGE was used to analyze the actin filament (16). The total protein extracts from the SM3 cells and the stable transfectants were subjected to dot blot and Western blot analyses with a polyclonal MLCK antibody to analyze MLCK protein accumulated in these cell lines. Dot blot analysis detected a higher level of MLCK in SM3 cell extract than in extracts from transfectants (Fig. 3B). The result was further confirmed by Western blot analysis (Fig. 3A). The MLCK protein was detected as a 152-kDa band in the SM3 cells (Fig. 3A). The same size protein band was also detected in the total protein extracts from rabbit aorta (data not shown). The signal of the 152-kDa band was dramatically suppressed in the transfectants; the signals from A3, A8, and C1 are only 19.4, 2.9, and 12.4% of the signal from SM3 (Fig. 3A). These results indicate that A3, A8, and C1 cell lines down-regulate the level of MLCK transcript and protein.

Inhibition of Lamellipodia Formation—The effect of the down-regulated MLCK on VSMC was examined by monitoring lamellipodia formation as a prerequisite for cell migratory activity (Fig. 4). Lamellipodia formation was induced by exposing cells to PDGF-BB. Staining with rhodamine-phalloidin revealed that the control SM3 cells were induced to initiate lamellipodia within 10 min after exposure to PDGF-BB (Fig. 4, A–C). In striking contrast, activated lamellipodia was not evident in the stable transfectants of C1, even after prolonged exposure to PDGF-BB (Fig. 4, D–F). A3 and A6 also failed to develop lamellipodia (not shown). This observation was further confirmed by quantitative analysis. Without PDGF-BB added, only 6.0 ± 0.6% of the control SM3 cells were scored as lamellipodia-positive. Upon PDGF-BB stimulation, 26.5 ± 13.6% of the SM3 cells became lamellipodia-positive within 10 min after stimulation with PDGF-BB. At 30 min after stimulation, 21.4 ± 11.4% of cells were still detected as lamellipodia-positive (Fig. 5). This activated lamellipodia was not evident when stable transfectants were exposed to PDGF-BB (Fig. 5). For example, without PDGF-BB activation, 2.97 ± 0.26% of C1 cells were scored as lamellipodia-positive (Fig. 5). The lamellipodia-positive population of A3 was increased only to 8.72 ± 8.27% at 10 min and to 5.77 ± 8.33% at 30 min after exposure to PDGF-BB, respectively. Similar results were obtained in A3 and A8 cell lines (Fig. 5).

We must look more carefully into the basal level of lamellipodia in SM3. We obtained three clones by subjecting SM3 to repeated subcloning as described under “Experimental Procedures.” Their levels of lamellipodia-positive cells were 6.1 ± 6.8, 16.6 ± 10.8, and 8.7 ± 1.0%. This intrinsic clonal variation was highly significant.
the amount of total protein in each dot. Samples were stained with 0.1% Amide Black in 50% ethanol to confirm MLCK was detected by the same antibody. Samples were dot-blotted onto nitrocellulose membrane in duplicate. Bands stained with Coomasie brilliant blue on the SDS-polyacrylamide gel were almost equal. Upper panel, dot blot analysis. The same samples were dot-blotted onto nitrocellulose membrane in duplicate. MLCK was detected by the same antibody. Lower panel, actin protein bands stained with Coomasie brilliant blue on the SDS-polyacrylamide gel, indicating that the contents of the protein samples loaded onto the gel were almost equal. B, upper panel, dot blot analysis. The same samples were dot-blotted onto nitrocellulose membrane in duplicate. MLCK was detected by the same antibody. Lower panel, dot-blotted samples were stained with 0.1% Amide Black in 50% ethanol to confirm the amount of total protein in each dot.

FIG. 3. Western and dot blot analyses of A3, A8, C1, and control SM3. A, Western blot analysis of MLCK expression level in SM3, A3, A8, and C1. The extracts from 2.5 × 10^6 cells/lane were subjected to SDS-PAGE and then transferred on a polyvinylidene difluoride membrane. Upper panel, MLCK was detected by a polyclonal antibody raised against chicken gizzard MLCK (26, 27). Lower panel, actin protein bands stained with Coomasie brilliant blue on the SDS-polyacrylamide gel, indicating that the amounts of the protein samples loaded onto the gel were almost equal. B, upper panel, dot blot analysis. The same samples were dot-blotted onto nitrocellulose membrane in duplicate. MLCK was detected by the same antibody. Lower panel, dot-blotted samples were stained with 0.1% Amide Black in 50% ethanol to confirm the amount of total protein in each dot.

FIG. 4. Lamellipodia induced by PDGF-BB. Cells were incubated with 10 ng/ml PDGF-BB, fixed, permeabilized, and stained with rhodamine-phalloidin. Lamellipodia was seen more frequently in SM3 10 min (B) and 30 min (C) after stimulation by PDGF-BB than before stimulation (A). In contrast, no remarkable change was seen in C1 10 min (E) and 30 min (F) after stimulation by PDGF-BB compared with before (D). Bar, 50 μm.

indicates that the lamellipodia-positive population in A3, A8, and C1 is not altered by PDGF-BB. Therefore, we concluded that the down-regulation of MLCK causes poor responsiveness to PDGF-BB in inducing lamellipodia.

Rescue Experiments—To see whether or not the failure in inducing lamellipodia in the transfectants is solely attributable to the targeting of the endogenous mRNA by antisense mRNA produced in parent SM3, we transfected MLCKpME18S (see “Experimental Procedures”) to A3, A8, and C1 cells so that the plasmid neutralizes the antisense RNA by overexpressing mRNA in a sense direction. As a control, we transfected the empty vector of pME18S to the cells. The cells carrying these plasmids were marked by the fluorescence due to the co-transfected pEGFP-C1. We observed that the level of lamellipodia-positive cells in A8 was increased from 6.5 ± 1.2 to 32.8 ± 1.4% upon stimulation by PDGF-BB. The increase was not observed with the cells with the control vector, i.e. from 6.6 ± 0.72 to 6.7 ± 0.94%. Similar results were obtained with A3 cells (from 9.5 ± 0.8 to 25.0 ± 3.1%) and C1 cells (from 8.3 ± 1.3 to 24.8 ± 0.7%).

Decreased Cell Migration—We cultured cells in a modified Boyden chamber to more directly investigate the cell migratory activities of stable transfectants (29). The control SM3 cells were stimulated by PDGF-BB at various concentrations, such as 0, 1, 10, and 100 ng/ml, and the cells migrated through the membrane pores were scored. Since activated migration was detectable above 10 ng/ml PDGF-BB (data not shown), all quantitative analyses were conducted using 10 ng/ml PDGF-BB. Within 4 h, 108.3 ± 61.1 cells (mean ± S.D., n = 6 wells) out of 2 × 10^3 SM3 cells plated had migrated from the upper chamber to the lower chamber, while only 5.7 ± 10.5 A3 cells, 4.3 ± 2.2 A8 cells, and 2.5 ± 4.3 C1 cells were detected as migratory (Fig. 6). These results indicate that cell migration activity of the A3, A8, and C1 lines was significantly lower than the control SM3 cells.

Myosin Phosphorylation as Examined by Western Blots of Control and Transfected Cells—To assess the specificity of our antisense approach, we addressed two questions: (i) whether myosin phosphorylation at MLC20 is inhibited by the down-regulation of MLCK and (ii) whether the inhibition involves the phosphorylation of Ser19 and Thr18 of MLC20, which play key roles in the activation of myosin. As shown in Fig. 7, we subjected control SM3 and transfected cells to Western blot analysis for MLC20 phosphorylation. The basal phosphorylation as expressed by (MLC-2P/total MLC20 of SM3 in the absence of PGF2α was 30.5% (Fig. 7A, lane a). PGF2α enhanced the extent of phosphorylation to 83.4%. These values are consistent with previous reports (21, 31). Similar results
were obtained with the transfected cells, where MLCK expression was down-regulated (Figs. 2 and 3); PGF2α stimulated phosphorylation levels in A3, A8, and C1 cells from 32.4, 32.0, and 29.5% (Fig. 7A, upper panel, lanes d, g, and h) to 81.1, 78.9, and 64.4% (Fig. 7A, upper panel, lanes e, h, and k).

The phosphorylation sites were determined using specific anti-MLC20 and anti-MLC20 phosphorylated at Ser19 and by anti-MLC20-2P antibody raised against the peptide diphosphorylated at Thr18 and Ser19 (Fig. 7A, middle and lower panels) (30). Western blot analysis revealed that PGF2α treatment increased the level of both monophosphorylated and diphosphorylated MLC20. There was no significant difference in the phosphorylation level between control SM3 cells and cloned transfectants.

The similar extent of phosphorylation at Thr18/Ser19 of MLC20 between the control SM3 cells and the transfectants was unexpected. Since Rho kinase was recently reported to phosphorylate those residues as does MLCK (32), we examined the effect of Y-27632, a specific inhibitor for Rho kinase (33). PGF2α-induced phosphorylation was neutralized by Y-27632, as shown in Fig. 7B. The phosphorylated population declined from 86.3 to 36.3% (Fig. 7B, lanes b and c) in the SM3 cells, from 65.8 to 22.7% in the A3 cells, from 76.4 to 29.6% in the A8 cells, and from 78.7 to 30.3% in the C1 cells (Fig. 7B, lanes e, f, h, i, k, and l). These results are consistent with the concepts that MLCK is specifically targeted and that Rho kinase activity is preserved in cloned cell lines in which MLCK is down-regulated.

We used a similar approach to examine the effect of PDGF-BB on MLC20 phosphorylation (Fig. 7A, upper panel). The changes in phosphorylation of the typical experiment were from 30.5 ± 5.7% (mean ± S.D., n = 5) to 25.9 ± 8.1% (mean ± S.D., n = 5) in SM3 (lanes a and c), from 32.4 to 24.2% in A3 (lanes d and f), from 32.0 to 29.4% in A8 (lanes g and i), and from 29.5 to 25.0% in C1 (lanes j and l). Because these values are within the confidential limits at 95% of SM3 (i.e. 24.8 and 39.0% before PDGF stimulation and 17.3 and 37.3% after PDGF stimulation), we concluded the absence of stimulation. Further, the sites of phosphorylation, i.e. Thr18/Ser19, were not altered as determined by anti-MLC-P and anti-MLC-2P antibodies (Fig. 7A, middle and lower panels). Thus, MLC20 phosphorylation was not affected by treatment of PDGF-BB.

It is worthwhile reiterating that the essential role of MLCK in SM3 was for PDGF-BB to induce chemotactic activity, presumably in association with lamellipodia (Figs. 4 and 5). Nevertheless, MLC20 phosphorylation of SM3 remained as low as that of transfectants even when PDGF-BB was present (Fig. 7A, upper panel). MLC20 phosphorylation is not the sole role of MLCK in regulating the actomyosin motor. Our in vitro study shows that MLCK binds myosin at its C terminus to activate it without phosphorylating MLC20 (20). We expect that such an activating role may cause motility (see "Discussion").

**DISCUSSION**

We established MLCK-deficient transfectants by targeting MLCK in VSMCs and found that lamellipodia, induced by PDGF-BB, was absent in the transfectants. Therefore, MLCK is likely to be actively involved in the membrane ruffling. Shoemaker et al. (34) allowed an antisense oligonucleotide for MLCK to incorporate into cultured fibroblasts and observed their morphological change; the rounding of their shape was associated with the down-regulation of MLCK. The change could be related to the decreased lamellipodia observed in the present study.

Most migrating cells show lamellipodia at one side of the cell periphery, causing cell migration (reviewed in Ref. 35). Upon the addition of PDGF-BB to the medium culturing SM3, it developed lamellipodia at all sides of the cell periphery (Fig. 4). Such cells would not move. However, the Boyden chamber method (Fig. 6), which creates a gradient of PDGF-BB, would induce lamellipodia at one side so that it causes migration. The absence of cell migration in the transfectants suggests that the down-regulation of MLCK should affect the induction of the polarized lamellipodia.

The induction of stress fibers in cells is also involved in the cell migration (reviewed in Ref. 36). Prominent stress fibers were found in the cells, the motility of which was activated by the transfection of constitutively active, p21-activated kinase (37, 38). In accordance, stress fibers neighboring lamellipodia in SM3 became less prominent when its motility was induced by PDGF-BB (Fig. 4, a–c). However, the organization of stress fibers in the transfectant was not altered by PDGF-BB (Fig. 4,
Involvement of MLCK in the p21-activated kinase-induced migration is now controversial (37, 38). The transfectants down-regulating MLCK should be a useful tool to solve the controversy. One might consider the argument that the failure of lamellipodia induction in the transfectants may be caused by non-specific changes during the repeated subculture and that the observed effects are not due to the down-regulation of MLCK. However, this possibility can be ruled out by the rescue experiments indicating that the neutralization of transfected antisense mRNA caused the recovery in the lamellipodia induction. Further, we carried out an experiment to see whether the expression of PDGF-BB receptor was affected or not in the transfectants as described under "Experimental Procedures." We detected PCR products in both SM3 and transfectants to a similar extent (data not shown). Together with the preservation of Rho kinase activity in the transfectants (Fig. 7B), this result gives additional support to the specificity of targeting.

Sakurada et al. (30) and Matsumura et al. (10) raised antibodies against phosphorylated MLCK20 and stained SM3 and fibroblasts. Myosin with phosphorylated MLCK20 was located not only in stress fiber but also in the cellular periphery, including membrane ruffling, suggesting myosin involvement. We obtained a similar suggestion. However, the contribution of MLCK to MLCK20 phosphorylation should be slight in SM3, because the phosphorylated population upon PGF2α treatment was mostly inhibited by Y-27632 (Fig. 7B, a–c), which hardly inhibits MLCK activity (33).

Under the assumption that the only possible target for MLCK is myosin, we failed to make it clear how MLCK activates motility via myosin in SM3. PDGF-BB did not elevate MLCK20 phosphorylation (Fig. 7A), while it does activate chemotaxis (Fig. 6). The failure agrees with the report of p21-activated kinase-induced motility, which is not necessarily associated with MLCK20 phosphorylation (37, 38). These data indicate a minimal role for MLCK20 phosphorylation with a reservation that the PDGF-induced motility results from MLCK20 phosphorylation only at cellular periphery, for which the sensitivity of the immunoblot analysis (Fig. 7A) is not high enough. Similarly, the low sensitivity of the assay should be noted from the observations that the population of SM3 cells responding to the PDGF stimulation was only about 20% (Fig. 5, SM3). The changes in MLCK20 phosphorylation would be diluted by the large unresponsive population.

Does a minimal role for MLCK20 phosphorylation rule out involvement of the MLCK molecule in motility? MLCK is furnished not only with the kinase activity but also with the binding activity to myosin (19, 20) and actin (15, 17, 18). An in vitro experiment shows that smooth muscle myosin is activable by the myosin-binding fragment of MLCK, while MLCK20 remains unphosphorylated (20). We expect that such a role of myosin also works in vivo to cause migration of SM3 cells. MLCK also functions as an actin-bundling protein (39, 40). It binds to actin filaments at its Ca/CaM-sensitive and Ca/CaM-insensitive site for actin to cross-link them into bundles. Such a mechanism also may support motility by inducing lamellipodia.

The Ca/CaM-sensitive site also exerts an inhibitory effect on actin-myosin interaction (18). However, the inhibition is evident only when myosin is fully phosphorylated (20). The myosin under the PDGF-stimulated culture remains unphosphorylated (Fig. 7). Therefore, the actin-linked inhibition may not cause migration.

We chose 199 bp (bp 3052–3252) of rabbit smooth muscle MLCK cDNA as the probe for Northern blots. The probe corresponds to the 3′-flanking region of endogenous MLCK mRNA. MLCK cDNA introduced through a plasmid vector is expected to produce antisense mRNA of 1366 bases, corresponding to bp 1666–3032 of the MLCK cDNA. Therefore, the probe will hybridize with endogenous MLCK mRNA. The mode by which antisense RNA inhibits synthesis of the target protein is controversial (41). The failure to detect endogenous MLCK mRNA (Fig. 2) in the transfectants suggests that it was degraded, presumably by ribozyme activity, in the complex of endogenous MLCK mRNA with the antisense mRNA.

We detected a 5.8-kilobase band as mRNA for MLCK in SM3 (Fig. 2), which is in accordance with the 5.8-kilobase mRNA in rabbit uterine smooth muscle (22). However, we did not detect an 8.7-kilobase mRNA for embryonic MLCK, as reported by Gallagher et al. (42). The size of MLCK of SM3 was 152 kDa in the Western blots with an antibody against MLCK (26, 27), which is also in agreement with their report (22). However, we can detect another broad, faint bands in the Western blots of SM3 and its transfectants at the position of higher molecular weight (Fig. 3A). We could not identify them.

The migration of VSMCs is a critical step in developing some vascular diseases, such as atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty. The VSMCs that were transfected by MLCK cDNA in an antisense direction did not migrate. Thus, our experimental methods are potentially applicable to a living animal model of such vascular diseases. We chose a plasmid vector pCXLP–(23) to construct the plasmid that produces antisense MLCK mRNA. pCXLP–has an encapsidation sequence, so it is able to produce a recombinant retrovirus to introduce a gene of interest into living tissue (23). To gain further insight, we are going to use a recombinant retrovirus vector that has antisense MLCK mRNA to infect an animal model subjected to endothelium injury by balloon angioplasty. If the development of atherosclerosis can be prevented by infection of the virus, it would be potentially beneficial for treatment of atherosclerosis or restenosis after percutaneous transluminal coronary angioplasty.

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