The Phosphorylation of Myosin II at the Ser1 and Ser2 Is Critical for Normal Platelet-derived Growth Factor–induced Reorganization of Myosin Filaments

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Phosphorylation of the regulatory light chain of myosin II (MLC20) at the activation sites promotes both the motor activity and the filament formation of myosin II, thus playing an important role in various cell motile processes. In contrast, the physiological function of phosphorylation of MLC20 at the inhibitory sites is unknown. Here we report for the first time the function of the inhibitory site phosphorylation in the cells. We successfully produced the antibodies specifically recognizing the phosphorylation sites of MLC20 at Ser1, and the platelet-derived growth factor (PDGF)-induced change in the phosphorylation at the Ser1 was monitored. The phosphorylation of MLC20 at the Ser1 significantly increased during the PDGF-induced actin cytoskeletal reorganization. PDGF disassembled the stress fibers, and this was attenuated with the expression of unphosphorylatable MLC20, at the Ser1/Ser2 phosphorylation sites. The present results suggest that the down-regulation of myosin II activity achieved by the phosphorylation at the Ser1/Ser2 sites plays an important role in the normal reorganization of actomyosin filaments triggered by PDGF receptor stimulation.

INTRODUCTION

Cell migration plays a key role in both the physiological and the pathophysiological function of the cells including development, wound healing, immunity, and metastasis (Lauffenburger and Horwitz, 1996). Reorganization of actomyosin filaments is an essential process for these cell behaviors. It has been thought that myosin II plays a fundamental role in various types of cellular motility.

In vitro biochemical studies have revealed that the function of smooth muscle and nonmuscle myosin II is regulated by the phosphorylation of MLC20 (Sellers, 1991; Tan et al., 1992). A number of studies have shown that the phosphorylation of MLC20 at Thr18 and Ser19 activates its motor activity and increases filament stability. On the other hand, it has been known that MLC20 can be phosphorylated at other sites different from the activation sites. Originally, it was found that protein kinase C (PKC) phosphorylates Ser1/Ser2 and Thr9 of MLC20. This phosphorylation decreases the affinity of myosin II phosphorylated at the activation sites for actin and the affinity of MLC20 for myosin light-chain kinase (MLCK; Nishikawa et al., 1984; Bengur et al., 1987; Ikebe et al., 1987; Ikebe and Reardon, 1990). In other words, the phosphorylation of MLC20 at these sites inhibits rather than activates myosin II.

It was reported that the phosphorylation of MLC20 at the Thr9 inhibits myosin II motor activity and the phosphorylation of MLC20 by MLCK (Nishikawa et al., 1984; Turbedsky et al., 1997); however, phosphorylation of the inhibitory sites in nonmuscle cells has only been observed on Ser1 and/or Ser2, but not on Thr9 in vivo (Kawamoto et al., 1989; Yamakita et al., 1994), and this is because the Ser1/Ser2 sites are resistant to dephosphorylation by myosin light chain phosphatases while phospho-Thr9 is readily dephosphorylated (Ikebe et al., 1999). Furthermore, it has been demonstrated that the phosphorylation of Ser1/Ser2 sites significantly inhibits its motor activity (Ikebe et al., 1987). Therefore, it is anticipated that the phosphorylation of Ser1/Ser2 sites has a role in regulating myosin II function in cells. The phosphorylation of MLC20 at Ser1/Ser2 but not Thr9 was observed during mitosis in mammalian cultured cells (Yamakita et al., 1994). However, the expression of unphosphorylatable MLC20 at the inhibitory sites did not affect the progression of oogenesis in Drosophila embryos (Rouy et al., 2002). Therefore, it is unclear whether phosphorylation of MLC20 at the inhibitory sites is involved in down-regulation of myosin II activity during Drosophila oogenesis. Although it is anticipated that the down-regulation of myosin II activity by the phosphorylation at the inhibitory sites may be important for cell motile events, the physiological significance of this inhibitory phosphorylation of myosin II on myosin function in the cells has not been well understood.

To clarify the cellular functions of the inhibitory site phosphorylation of myosin II, we developed a site-specific anti-phosphoamino acid antibody (pSer1 Ab) that specifically recognizes the phosphorylated MLC20 at the inhibitory sites (Ser1) but not the activation sites. By using this probe, we succeeded in monitoring the spatio-temporal change in myosin II phosphorylated at the Ser1/Ser2 site(s) after external stimuli. We found that the stimulation of platelet-derived...
growth factor (PDGF) mediated a transient phosphorylation of MLC20 at the Ser1/Ser2 site(s). The increase in phosphorylation at the inhibitory sites coincided with the PDGF-induced disassembly of stress fibers. Expression of unphosphorylatable MLC20 at the Ser1/Ser2 sites diminished the disassembly of stress fibers and focal adhesions in 3T3 fibroblasts. These results demonstrate that the phosphorylation at the Ser1/Ser2 sites of MLC20 regulates the dynamics of actomyosin filament formation in cells.

**MATERIALS AND METHODS**

**Materials**
Smooth muscle myosin II (Ikebe and Hartshorne, 1985a), MLCK, and protein kinase C (PKC; Ikebe et al., 1987) were prepared as described previously. Actin was prepared from rabbit skeletal muscle according to the method of Spudich and Watt (1971). LY294002, PD98059, and the PKC inhibitors, GF109203X, Go6987, and Rottlerin, were purchased from EMD Chemicals, Inc. (San Diego, CA).

**Antibodies**
The N-terminus acetylated phosphoepitope phosphohepSKRAKTC was coupled to keyhole limpet hemocyanin at the C-terminal cysteine residue by Genemed Synthesis (South San Francisco, CA). A pSer1 antibody (Ab) was affinity-purified using the phosphopeptide and then absorbed with an unphosphopeptide. A pSer19 Ab and pTS Ab specifically recognized the phosphorylated MLC20 at Ser19 and di-phosphorylated MLC20 at Thr18 and Ser19, respectively (Komatsu et al., 2000; Komatsu and Ikebe, 2004). Anti-MLC20 antibodies were from Sigma (M4401, St. Louis, MO) and Santa Cruz Biotechnology (sc-15370, Santa Cruz, CA), and anti-Myc, -paxillin, -myosin II Abs were purchased from Sigma-Aldrich, Santa Cruz Biotechnology, Transduction Laboratories (Lexington, KY), and Covance Research Products (Madison, WI). A rabbit Ab against heavy chain of myosin IIB and MLC20 were kindly provided by Dr. R. Adelstein (National Institutes of Health, Bethesda, MD) and Dr. J. Stull (University of Texas SW Medical Center).

**Cell Stimulation**
NIH3T3 fibroblast cells were maintained in DMEM containing 10% newborn calf serum. For PDGF and 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulations, NIH3T3 or MEFT3T3 Tet-Off cells were cultured for 18 h in DMEM supplemented with 0.1% newborn calf serum or 0.1% fetal bovine serum (FBS). Serum-starved cells were treated with either PDGF or TPA.

**Biochemical Procedures**
Urea/glycerol gel electrophoresis (Perrie and Perry, 1970) and SDS-PAGE (FBS). Serum-starved cells were treated with either PDGF or TPA.

**RESULTS**

Quantification Analysis
For quantification of stress fiber formation, more than 100 cells in the randomly chosen fields were recorded blindly by a confocal fluorescence microscope, and the percentage of cells having stress fibers was calculated. The values shown are means ± SD from three independent experiments (more than 100 cells/experiment). For quantification of focal adhesions (FAs), the number and the size (area) of FAs were determined by “Particle analysis” function in ImageJ (Caï et al., 2006). The FAs were determined by quantifying pixels in cells stained with Ab against paxillin. We defined paxillin foci as small FAs.

**Statistical Analysis**
All statistical analyses were performed with a Student’s t test tool.

**Plasmid Construction, Conditional Cell Lines, and Transfection**
Mutant MLC20 in which PKC phosphorylation sites (Ser1 and Ser2) were mutated to Ala was made by site-directed mutagenesis (Yano et al., 1993). Nonmuscle human myosin IIB heavy-chain cDNA was received as a gift from Dr. R. Adelstein (National Institutes of Health) and cloned into pEYFP-C1 plasmid (Clontech, Palo Alto, CA). To delete the C-terminal amino acid residues of the human myosin IIB heavy chains, a stop codon was created at the codon 1898 for 4C-Myc-Myosin IIB.

MEF/T3T3 Tet-Off cells, which can be suppressed expression of fusion proteins in the presence of doxycycline, were grown in DMEM supplemented with 10% FBS. MLC20swere subcloned into pTRE2-Hyg (Clontech) containing a myc tag sequence. The myc tag sequence was introduced at the C-terminal end of MLC20. To obtain stably expressed MLC20-myc cell lines, colonies were maintained in 0.25 mg/ml G418, 0.25 mg/ml hygromycin, and 2 µg/ml doxycycline (Invitrogen Invitrogen, BD Biosciences, Clontech).

For transient transfection, NIH3T3 cells on a coverslip were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the protocol provided by the manufacturer. For transfection of stable cell lines, MEFT3T3 Tet-Off cells were transfected with electroporation using a Gene Pulser II (Bio-Rad Laboratories, Richmond, CA; Komatsu et al., 2000).

**Production of Inhibitory Site-specific Antibody**
To monitor the extent and the spatial distribution of MLC20 phosphorylation at the inhibitory sites, we generated the phosphorylation site-specific antibodies. Because the phosphorylation of MLC20 at the inhibitory sites has only been observed on Ser1/2 but not on Thr9 in cells (Kawamoto et al., 1989; Yamakita et al., 1994), we produced the antibodies (pSer1 Ab) that raised against the N-terminal serine phosphorylation. The specificity of pSer1 Ab was examined by immunoblot analysis. Myosin II was phosphorylated by either MLCK or PKC, and the unphosphorylated, mono-, and di-phosphorylated MLC20 were separated by urea/glycerol gel electrophoresis (Figure 1A, left), followed by immunoblotting with pSer1 Ab (Figure 1A, right). MLCK phosphorylated MLC20 to yield monophosphorylated MLC20 with minor di-phosphorylated MLC20. This represents the phosphorylation at Ser19 and Ser19/Thr18, respectively (Ikebe and Hartshorne, 1985b). On the other hand, PKC produced monophosphorylated MLC20 that is composed of MLC20 containing phospho-Thr9, phospho-Ser1, and phospho-Ser2, respectively (Ikebe et al., 1987). The pSer1 Ab only recognized the phosphorylated MLC20 of myosin II by PKC, but not phosphorylated MLC20 by MLCK and unphosphorylated MLC20. To examine whether pSer1 antibodies are specific to the N-terminal serine sites, we produced a S1A/S2A MLC20 mutant. The mutant was incubated with PKC for phosphorylation and subjected to Western blot analysis using pSer1 Ab. Although PKC phosphorylated the mutant MLC20 presumably at Thr9, the pSer1 Ab did not react with the S1A/S2A MLC20 mutant incubated with PKC (Figure 1B), indicating that pSer1 Ab recognized the phosphorylated MLC20 at the N-terminal serine sites but not Thr9 (Turbedsky et al., 1997; Varlamova et al., 2001). Figure 1C shows the Western blot of whole cell lysates of mammalian cultured
activation of PKC pathway (Heldin et al., 1998). The level of MLC20 phosphorylation at Ser1 was examined by both immunostaining and immunoblotting of NIH3T3 cells with pSer1 Ab (Figure 2, A–C). Immunofluorescence images of the serum-starved NIH3T3 cells probed by pSer1 Ab showed the weak filamentous localizations of myosin II phosphorylated at Ser1/Ser2 sites of MLC20, and this was colocalized with myosin II filaments as well as actin stress fibers (Figure 2A, a, e, and i). It should be noted that the staining of the nucleus, in addition to the filamentous structures, was also observed in the serum-starved cells, suggesting that the phosphorylated MLC20 at Ser1/Ser2 seems to be present in the nucleus. This observation is similar to the previous reports that the phosphorylated MLC20 was found in the nucleus (Matsumura et al., 1998; Nagano et al., 2006). After PDGF stimulation, the disassembly of the stress fibers was observed (Figure 2A, j–l). Interestingly, the MLC20 phosphorylation at Ser1/Ser2 sites was markedly increased by PDGF and reached the maximum at 30 min after the stimulation (Figure 2B, top panel). The majority of the cells showing the disassembly of stress fibers is consistent with the time course of maximum phosphorylation of Ser1/Ser2 sites determined by Western blot analysis (Figure 2B). It should be mentioned that some cells showed the disassembly of stress-fibers at earlier times. These cells showed a relatively high level of phosphorylation of MLC20 at Ser1/Ser2 sites revealed by pSer1 Ab staining. The level of phosphorylated MLC20 at the activation sites was detected by two phospho-antibodies (pSer19 Ab and pTS Ab), which specifically recognized the phosphorylated MLC20 at Ser19 and diphosphorylated MLC20 at Thr18 and Ser19, respectively (Komatsu et al., 2000; Komatsu and Ikebe, 2004). Phosphorylation of MLC20 at the Ser19 did not increase after PDGF stimulation. The di-phosphorylated MLC20 at the activation sites was slightly elevated upon PDGF stimulation, but it was sustained thereafter (Figure 2B, middle panels). To monitor the level of phosphorylated MLC20 at the inhibitory and activation sites, the total homogenates were subjected to alkali-urea and glycerol gel electrophoresis (Figure 2C). The amount of phosphorylated MLC20 at each site was estimated from the PDGF-induced change in the amount of phosphorylation at each site (Figure 2B) and the change in the singly and doubly phosphorylated MLC20 determined by the alkali-urea/glycerol PAGE (Figure 2C). The total amount of phosphorylated MLC20 before PDGF stimulation was ~7.4 ± 1.6% of the total MLC20 estimated by alkali/urea gel (Figure 2C, lane 1). On the other hand, the total amount of phosphorylated MLC20 (monophosphorylated MLC20 + diphosphorylated MLC20) after PDGF stimulation was ~38.7 ± 2.2% of the total MLC20 (Figure 2C, lane 2). Although PDGF stimulation significantly increased the phosphorylated MLC20 at Ser1, it rather decreased the phosphorylated MLC20 recognized by pSer19Ab (Figure 2B). It should be noted that the pSer19Ab recognizes both singly and doubly phosphorylated MLC20, at the activation sites (Komatsu et al., 2000); therefore, the result suggests that the total extent of phosphorylation at the activation sites was relatively decreased after PDGF stimulation. Because the amount of phosphorylation at Ser1 before PDGF stimulation was at a negligible level, the observed mono-phosphorylated MLC20 at rest should be predominantly attributed to phosphorylated MLC20 at Ser19. The amount of phosphorylated MLC20 at the activation site (Ser19) at 30 min after PDGF stimulation was ~80% of that before PDGF stimulation (7.4 ± 1.6%; Figure 2B); therefore, it is calculated that more than 30% of the total MLC20 was phosphorylated at the Ser1/Ser2 sites after PDGF stimulation (38.7 ± 2.2 [%PDGF] – 0.8 × (7.4 ± 1.6) [Control]). The results suggest

**Phosphorylation of MLC20 at the Ser1/Ser2 Sites Correlates with PDGF-induced Disassembly of the Stress Fibers**

To investigate the role of phosphorylation of myosin II at the inhibitory sites on myosin function during cell motility, the serum-starved NIH3T3 cells were stimulated with PDGF, a potent stimulator of cell motility that is implicated in the activation of PKC pathway (Heldin et al., 1998). The level of MLC20 phosphorylation at Ser1 was examined by both immunostaining and immunoblotting of NIH3T3 cells with pSer1 Ab (Figure 2, A–C). Immunofluorescence images of the serum-starved NIH3T3 cells probed by pSer1 Ab showed the weak filamentous localizations of myosin II phosphorylated at Ser1/Ser2 sites of MLC20, and this was colocalized with myosin II filaments as well as actin stress fibers (Figure 2A, a, e, and i). It should be noted that the staining of the nucleus, in addition to the filamentous structures, was also observed in the serum-starved cells, suggesting that the phosphorylated MLC20 at Ser1/Ser2 seems to be present in the nucleus. This observation is similar to the previous reports that the phosphorylated MLC20 was found in the nucleus (Matsumura et al., 1998; Nagano et al., 2006). After PDGF stimulation, the disassembly of the stress fibers was observed (Figure 2A, j–l). Interestingly, the MLC20 phosphorylation at Ser1/Ser2 sites was markedly increased by PDGF and reached the maximum at 30 min after the stimulation (Figure 2B, top panel). The majority of the cells showing the disassembly of stress fibers is consistent with the time course of maximum phosphorylation of Ser1/Ser2 sites determined by Western blot analysis (Figure 2B). It should be mentioned that some cells showed the disassembly of stress-fibers at earlier times. These cells showed a relatively high level of phosphorylation of MLC20 at Ser1/Ser2 sites revealed by pSer1 Ab staining. The level of phosphorylated MLC20 at the activation sites was detected by two phospho-antibodies (pSer19 Ab and pTS Ab), which specifically recognized the phosphorylated MLC20 at Ser19 and diphosphorylated MLC20 at Thr18 and Ser19, respectively (Komatsu et al., 2000; Komatsu and Ikebe, 2004). Phosphorylation of MLC20 at the Ser19 did not increase after PDGF stimulation. The di-phosphorylated MLC20 at the activation sites was slightly elevated upon PDGF stimulation, but it was sustained thereafter (Figure 2B, middle panels). To monitor the level of phosphorylated MLC20 at the inhibitory and activation sites, the total homogenates were subjected to alkali-urea and glycerol gel electrophoresis (Figure 2C). The amount of phosphorylated MLC20 at each site was estimated from the PDGF-induced change in the amount of phosphorylation at each site (Figure 2B) and the change in the singly and doubly phosphorylated MLC20 determined by the alkali-urea/glycerol PAGE (Figure 2C). The total amount of phosphorylated MLC20 before PDGF stimulation was ~7.4 ± 1.6% of the total MLC20 estimated by alkali/urea gel (Figure 2C, lane 1). On the other hand, the total amount of phosphorylated MLC20 (monophosphorylated MLC20 + diphosphorylated MLC20) after PDGF stimulation was ~38.7 ± 2.2% of the total MLC20 (Figure 2C, lane 2). Although PDGF stimulation significantly increased the phosphorylated MLC20 at Ser1, it rather decreased the phosphorylated MLC20 recognized by pSer19Ab (Figure 2B). It should be noted that the pSer19Ab recognizes both singly and doubly phosphorylated MLC20, at the activation sites (Komatsu et al., 2000); therefore, the result suggests that the total extent of phosphorylation at the activation sites was relatively decreased after PDGF stimulation. Because the amount of phosphorylation at Ser1 before PDGF stimulation was at a negligible level, the observed mono-phosphorylated MLC20 at rest should be predominantly attributed to phosphorylated MLC20 at Ser19. The amount of phosphorylated MLC20 at the activation site (Ser19) at 30 min after PDGF stimulation was ~80% of that before PDGF stimulation (7.4 ± 1.6%; Figure 2B); therefore, it is calculated that more than 30% of the total MLC20 was phosphorylated at the Ser1/Ser2 sites after PDGF stimulation (38.7 ± 2.2 [%PDGF] – 0.8 × (7.4 ± 1.6) [Control]). The results suggest
that a significant fraction of MLC$_{20}$ was phosphorylated at the Ser1/Ser2 sites after PDGF stimulation.

Consistent with the Western blot data, the intensity of immunofluorescence signals of pSer1 Ab in the whole cells areas was significantly increased after PDGF stimulation (Figure 2A). The increase in the signal intensity was 1.4-, 3.5-, and 2.2-fold, at 10, 30, and 60 min after the stimulation ($n=10$), respectively. It should be noted that the signal intensity observed in Figure 2, A–D, looks high, but this is because the cells changed their shapes and significantly decreased their cell volumes. These results suggest that the phosphorylation of the Ser1/Ser2 sites of MLC$_{20}$ is involved in the PDGF-induced reorganization of actomyosin filaments.

PKC$_{a/b}$ Is Required for the PDGF-mediated Inhibitory Phosphorylation of MLC$_{20}$

The PDGF signaling pathways have been implicated in cell growth and motility coupling with the activation of protein kinases such as phosphatidylinositol 3 kinase (PI3K), p42/p44 mitogen-activated protein kinases (MAPKs), and the PKC family (Heldin et al., 1998). To determine which of the signaling pathways triggered by PDGF stimulation are responsible for MLC$_{20}$ phosphorylation at the inhibitory sites, NIH3T3 cells were pretreated with various kinase inhibitors and the effect on the MLC$_{20}$ phosphorylation at the inhibitory sites was examined. Among the inhibitors tested, only the PKC inhibitor GF109203X, having a broad inhibitory spectrum against PKC isoforms, diminished the PDGF-mediated MLC$_{20}$ phosphorylation at Ser1 (Figure 3A). This result indicates that PKC pathways are responsible for the PDGF-mediated phosphorylation of MLC$_{20}$ at the inhibitory sites.

To verify which PKC isoforms are responsible for the PDGF-mediated phosphorylation of MLC$_{20}$ at the inhibitory sites, NIH3T3 cells were pretreated with various PKC inhibitors having distinct isoenzyme specificities. The cells were examined either by Western blot or under a fluorescent microscope. The decrease in MLC$_{20}$ phosphorylation at the inhibitory sites by PKC$_{a/b}$ specific inhibitor (Go6976) as
Figure 3. Effect of the protein kinase inhibitors on the PDGF-mediated Ser1 phosphorylation. (A) Immunoblotting of PDGF-stimulated NIH3T3 cells in the presence of various kinase inhibitors. Serum-starved NIH3T3 cells were preincubated with the protein kinase inhibitors for 10 min (3 μM GF109203X, PKC inhibitor; 10 μM LY294002, PI3K inhibitor; 50 μM PD98059, MEK inhibitor; and 100 nM Go6976, p38 MAP kinase inhibitor, respectively) before PDGF (20 ng/ml) stimulation. The reaction was stopped at 30 min after stimulation, and the whole cell lysates were subjected to immunoblot analysis with pSer1 Ab (top panel) and MLC20 Ab (bottom panel), respectively. NC, no treatment with PDGF; control, 0.1% DMSO. (B) Immunoblotting of PDGF-stimulated NIH3T3 cells in the presence of PKC inhibitors. Serum-starved NIH3T3 cells were treated with 20 ng/ml PDGF for 30 min in the presence of 0.1% DMSO (control), 3 μM GF109203X, 1 μM or 100 nM Go6976, or 1 μM Rottlerin. Cells were preincubated with the PKC inhibitors for 10 min before cell stimulation. The whole cell lysates were subjected to Western blotting with pSer1 Ab (top panel) and MLC20 Ab (bottom panel), respectively. NC, no treatment with PDGF. (C) Immunostaining of PDGF-stimulated NIH3T3 cells in the presence of PKC inhibitors. Serum-starved NIH3T3 cells were treated with 20 ng/ml PDGF for 30 min under the same condition as in B. The cells were stained with pSer1 Ab (a–d), MLC20 Ab (e–h) and Alexa Fluor546-phalloidin (i–l). Bar, 25 μm.

well as GF109203X was found by Western blot analysis (Figure 3B). Consistent with this result, the decrease in the stress fibers after 30 min of PDGF stimulation was rescued by these inhibitors (Figure 3C). In contrast, Rottlerin, a PKCδ preferential inhibitor, failed to decrease the MLC20 phosphorylation (Figure 3B) and did not rescue the stress fiber disassembly induced by PDGF (Figure 3C). These results strongly suggest that Ca²⁺/CaM-dependent PKC isoforms (PKCα/β) play a critical role in the PDGF-induced disassembly of stress fibers via myosin II phosphorylation at the inhibitory sites.

Phosphorylation of MLC20 at Ser1/Ser2 Sites Is Involved in the Regulation of the Disassembly of Stress Fibers Induced by PDGF Receptor Stimulation

To study the role of phosphorylation of MLC20 at the inhibitory sites on the disassembly of stress fibers, we produced stably transfected MEF/3T3 Tet-Off cell lines expressing myc-tagged MLC20. The reconstitution of myc-tagged MLC20 to myosin II was analyzed by using an ATP-dependent actin-binding property of myosin II (see Materials and Methods for details). As shown in Figure 4, both wild-type and S1A/S2A MLC20 stable cell lines were cultured in the presence or absence of doxycycline (Dox) and were subjected to an actin-binding assay. The expression level of myc-tagged MLC20 in each clone was ~80% of the total MLC20, respectively (Figure 4, left panel: Cell lysates). The amount of myc-tagged MLC20 incorporated into myosin II was ~3.5 times higher than that of endogenous MLC20 (Figure 4, right panel). Furthermore, the localization of the myc-tagged MLC20 signal showed filamentous localization that coincides with the localization of F-actin (Figure 5, A and B). The result indicates that myc-tagged MLC20 was effectively incorporated into myosin II in the stress fibers.

To evaluate the role of the phosphorylation of MLC20 at the inhibitory sites on the disassembly of the stress fibers, the MEF/3T3 Tet-Off cells, expressing either wild-type or S1A/S2A MLC20, were cultured for 3 d without Dox to induce the expression of myc-tagged MLC20. As shown in Figure 5, the cells expressing S1A/S2A MLC20 (Figure 5B, a–c) as well as those expressing wild-type MLC20 (Figure 5A, a–c) showed filamentous localization of MLC20. This coincides with the localization of F-actin, indicating that the mutation at Ser1/Ser2 sites of MLC20 does not change the myosin II localization before PDGF stimulation. After 40 min of PDGF stimulation, the cells expressing the wild-type MLC20 showed the disassembly of stress fibers, accompanied by the decrease in the number of focal adhesion (Figure 5A, d–f). In contrast, the PDGF-induced disassembly of stress fibers was attenuated in the cells expressing the unphosphorylatable S1A/S2A MLC20 (Figure 5B, d–f). On the other hand, the majority of the cells in the presence of Dox, thus inhibiting the expression of S1A/S2A MLC20 showed the disassembly of stress fibers (Figure 5B, g–i). The fraction of the cells forming the stress fibers in the control cells was 32.7 ± 2.0 and 29.6 ± 3.6% in the presence and absence of Dox, respectively, after PDGF stimulation (Figure 5C).

On the other hand, a significant increase in the stress fiber formation was observed for the cells expressing S1A/S2A MLC20 at 40 min after PDGF stimulation. The fraction of the cells forming the stress-fibers was 21.0 ± 5.3% before the expression of S1A/S2A MLC20, and the value was significantly increased to 45.3 ± 1.5% (p < 0.003, t test; Figure 5C). We also quantified focal adhesions in controls versus S1A/S2A MLC20-expressing cells and found that the number of large focal adhesions was significantly different between the controls and S1A/S2A MLC20-expressing cells after PDGF stimulation. PDGF stimulation decreased the number of the large focal adhesions (3.22–16.05 μm²), but the decrease in the number was reduced by the expression of S1A/S2A MLC20 (p < 0.02, t test; Figure 5D).
On the other hand, the decrease in the small focal adhesions (0.535–2.675 μm²) after the stimulation was not significantly different between controls and S1A/S2A MLC20 expressing cells (p = 0.2870, 0.168, and 0.150 [Wt (Dox +/−) vs. S1A/S2A and S1A/S2A; Dox (+) versus (−)], test; Figure 5D). Similar results were obtained when NIH3T3 cells were transiently transfected with S1A/S2A MLC20-expressing vector. At 40 min after PDGF stimulation, the wild-type MLC20-transfected cells showed the disassembly of stress fibers accompanied with the decrease in the number of focal adhesion (not shown). In contrast, the expression of S1A/S2A MLC20 significantly attenuated the PDGF-induced disassembly of the stress fiber compared with the cells transfected with the wild-type MLC20 (not shown). The fraction of the cells forming the stress fibers in the wild-type transfected cells was 3.4 ± 1.5% (n = 150; 40–60 transfected cells analyzed in three independent experiments) at 40 min after PDGF stimulation. The value was the same as that of the nontransfected cells (4.0 ± 2.5%, n = 174). On the other hand, a significant increase in the stress fiber formation was observed for the cells transfected with S1A/S2A MLC20 (17.4 ± 0.7%; n = 150, p < 0.008, test). These results further support the idea that the phosphorylation of MLC20 at Ser1/Ser2 sites affects the stability of stress fibers, thus contributing to the morphological changes induced by PDGF.

Effect of Heavy-Chain Phosphorylation on the Disassembly of Stress Fibers

Previous studies have reported that PKC phosphorylates the heavy chains of nonmuscle myosin II in the nonhelical tailpiece, resulting in the inhibition of the assembly of myosin II into filaments in vitro (Murakami et al., 1998; Bresnick, 1999). Although it is not known if PDGF stimulation induces the phosphorylation of myosin II heavy chain, we examined whether the heavy-chain phosphorylation is involved in the regulation of the disassembly of stress fibers in cells. NIH3T3 cells were transfected with yellow fluorescent protein (YFP)-tagged ΔC-Myosin II B in which the nonhelical tail sequence containing multiple phosphorylation sites is deleted (Murakami et al., 1990, 1998). The expression level of wild-type and ΔC-Myosin II B in cells was estimated by measuring the fluorescence intensity of YFP signals, and the cells that expressed a similar level of the wild-type or ΔC-Myosin II B were used for the experiment. The amount of myosin II filaments in the cell expressing exogenous wild-type myosin II B was estimated by staining with anti-myosin heavy-chain II B antibodies. The signal intensities were about three times higher than that of endogenous myosin II B in the untransfected cells, suggesting that the expressed wild-type myosin II B was incorporated into the myosin filaments. We could not use the myosin IIB–specific antibodies to detect the incorporation of ΔC-Myosin II B into filaments, because the antibodies recognize the nonhelical tail domain (Phillips et al., 1995). However, because YFP-mycosin IIB with and without the nonhelical tail were incorporated into the filaments with similar extent, it is expected that ΔC-Myosin II B is also incorporated into the filaments. Before the PDGF stimulation, both the wild-type and ΔC-Myosin II B–transfected cells showed filamentous localization that coincided with the stress fibers (Figure 6A, a, e, and c, g). After 30 min of PDGF stimulation, the filamentous localization of myosin II B was diminished that correlated with the disassembly of the stress fibers (Figure 6A, b, f, and d, h). The number of cells showing PDGF-induced disassembly of stress fibers was not significantly different between wild-type and ΔC-Myosin II B–expressing cells (p > 0.98, test; Figure 6B). The role of the phosphorylation at the inhibitory sites of MLC20 and the heavy-chain phosphorylation at the tail nonhelical piece on the formation of the myosin filament structures is discussed in the Discussion.

DISCUSSION

Actomyosin contractility in nonmuscle cells plays a fundamental role in various types of cellular motility including cell migration and cell division (Lauffenburger and Horwitz, 1996; Geiger and Bershadsky, 2001; Matsumura, 2005). Despite the abundant research of myosin II functions coupled with phosphorylation of MLC20 at the activation sites, little is known about the phosphorylation of MLC20 at the inhibitory sites. The present study provides the first evidence that the phosphorylation of MLC20 at Ser1/Ser2 sites plays an important role in the normal reorganization of actomyosin structures induced by the stimulation of PDGF signaling.

Because PDGF stimulation could activate various protein kinase pathways (Heldin et al., 1998), we attempted to identify the kinase responsible for the PDGF-mediated phosphorylation of MLC20 at the inhibitory sites in cells. Using the various types of kinase-specific inhibitors, we found that PKC but not PI3 kinase and MAPKs is responsible for the phosphorylation. Furthermore, our results suggest that the phosphorylation of MLC20 at the inhibitory sites is mediated by conventional PKC isoforms α/β (Figure 3). Supporting this observation, as previously reported, conventional PKC is the major kinase responsible for the inhibitory phosphorylation of MLC20 in mitotic extracts (Varlamova et al., 2001). It was originally reported that the cdc2 kinase is responsible for the phosphorylation of MLC20 at the inhibitory sites in mitosis (Satterwhite et al., 1992); however, it was subsequently reported that there are kinases other than cdc2 kinase responsible for the phosphorylation of MLC20 at the inhibitory sites in mitotic cells from mammalian cultured cells (Yamakita et al., 1994) and sea urchin eggs (Komatsu et al., 1997). An in vitro biochemical study has shown that
PKC\textsubscript{H9251} has approximately threefold greater catalytic activity than PKC\textsubscript{H9252} for MLC\textsubscript{20} as a substrate and that PKC\textsubscript{H9251} phospho-
phorylates Ser1/2 and Thr9 of MLC\textsubscript{20}, whereas PKC\textsubscript{H9252} predominantly phosphorylated Thr9 of MLC\textsubscript{20} (Varlamova \textit{et al.}, 2001). Furthermore, PKC\textsubscript{H9251} is the most abundant conventional PKC isoforms in the NIH3T3 cells (Goodnight \textit{et al.}, 1995). Therefore, we concluded that the phosphorylation of MLC\textsubscript{20} at the inhibitory sites upon PDGF stimulation is predominantly catalyzed by conventional PKC\textsubscript{H9251}.

It was previously reported that expression of the charge reversal form of the MLC\textsubscript{20} mutant at the activation sites (substitution of Thr18 and Ser19 by Asp) promotes stable stress fiber formation in NIH3T3 cells (Amano \textit{et al.}, 1998) and that the increase in MLC\textsubscript{20} phosphorylation at the activation sites, via inhibition of myosin phosphatase, induces both formation of stress fibers and focal adhesions (Totsukawa \textit{et al.}, 2000). In addition, the mutation of the activation sites of MLC\textsubscript{20} to unphosphorylatable residues (S19A/Thr18/A) results in the inhibition of the myosin II contractile activity (Komatsu \textit{et al.}, 2000) and the reduction of the number of actomyosin filaments (Komatsu and Ikebe, unpublished observations). These results suggest that the activation of the myosin activity is critical to induce the formation of myosin filaments and necessary to maintain the structure of stress fibers and focal adhesions (Figure 7A).

Recently it was reported that PDGF stimulation triggers the transient phosphorylation of Rho GTPase family member RhoE in NIH3T3 cells (Riento \textit{et al.}, 2005) and proposed that RhoE phosphorylation increases the stability of RhoE protein resulting in the disruption of stress fibers through motor activity.
the inhibition of signaling downstream of RhoA (Guasch et al., 1998; Riento et al., 2005). Other Rho family members Rac and Cdc42 have also been shown to be transiently activated by PDGF stimulation thus attenuating the stress fiber formation (Sander et al., 1999; Jimenez et al., 2000). On the basis of these observations together with our present data, we propose that the PKC-dependent down-regulation of myosin motor activity and the down-regulation of RhoA concertedly control the change in actin cytoskeletal structure upon PDGF stimulation (Figure 7B).

How does the phosphorylation at the inhibitory sites induce disassembly of stress fiber and the decrease in the focal adhesion? It was previously reported that the phosphorylation at the inhibitory sites inhibits the motor activity of myosin II phosphorylated at the activation sites, but not the myosin filament formation (Nishikawa et al., 1984; Ikebe et al., 1987; Ikebe and Reardon, 1990). Therefore, we think that the inhibition of myosin II motor activity by the phosphorylation of MLC20 at the inhibitory sites is in part responsible for the PDGF-induced disassembly of stress fibers and the decrease in the focal adhesion. Supporting this view, blebbistatin, a specific inhibitor of actin-activated myosin II ATPase activity but not the filament formation, blocked the formation of actomyosin stress fibers and focal adhesions (Straight et al., 2003; Kovacs et al., 2004; Hotulainen and Lappalainen, 2006). This result suggests that the myosin motor activity is critical for the formation of stress fibers and focal adhesions. In favor of this view, Burridge and coworkers suggested that the activation of myosin II activity produces the force driving the formation of stress fibers and focal adhesions (Chrzanoswka-Wodnicka and Burridge, 1996). Because stress fibers are thought to attach to the focal adhesions that provide the force required for anchoring them to the cell matrix (Geiger and Bershadsky, 2001; Geiger et al., 2001), it is therefore likely that the decrease in the myosin II-driven force by the phosphorylation of MLC20 at the inhibitory sites influences the formation of focal adhesions as well as the stress fibers.

Previous in vitro biochemical studies have suggested that the phosphorylation of myosin II at the nonhelical tail plays a role in the disassembly of myosin filamentous structures (Murakami et al., 1998, 2000; Dulyaninova et al., 2005). In the present study, the expression of S1A/S2A MLC20 attenuated the PDGF-induced disassembly of stress fibers (Figure 5), whereas the expression of ΔC-Myosin IIB did not significantly change the disassembly of stress fibers (Figure 6). It was recently reported that phosphorylation of nonmuscle myosin heavy-chain (MHC)-II-B at the tail by atypical PKC zeta (αPKCζ) destabilizes the myosin IIB filaments in an epidermal growth factor (EGF)-dependent manner (Even-Faitelson and Ravid, 2006). However, αPKCζ did not phosphorylate the MLC20 (Varlamova et al., 2001). It is therefore likely that the phosphorylation of MLC20 and MHC by PKC isoforms are differentially involved in the reorganization of actomyosin filaments during the cell motile events modulated based on the types of extracellular stimuli. Interestingly, it was reported that bradykinin and EGF-mediated MHC-IIA and -IIB phosphorylation is associated with a loss of cortical myosin II (van Leeuwen et al., 1999; Straussman et al., 2001). These previous works also suggest that MHC phosphorylation is involved in the regulation of microfilament disassembly in the cell cortex. Taking these findings together, it is plausible that the phosphorylation of MLC20 at the inhibitory sites and MHC phosphorylation at the nonhelical tail play distinct roles in the actomyosin dynamics in different spatial domains in cells. This view is supported by the recent report showing that the expressed MHC-IIB, in
which the PKC phosphorylation sites were converted to Asp residues, was able to localize at the stress fibers, but this mutation attenuated the localization of the mutant MHC-IIB in the cells. Previous studies, we propose a model for reorganization of the actomyosin structure upon PDGF stimulation in the cells. The initial stage of the PDGF receptor stimulation is the dynamic cytoskeletal rearrangement involving a decrease in the stress fiber and a reduction in the focal adhesion complexes. Present results suggest that the down-regulation of myosin II activity via the phosphorylation of MLC20 at the Ser1/Ser2 sites is the factor determining reorganization of actomyosin structure in this process. It has been thought that the mechanical force linking the focal adhesion and the cytoskeleton influences the stress fiber formation, and we think that the down-regulation of the contractile activity of myosin II by PDGF-mediated phosphorylation at the Ser1/Ser2 sites facilitates the normal disassembly of the stress fibers and the actin cytoskeletal reorganization. The later stage of PDGF receptor stimulation is the promotion of cell motility. The force generated by the activation of myosin II motor activity is thought to be essential for this motile process. Recently, we found that the disruption of zipper-interacting protein (ZIP) kinase by siRNA decreases the myosin II phosphorylation at the activation sites and leads to the inhibition of PDGF-induced cell migration as well as wound healing of NIH3T3 cells.

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