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ZIP kinase is responsible for the phosphorylation of myosin II and necessary for cell motility in mammalian fibroblasts

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Reorganization of actomyosin is an essential process for cell migration and myosin regulatory light chain (MLC_{20}) phosphorylation plays a key role in this process. Here, we found that zipper-interacting protein (ZIP) kinase plays a predominant role in myosin II phosphorylation in mammalian fibroblasts. Using two phosphorylation site-specific antibodies, we demonstrated that a significant portion of the phosphorylated MLC_{20} is diphosphorylated and that the localization of mono- and diphosphorylated myosin is different from each other. The kinase responsible for the phosphorylation was ZIP kinase because (a) the kinase in the cell extracts phosphorylated Ser19 and Thr18 of MLC_{20} with similar potency; (b) immunodepletion of ZIP kinase from the cell extracts markedly diminished its myosin II kinase activity; and (c) disruption of ZIP kinase expression by RNA interference diminished myosin phosphorylation, and resulted in the defect of cell polarity and migration efficiency. These results suggest that ZIP kinase is critical for myosin phosphorylation and necessary for cell motile processes in mammalian fibroblasts.

Introduction

Phosphorylation of the myosin regulatory light chain (MLC_{20}) is critical for the contractile activity in smooth muscle and motile events in nonmuscle cells (Hartshorne, 1987; Kamm and Stull, 1989; Tan et al., 1992). The phosphorylation of MLC_{20} at Ser19 and Thr18 activates the motor activity of smooth muscle and nonmuscle myosin II (Ikebe and Hartshorne, 1985b; Ikebe et al., 1986; Ikebe and Reardon, 1990), whereas the phosphorylation of Ser1/Ser2 and Thr9 rather inhibit the actomyosin ATPase activity due to the decrease in the affinity for actin (Nishikawa et al., 1984; Ikebe et al., 1987a). Although Ca^{2+}/CaM-dependent myosin light chain kinase (MLCK) can phosphorylate Thr18 of MLC_{20} in addition to Ser19, the rate of phosphorylation of the former site is much slower than the latter site (Ikebe and Hartshorne, 1985b); therefore, it has been thought that the phosphorylation at Ser19 is physiologically an important site for smooth muscle myosin II, thus the regulation of smooth muscle contraction. On the other hand, the physiological significance of Thr18 phosphorylation is obscure. Nevertheless, Thr18 phosphorylation occurs in vivo and it has been reported that diphosphorylation of MLC_{20} at Thr18 and Ser19 occurs in smooth muscle induced by external stimuli (Colburn et al., 1988; Singer, 1990) and in nonmuscle cells in conjunction with the cellular shape change and exocytosis (Itoh et al., 1992; Choi et al., 1994). It has been demonstrated that diphosphorylation of MLC_{20} at Thr18 and Ser19 increases actomyosin ATPase activity more than that of monophosphorylation at Ser19 (Ikebe and Hartshorne, 1985b). Furthermore, the phosphorylation of myosin at Thr18 in addition to Ser19 significantly stabilizes the filament formation of myosin II in vitro (Ikebe et al., 1988). It is likely that Thr18 phosphorylation of MLC_{20} plays a more significant role in nonmuscle cells where the polymerization–depolymerization of myosin is thought to be dynamically regulated.

Although MLCK is thought to be responsible for myosin II phosphorylation, recent studies have suggested that other protein kinases might also contribute to phosphorylation of myosin II. Amano et al. (1996) showed that Rho-associated kinase (Rho-kinase) phosphorylates MLC_{20} at Ser19. Recently it was reported that zipper-interacting protein (ZIP) kinase

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Abbreviations used in this paper: DIC, differential interference contrast; MBS, myosin binding subunit; MLC_{20}, myosin regulatory light chain; MLCK, myosin light chain kinase; MLCP, myosin phosphatase; Rho-kinase, Rho-associated kinase; RNAi, RNA interference; siRNA, small interfering RNA; ZIP, zipper-interacting protein.

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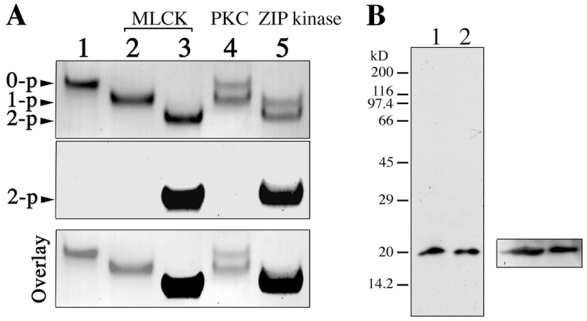

Figure 1. **Specificity of pTS Ab against the diphosphorylated regulatory light chain of myosin.** (A) Unphosphorylated or phosphorylated MLC_{20} either by MLCK or PKC were separated by urea/glycerol gel (top), followed by immunoblotting with pTS Ab (middle). Lane 1, unphosphorylated MLC_{20}; lane 2, monophosphorylated MLC_{20} by MLCK; lane 3, diphosphorylated MLC_{20} by MLCK; lane 4, mono- and diphosphorylated MLC_{20} by PKC; lane 5, mono- and diphosphorylated MLC_{20} by ZIP kinase. Note that pTS Ab reacted with diphosphorylated MLC_{20}, but not monophosphorylated MLC_{20} and phosphorylated MLC_{20} by PKC. (B) Immunoblot of whole cell lysates with pTS Ab. The whole cell lysates of (lane 1) REF-2A fibroblast or (lane 2) NRK epithelial cells were analyzed by immunoblotting with pTS Ab. (Right) Immunoblot with anti-MLC_{20} mAb, showing the total amount of MLC_{20}.

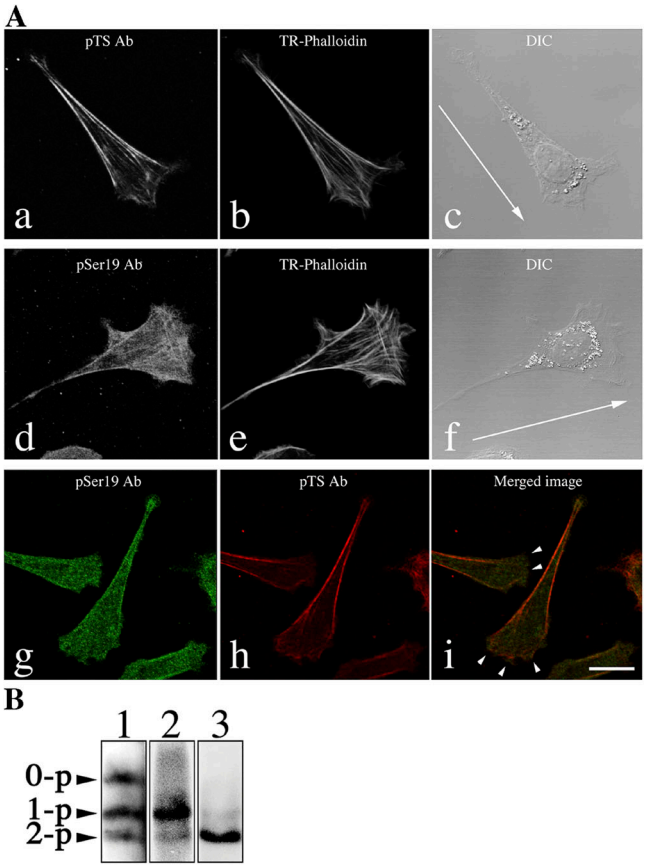

Figure 2. **Distribution of mono- and diphosphorylated myosin II in motile fibroblasts.** (A) REF-2A fibroblasts were stained with pTS Ab and pSer19 Ab. Panels a–i show the immunofluorescent images of motile fibroblasts. (a and h), pTS Ab; (d and g), pSer19 Ab; (b and e) Texas red–phalloidin; (c and f) DIC image. A motile REF-2A cells were subjected to double staining with (g) pSer19 Ab and (h) pTS Ab. (i) was merged image of g and h. Arrows in c and f show the direction of cell locomotion. Bar, 20 μm. Note the strong signal of pTS Ab at the tail portion of the motile cells and relatively weak at the anterior region, whereas the signals of pSer19 Ab at the posterior and anterior regions are nearly the same. (B) The whole cell lysates of REF-2A fibroblast were subjected to urea/glycerol gel electrophoresis, followed by immunoblotting with (lane 1) anti-MLC_{20} pAb, (lane 2) pSer19 Ab, and (lane 3) pTS Ab.

(Murata-Hori et al., 1999) and integrin-linked kinase (Deng et al., 2001) phosphorylate MLC_{20} at Ser19 and Thr18. Interestingly, ZIP kinase phosphorylate Ser19 and Thr18 of MLC_{20} with similar potency in contrast to MLCK (Niiro and Ikebe, 2001). These findings have raised a hypothesis that myosin II can be phosphorylated by various protein kinases in cells by diverse stimulations. On the other hand, it has been realized that the phosphorylation level of myosin II is also controlled by regulating myosin phosphatase (MLCP). Kimura et al. (1996) showed that the myosin binding subunit (MBS) of MLCP is phosphorylated by Rho-kinase and the phosphorylation down-regulates MLCP activity. The phosphorylation site of MBS responsible for the down-regulation of MLCP is Thr641 (rat sequence; Feng et al., 1999) and it was found subsequently that MBS can be phosphorylated at Thr641 by various kinases including ZIP kinase like kinase (MacDonald et al., 2001) and integrin-linked kinase (Kiss et al., 2002; Muranyi et al., 2002) suggesting that MLCP activity is regulated via multiple signaling pathways.

Phosphorylation of myosin II has been thought to be critical for the various actin-based contractile events in nonmuscle cells (Tan et al., 1992; Komatsu et al., 2000). Because the assembly and the motor activity of myosin II is regulated by MLC_{20} phosphorylation, the localization of phosphorylated MLC_{20} would reflect the distribution of activated phosphorylated myosin II in motile cells. A critical question is whether the localization of myosin II phosphorylated at only Ser19 and at both Ser19 + Thr18 of MLC_{20} are different from each other and whether this is related to the function of myosin at particular cellular compartment, because the diphosphorylation of MLC_{20} significantly facilitates the formation of stable myosin filaments (Ikebe et al., 1988; Kamisoyama et al., 1994).

Here, we identified that ZIP kinase is responsible for myosin phosphorylation in motile fibroblasts. The present paper also showed that there is a significant amount of diphosphorylated myosin in motile cells whose localization is different from the monophosphorylated one and plays an important role in the maintenance of cell morphology and migration. ZIP kinase phosphorylating Thr18 and Ser19 of MLC_{20} with the same potency is primarily responsible for this event.

Results

Production of the antibodies that specifically recognize diphosphorylated MLC_{20}

Different states of myosin phosphorylation (single Ser19 or both Ser19 and Thr18 sites) exhibited considerable differences both in the actin activated ATPase activity and filament stability of myosin in vitro (Ikebe and Hartshorne, 1985b). To investigate the functional difference in diphos-

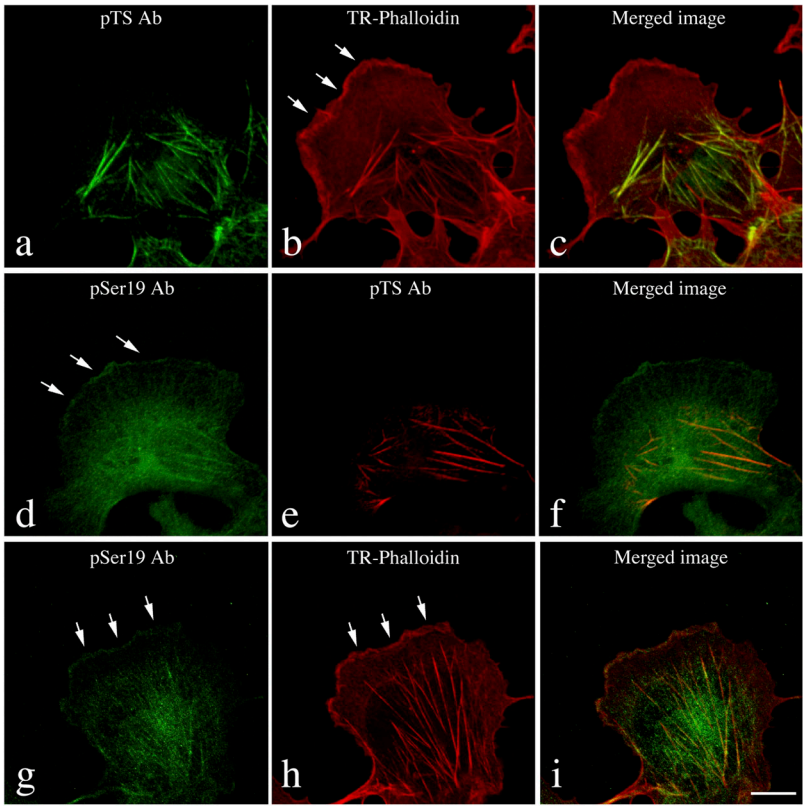

Figure 3. **Differential localization of mono- and diphosphorylated myosin II in migrating COS 7 cells.** COS 7 cells were stained with pTS Ab (a, green) and pSer19 Ab (g, green), and with Texas red–phalloidin (b and h, red). Panels c and i show merged image of a and b, and g and h, respectively. Panels d–f were double staining with pSer19 Ab (d, green) and pTS Ab (e, red). Panel f show merged image of d and e. Arrows indicate the membrane ruffling area in the leading edge. Bar, 16 μm.

phorylated and monophosphorylated myosin II in cell motile process, we developed the specific antibodies differentially recognizing the mono- and diphosphorylated myosin. Previously, we produced the antibodies (pSer19 Ab) that recognized the phosphorylated MLC_{20} of myosin II at Ser19 (Komatsu et al., 2000). Although this antibody is useful to detect the Ser19 phosphorylated myosin II, it cannot distinguish between the singly phosphorylated MLC_{20} and diphosphorylated MLC_{20} at both Ser19 and Thr18, although the antibodies recognizes the single phosphorylated MLC_{20} at Ser19 stronger than the diphosphorylated MLC_{20}. To overcome this problem, we developed the antibodies that recognize diphosphorylated MLC_{20} but not MLC_{20} phosphorylated at only Ser19.

The specificity of pTS Ab was examined by immunoblot analysis. MLC_{20} was phosphorylated by MLCK, PKC, and ZIP kinase. The unphosphorylated, mono-, and diphosphorylated MLC_{20}s were separated on an urea/glycerol gel (Fig. 1 A, top), followed by immunoblotting with pTS Ab (Fig. 1 A, middle). The pTS Ab only recognized the diphosphorylated MLC_{20} by MLCK and ZIP kinase, but did not recognized unphosphorylated, monophosphorylated by MLCK and ZIP kinase, or monophosphorylated (Thr9) MLC_{20} by PKC. It was shown previously that ZIP kinase phosphorylates Ser19 and Thr18 of MLC_{20} with same rate constant thus yielding the same amount of Ser19 phosphorylated MLC_{20} and Thr18 phosphorylated MLC_{20} (Niiro and Ikebe, 2001). Therefore, the results shown in Fig. 1 indicate that pTS Ab specifically recognizes diphosphorylated MLC_{20}. Fig. 1 B shows the immunoblotting of whole cell lysates of REF-2A fibroblast and NRK epithelial cells. The result indicates that the antibodies specifically recognize MLC_{20} but not other proteins. pTS Ab and pSer19 Ab recognizing the Ser19 phosphorylated MLC_{20} were used as probes to determine the distribution of di- and monophosphorylated myosin II at MLC_{20} in motile fibroblasts.

Distribution of di- and monophosphorylated MLC_{20} in motile fibroblasts

Fig. 2 A shows the immunofluorescent images of motile fibroblast cells. A REF-2A fibroblast cell exhibits a polarized cell shape that is characteristic of the motile cells (Fig. 2 A, c). The staining with pTS Ab (Fig. 2 A, a) revealed filamentous localizations of diphosphorylated MLC_{20} and this was superimposed with F-actin localization (Fig. 2 A, b). The strong signal was detected at the peripheral tail portion in motile cells, whereas the signal at the anterior region was weak (Fig. 2 A, a). On the other hand, the signals by pSer19 Ab that recognized the phosphorylated MLC_{20} of myosin at Ser19 appeared to be strong at both posterior and anterior regions (Fig. 2 A, d). This observation is consistent with that of Matsumura et al. (1998). To further clarify the differential localization of the mono- and diphosphorylated MLC_{20}, the motile cells were subjected to dual immunostaining with pTS Ab and pSer19 Ab. The cells were first stained with the polyclonal pTS Ab and then followed by monoclonal pSer19 Ab. The merged image of pSer19 Ab (Fig. 2 A, g, green) and pTS Ab (Fig. 2 A, h, red) signals shows that the diphosphorylated MLC_{20} was accumulated at the tail (Fig. 2 A, i). In contrast, the ruffling membrane area of anterior region (Fig. 2 A, i, arrowheads) appeared greenish, indicating that monophosphorylated MLC_{20} is enriched more than diphosphorylated MLC_{20} in this area. It should be noted

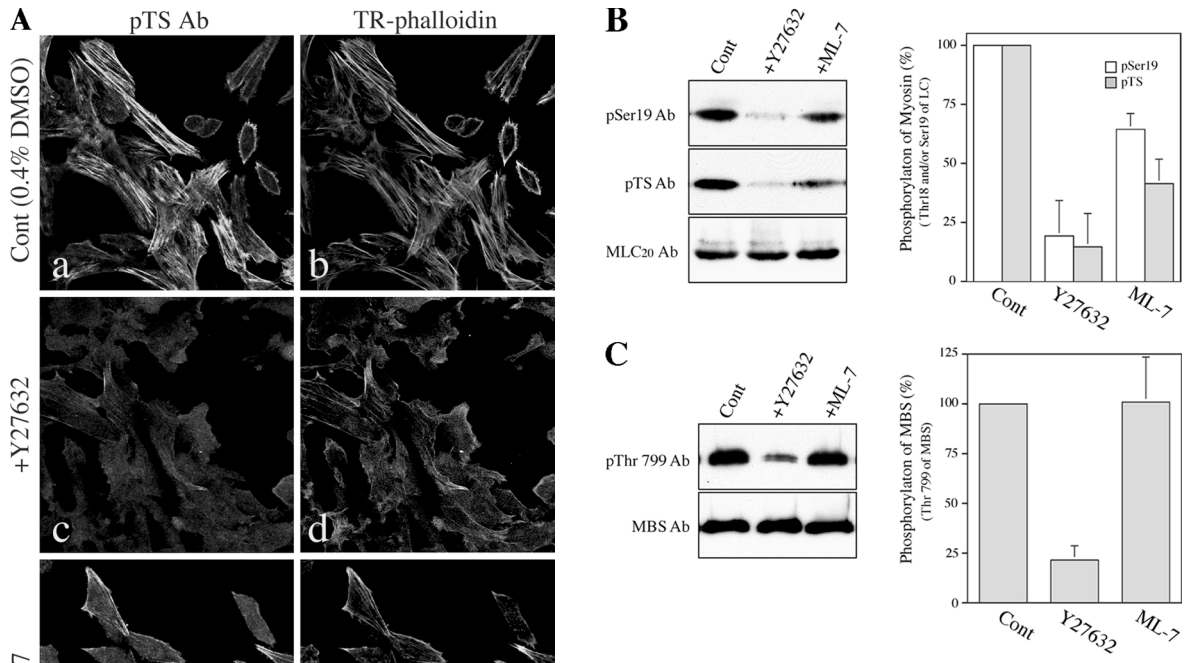

Figure 4. **Effect of the protein kinase inhibitors on the localization of diphosphorylated myosin II in REF-2A cells.** (A) REF-2A cells treated with 0.4% DMSO (a and b), 10 μM Y27632 (c and d) or 40 μM ML-7 (e and f). The cells were stained with pTS Ab (a, c, and e) and with Texas red–phalloidin (b, d, and f). Bar, 20 μm. The whole cell lysates treated with 10 μM Y27632, 40 μM ML-7 or 0.4% DMSO were subjected to Western blotting with (B) pSer19 Ab (top), pTS Ab (middle), or MLC_{20} Ab (bottom), respectively. (C) Immunoblotting with pThr 799 Ab (top) or MBS Ab (bottom). The amount of phosphorylated myosin II (B) or MBS (C) was determined by scanning densitometry (NIH image program). The densities of the phospho-antibody bands were normalized with those of pan antibody bands. The values shown are means ± SD from three independent experiments.

that diphosphorylated MLC_{20} shows strong stress fiber localization. To monitor the level of mono- and diphosphorylated MLC_{20} in cells, the total homogenates were subjected to urea/glycerol gel electrophoresis. As shown in Fig. 2 B, significant level of diphosphorylated MLC_{20} was observed. It should be noted that a significant level of unphosphorylated MLC_{20} was present, whereas a significant level of MLC_{20} was diphosphorylated. pTS antibody only recognized the diphosphorylated MLC_{20} of total cell homogenates. The result is consistent with Fig. 1 and further warrants that pTS antibody staining shows the localization of diphosphorylated MLC_{20} in cells.

The subcellular distribution of MLC_{20} in different phosphorylation states was also observed with spreading COS 7 cells. The diphosphorylated MLC_{20} was colocalized with actin stress fibers (Fig. 3, b and c), but not the membrane ruffling area at the leading edge (Fig. 3, a and c). In contrast, the significant level of pSer19 Ab immunoreactivity was observed at the membrane ruffling area in addition to the stress fibers (Fig. 3, d–i). The fluorescence signal of monophosphorylated myosin II at the membrane ruffling area divided by entire signal was approximately six times higher than that of diphosphorylated myosin II. These observations indicate that the localization of mono- and diphosphorylated myosin in migrating cells is different to each other. Because diphosphorylation of MLC_{20} stabilizes thick filaments (Ikebe et al., 1988), the result suggests that diphosphorylated myosin forming stable filaments are incorporated into large stress fiber structure, whereas monophosphorylated myosin is present at the area where dynamic rearrangement of myosin structure takes place.

Inhibition of myosin phosphorylation and disruption of myosin filaments in vivo

Previously, it was reported for NIH3T3 fibroblasts that MLCK is responsible for the phosphorylation of myosin at the peripheral region and that the assembly of stress fiber at the central region is mediated by Rho-kinase based upon the use of the specific kinase inhibitors (Totsukawa et al., 2000). Thus, we wondered whether the level of diphosphorylated myosin at both regions is reduced by inhibition of these kinases. The effect of kinase inhibitors on the localization of diphosphorylated myosin in REF-2A cells is shown in Fig. 4. In control cells, diphosphorylated myosin was strongly localized at the thick stress fibers in nonmotile cells (Fig. 4 A, a; Fig. 2). Rho-kinase inhibitor, Y27632, induced to disassemble stress fibers (Fig. 4 A, d) and simultaneously decreased the extent of the diphosphorylated myosin filaments (Fig. 4 A, c). On the other hand, ML-7 significantly decreased the diphosphorylation of myosin at the central region of the cell (Fig. 4 A, e), but a significant level of diphosphorylated myosin II was observed at the cell peripheral region. Consistent with these observations, myosin phosphorylation was diminished by ~60% for monophosphory-

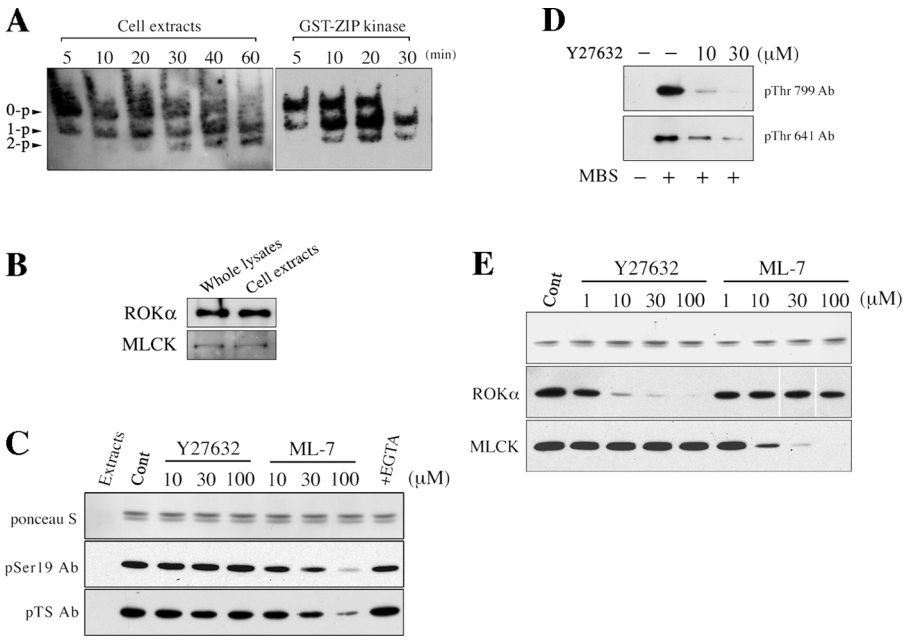

Figure 5. **Myosin phosphorylation activity in cell extracts.** (A) Time course of myosin phosphorylation by cell extracts and GST-ZIP kinase. The reaction was terminated at indicated time. Phosphorylated myosin was subjected to urea/glycerol gel electrophoresis, followed by immunoblotting with anti-MLC_{20} Ab. (B) Extraction of ROKα and MLCK. Whole lysates and cell extracts were subjected to Western blotting with ROKα Ab and MLCK Ab. (C) Effects of kinase inhibitors and EGTA on myosin phosphorylation activity in cell extracts. Myosin was phosphorylated with cells extracts and the signals were detected with pSer19 Ab (middle) or pTS Ab (bottom). Transfer membranes were stained with ponceau S (top). Assay was done in 0.2 mM $CaCl_2$ except for lane 9. (D) Rho-kinase activity in cell extracts. MBS was phosphorylated with cell extracts in the presence of Y27632 and phosphorylated MBS was detected with pThr 641 Ab or pThr 799 Ab. (E) Effects of kinase inhibitors on myosin phosphorylation by Rho-kinase (middle) or MLCK (bottom). The signal was detected with pSer19 Ab.

lation and 40% for diphosphorylation by treatment with 40 μM ML-7 (Fig. 4 B). On the other hand, the treatment of the cells with 10 μM Y27632 decreased both the mono- and diphosphorylated MLC_{20} to 20% of that of control (Fig. 4 B). The phosphorylation level of MBS at Thr799, that was one of the major phosphorylation sites by Rho-kinase (Kawano et al., 1999), was also decreased to 21% (Fig. 4 C). In contrast, treatment with ML-7 had no detectable effect on MBS phosphorylation at Thr799 (Fig. 4 C). It was reported previously that Y27632 activates MLCP activity (Uehata et al., 1997) and the present result was consistent with this earlier result. Therefore, the results suggest that the decrease in MLC_{20} phosphorylation by Y27632 is due to the activation of MLCP by Rho-kinase–induced MBS dephosphorylation, whereas ML-7 decreases MLC_{20} phosphorylation due to the inhibition of myosin II kinase but not the change in MLCP activity because ML-7 did not change the phosphorylation level of MBS. To verify whether the decrease in MLC_{20} phosphorylation by ML-7 is due to the inhibition of MLCK, we examined the effect of wortmannin on MLC_{20} phosphorylation in cells. Wortmannin treatment did not change MLC_{20} phosphorylation level (unpublished data), suggesting that ML-7–induced decrease in MLC_{20} phosphorylation is due to the inhibition of other kinases.

Myosin phosphorylation activity in cell extracts

To identify the candidate kinases for MLC_{20} phosphorylation, myosin II kinase activity was measured with cell extracts of REF-2A cells. Purified myosin II was incubated with the cell extracts in the presence of MLCP inhibitor (Microcystin-LR) and then the phosphorylation of myosin II was examined by Western blot analysis using pSer19 Ab or pTS Ab. As shown in Fig. 5 A, the cell extracts produced significant level of diphosphorylated MLC_{20}. The extents of mono- and diphosphorylated MLC_{20} were estimated by using urea/glycerol gel electrophoresis. The diphosphorylated MLC_{20} appeared whereas a significant portion of MLC_{20} remained unphosphorylated (Fig. 5 A). The results suggest that the protein kinases responsible for MLC_{20} phosphorylation phosphorylate Ser19 and Thr18 with similar potency.

Because it is known that MLCK and Rho-kinase phosphorylate MLC_{20} in vitro, we examined whether these kinases are responsible for MLC_{20} phosphorylation. We first examined whether or not these kinases are extracted. The total cell homogenates and the cell extracts were subjected to Western blot analysis with ROKα Ab and MLCK Ab as probes (Fig. 5 B). The signals of whole lysates and cell extracts in Fig. 5 B are similar to each other, indicating that majority of these kinases are recovered in the cell extracts.

As shown in Fig. 5 C, Rho-kinase inhibitor, Y27632 (maximal 100 μM), had no significant effect on myosin phosphorylation in the cell extracts. To examine whether Rho-kinase in the cell extracts is active, we used MBS as a substrate for Rho-kinase (Kimura et al., 1996). MBS was incubated with the extracts and followed by immunoblotting with pThr 641 Ab or pThr 799 Ab that recognizes the phosphorylated MBS at the two Rho-kinase–induced phosphorylation sites, Thr641 or Ser799, respectively (Kawano et al., 1999). As shown in Fig. 5 D, the cell extracts phosphorylated MBS, and the phosphorylation was inhibited by Y27632. The results indicate that there is significant Rho-kinase activity in the cell extracts, but Rho-kinase does not significantly phosphorylate myosin.

In contrast, the MLCK inhibitor, ML-7, inhibited myosin II phosphorylation by the cell extracts (Fig. 5 C). Myosin phosphorylation in Ca^{2+} by the cell extracts was decreased by 50% with 30 μM ML-7 (Fig. 5 C), whereas the phosphorylation by isolated Rho-kinase was not significantly in-

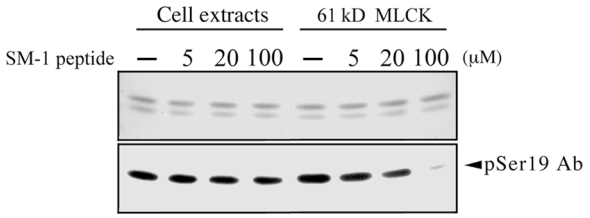

Figure 6. **Effects of SM-1 peptide inhibitor of MLCK on myosin MLC_{20} phosphorylation by cell extracts.** Myosin II was phosphorylated by cell extracts or Ca^{2+}/CaM-independent 61-kD MLCK fragment in the presence of SM-1 peptide. (bottom) pSer19 Ab; (top) ponceau S staining.

hibited by ML-7 even with 100 μM (Fig. 5 E, middle). However, myosin phosphorylation activity in the cell extracts was not affected by the elimination of Ca^{2+} (Fig. 5 C, +EGTA) and this is contradictory to the fact that MLCK requires Ca^{2+}/CaM for its activity. One possibility to account for this discrepancy is that MLCK might become the constitutively active form by proteolysis during the preparation steps of cell extracts (Ikebe et al., 1987b). To address this possibility, MLCK-specific peptide inhibitor, SM-1, which strongly inhibits both native and constitutively active MLCK (Ikebe et al., 1987b), was examined for the inhibition of the kinase activity in the cell extracts. As shown in Fig. 6, SM-1 peptide inhibitor did not inhibit the kinase activity in the cell extracts, whereas it significantly inhibited the 61-kD constitutively active form of MLCK. These results indicate that major myosin II kinase activity in the cell extract was neither Rho-kinase nor MLCK. These results together with the results of Figs. 4–6 suggest that ML-7 sensitive kinases other than Ca^{2+}/CaM-dependent MLCK are responsible for myosin phosphorylation in fibroblast cells.

ZIP kinase is involved in myosin phosphorylation activity in cell extracts

The above results suggest that the myosin II kinase responsible for the phosphorylation of myosin II in the motile cells can phosphorylate Ser19 and Thr18 with similar potency. Previously, it was reported that ZIP kinase phosphorylates MLC_{20} of myosin at Thr18 and Ser 19 with the same rate constant in a Ca^{2+}-independent manner and that ZIP kinase is not inhibited by Y27632 (Niiro and Ikebe, 2001). Therefore, we examined the time course of the production of mono- and diphosphorylated MLC_{20} by the cell extracts. This time course was compared with that of ZIP kinase. The myosin II kinase in the cell extracts showed the similar pattern of mono- and diphosphorylated MLC_{20} production as ZIP kinase (Fig. 5 A). The present results together with the previous findings raise a possibility that ZIP kinase is involved in the phosphorylation of myosin II. To address whether ZIP kinase activity is responsible for myosin II phosphorylation in cells, the cell extracts were immunodepleted by ZIP kinase Ab. As shown in Fig. 7 A, the immunodepletion markedly diminished the myosin II phosphorylation activity of the cell extracts as compared with the mock-treated extracts in both Ca^{2+} and EGTA. The immunodepletion eliminated 75% of the total myosin II kinase

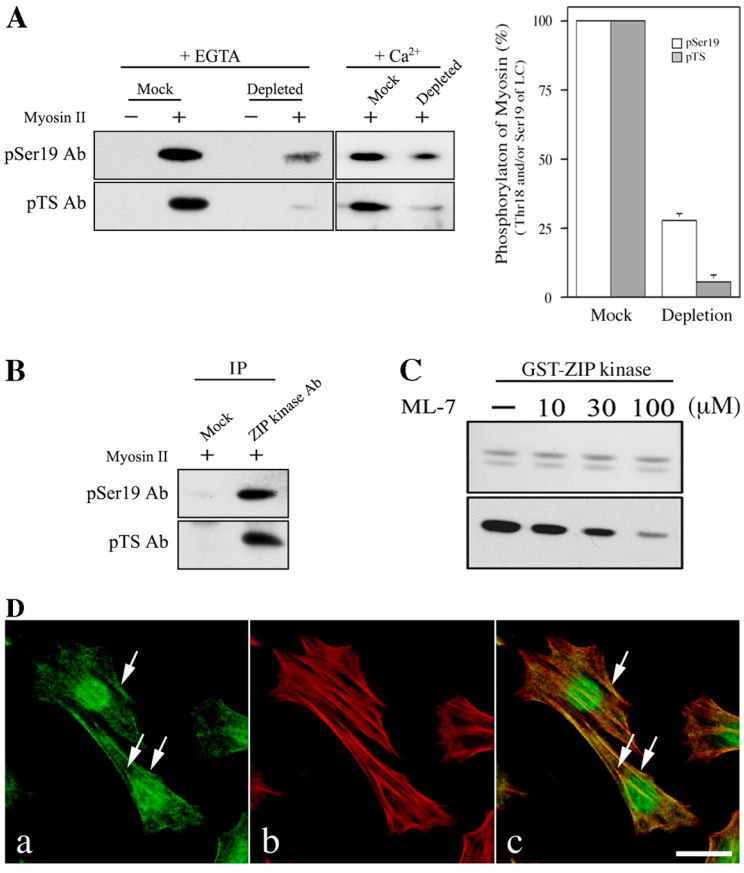

Figure 7. **Determination of ZIP kinase as myosin II MLC_{20} kinase in cultured cells.** (A) Cell extracts were subjected to immunodepletion with either nonspecific rabbit IgGs (mock) or anti-ZIP kinase Ab. (Left) Western blot of myosin II phosphorylated by either mock- or ZIP kinase–depleted cell extracts in EGTA or Ca^{2+} with pSer19 Ab or pTS Ab. (Right) Decrease in myosin II phosphorylation by immunodepletion in EGTA condition. The values shown are means ± SD from three independent experiments. (B) Myosin II was phosphorylated by immunoprecipitated ZIP kinase. (C) Effects of ML-7 on myosin II phosphorylation by ZIP kinase. Myosin phosphorylated by GST-ZIP kinase was examined with pSer19 Ab. (D) Localization of ZIP kinase in REF-2A fibroblasts. (a) ZIP kinase Ab; (b) Texas red–phalloidin; (c) merged image of a and b. Arrows indicate the colocalization of ZIP kinase with actin fibers. Bar, 20 μm.

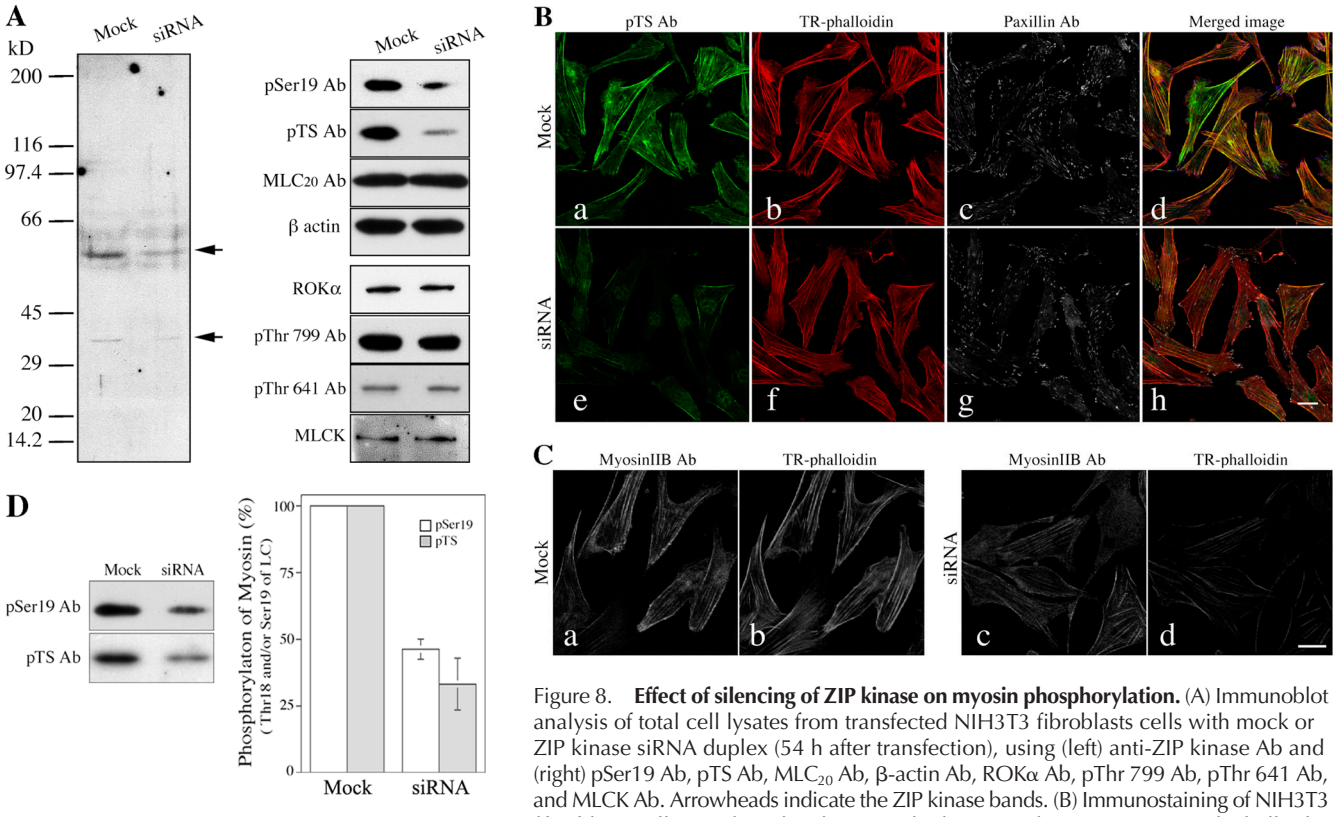

Figure 8. **Effect of silencing of ZIP kinase on myosin phosphorylation.** (A) Immunoblot analysis of total cell lysates from transfected NIH3T3 fibroblasts cells with mock or ZIP kinase siRNA duplex (54 h after transfection), using (left) anti-ZIP kinase Ab and (right) pSer19 Ab, pTS Ab, MLC_{20} Ab, β-actin Ab, ROKα Ab, pThr 799 Ab, pThr 641 Ab, and MLCK Ab. Arrowheads indicate the ZIP kinase bands. (B) Immunostaining of NIH3T3 fibroblasts cells transfected with siRNA duplex. pTS Ab (green), Texas red–phalloidin (red) and paxillin (gray). Bar, 20 μm. (C) Immunostaining of myosin heavy chain IIB. Bar, 20 μm. (D) Decrease in myosin kinase activity by suppression of ZIP kinase by RNAi. 54 h after transfection, cytoplasmic fractions from either mock- or siRNA-transfected cells were prepared and myosin kinase activity was measured as described in Materials and methods. The samples were separated by SDS-PAGE and followed by immunoblotting with pSer19 Ab and pTS Ab. The values shown are mean ± SD from three independent experiments.

activity of Ser19 phosphorylation and 90% of diphosphorylation in the cell extracts, respectively. The immunodepleted sample was examined for MLCK and Rho-kinase level by Western blot, but no detectable decrease in these kinases was observed (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200309056/DC1). Similar decrease in myosin II kinase activity by ZIP kinase immunodepletion was also found with NIH3T3 and COS 7 cells (Fig. S1 B). Fig. 7 B shows that ZIP kinase obtained by immunoprecipitation from the cell extracts phosphorylated MLC_{20} of myosin II. Interestingly, myosin phosphorylation by ZIP kinase was significantly inhibited by ML-7 (Fig. 7 C) with dose dependence similar to that for the inhibition of the myosin II kinase in the cell extracts (Fig. 5 C). Fig. 7 D shows the subcellular localization of ZIP kinase in REF-2A cells. ZIP kinase localized at stress fiber as well as cell cortical region where diphosphorylated myosin II is present and colocalized with actin structure. These results strongly support that ZIP kinase is a major kinase responsible for myosin II phosphorylation in mammalian cultured cells.

Microinjection and depletion of ZIP kinase result in the change in myosin phosphorylation in vivo

To test whether ZIP kinase is responsible for the change in myosin II diphosphorylation in cells, we microinjected exogenous ZIP kinase into REF-2A cells. Microinjection of GST-ZIP kinase into the serum-starved REF-2A cells markedly increased diphosphorylated MLC_{20} probed by pTS Ab (unpublished data).

To further evaluate the role of ZIP kinase in myosin II phosphorylation in mammalian cultured cells, we diminished ZIP kinase expression by using RNA interference (RNAi) technique. NIH3T3 cells were transfected with small interfering RNA (siRNA) oligoduplex corresponding to the coding region of the ZIP kinase mRNA. The siRNA-transfected cells were harvested and subjected to Western blot analysis using the specific antibodies as probes. Immunoblots showed that two molecular mass proteins recognized by ZIP kinase Ab (58 and 34 kD, respectively) were diminished in the cells transfected with siRNA (Fig. 8 A, left). It was reported that there are two ZIP kinase variants having different molecular weights (Kawai et al., 1998; Kogel et al., 1998; MacDonald et al., 2001), and it is expected that these two molecular weight bands are corresponding to the longer and shorter forms of ZIP kinase variants. Consistently, the myosin II kinase activity in cytosol fraction that is predominantly due to ZIP kinase activity was significantly decreased. Myosin II kinase activity in cytoplasmic fractions from cells transfected with either mock or ZIP kinase siRNA were measured. The activity was significantly diminished compared with that of mock-transfected cells (Fig. 8 D; 67% decrease). The extent of the decrease in activity was comparable to the decrease in the expression level of ZIP kinase (65% decrease). The siRNA specific to ZIP kinase attenu-

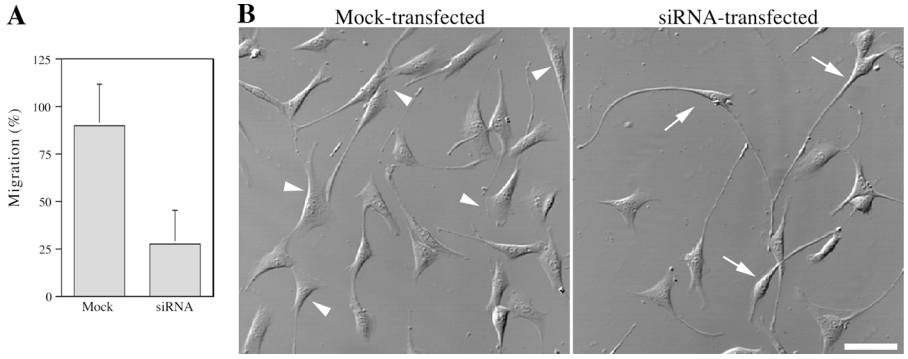

Figure 9. **ZIP kinase is necessary for establishment of cell polarity and migration.** (A) Inhibition of PDGF-induced migration rate by ZIP kinase siRNA. Figure shows the mean ± SD of three independent experiments. (B) Cell morphology of mock- or ZIP kinase siRNA-transfected cells. Arrowheads and arrows indicate the migrating cells and elongated cells, respectively. Bar, 80 μm.

ated neither Rho-kinase nor MLCK (Fig. 8 A, right). Consistently, MBS phosphorylation at Thr799, a Rho-kinase–specific site, was unaffected by the ZIP kinase–specific siRNA (Fig. 8 A, right). We also found that the siRNA treatment did not affect MBS phosphorylation at Thr641, another Rho kinase site. It was reported that ZIP kinase-like kinase can phosphorylate MBS at Thr641 in vitro (MacDonald et al., 2001). As shown in Fig. 8 A, both mono- and diphosphorylated MLC_{20} were markedly decreased in ZIP kinase siRNA-transfected cells compared with that of mock-transfected cells. These results suggest that the decrease in myosin phosphorylation via the suppression of ZIP kinase causes failure of direct myosin phosphorylation by ZIP kinase but not down-regulation of MLCP through the MBS phosphorylation. Fig. 8 B shows immunostaining of the siRNA-transfected cells with the phosphorylation site-specific antibodies. Consistent with the Western blot analysis, the intensity of immunofluorescence signals of pTS Ab was significantly reduced in the ZIP kinase siRNA-transfected cells (Fig. 8 B, e) compared with the control cells (Fig. 8 B, a). The decrease in MLC_{20} phosphorylation in the siRNA-transfected cells was observed at the central stress fibers (Fig. 8 B, e), similar to that observed with ML-7–treated cells (Fig. 4 A). It was also recognized that the filamentous structure of myosin II was low in the cells transfected with ZIP kinase siRNA (Fig. 8 C, c). This is consistent with the lower myosin II phosphorylation in siRNA-transfected cells because it is known that myosin II phosphorylation induces the filament formation of myosin (Ikebe and Hartshorne, 1985b; Sellers et al., 1985). To warrant that the effect of ZIP kinase siRNA on the decrease in MLC_{20} phosphorylation is due to the elimination of ZIP kinase, we examined whether human ZIP kinase that is resistant to siRNA can rescue the decrease in MLC_{20} phosphorylation of the cells treated with ZIP kinase siRNA. The cells treated by mouse ZIP kinase siRNA were transfected by human ZIP kinase and MLC_{20} phosphorylation level was monitored. The cells expressing human GFP-ZIP kinase showed strong diphosphorylation signals at the stress fiber (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200309056/DC1). The focal adhesion was also reduced in the siRNA-transfected cells (Fig. 8 B, g; decreased by 45.3 ± 9.6%). Consistent with this observation, a large number of nonadhesive cells were observed when siRNA-treated cells were cultured on noncoated or poly-D-lysine–coated glass bottom culture dishes (unpublished data). These results suggest that ZIP kinase plays a critical role in the change in MLC_{20} phosphorylation and the regulation of cytoskeletal structure in mammalian cultured cells.

Attenuation of ZIP kinase expression inhibits cell polarity and migration

It has been known that myosin II phosphorylation plays a role in cell migration, therefore, if ZIP kinase is a major factor regulating myosin II phosphorylation, it is anticipated that the attenuation of ZIP kinase expression decreases cell migration. First, we examined the migratory activity of ZIP kinase siRNA-transfected cells using fibronectin-coated microchambers. Migration efficiency of mock- and siRNA-transfected cells was estimated as ratio of cell migration observed in the presence and the absence of chemotactic factor, PDGF. siRNA-transfected cells consistently showed lower migration efficiency than mock-transfected cells (Fig. 9 A; Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200309056/DC1).

As shown in Fig. 9 B, significant number of fibroblasts transfected with ZIP kinase siRNA exhibited an elongated shape (Fig. 9 B, right, arrows). The elongated morphology of the siRNA-transfected cells was observed with differential interference contrast (DIC). We also examined for the extent of myosin phosphorylation of the elongated cells by immunostaining with pTS Ab and found that there was a dramatic decrease in myosin phosphorylation (unpublished data). On the other hand, mock-transfected cells displayed normal morphology (Fig. 9 B, left, arrowheads) and virtually no elongated cell shape was observed. Cells transfected with nonspecific siRNA (corresponding to human ZIP kinase sequence having four nucleotide substitutions) had also normal morphology and did not affect on myosin phosphorylation (unpublished data). These results suggest that the decrease in the motility activity of the ZIP kinase–depleted cells is due to the significant change in the cytoskeletal structure caused by the decrease in myosin II phosphorylation. These results also suggest that ZIP kinase plays a critical role during the cell spreading.

Discussion

Specific localization of mono- and diphosphorylated myosin II during cell migration

Biochemical studies have revealed that myosin II phosphorylated at both Ser19 and Thr18 of MLC_{20} is different from

that phosphorylated at only Ser19 in both actin activated ATPase activity and myosin filament formation (Ikebe and Hartshorne, 1985b; Sellers et al., 1985; Ikebe et al., 1986; Ikebe and Reardon, 1990). This raises the possibility that the diphosphorylated myosin II and monophosphorylated myosin II have a distinct role in cell motile and contractile processes. It is anticipated that the different properties of myosin II molecules is reflected by the distinct cellular localization. Using two phosphorylation site-specific antibodies, we found that mono- and diphosphorylated myosin II are differently localized in migrating cells. Diphosphorylated MLC_{20} was predominantly found at the posterior peripheral region, whereas monophosphorylated MLC_{20} was found at the anterior region of the cells. Furthermore, monophosphorylated myosin II localized at the membrane ruffle area of the leading edge, whereas diphosphorylated MLC_{20} did not. It is known that the rapid reorganization of actin takes place at the membrane ruffling area of the leading edge. Therefore, it is reasonable to assume that myosin II filaments at reading edge are also under rapid reorganization. On the other hand, diphosphorylated myosin II that forms stable filaments is recruited at the peripheral tail portion and develop the force necessary for the retraction of the tail region. This view is consistent with biochemical properties of myosin II that diphosphorylation of MLC_{20} stabilizes the formation of large myosin filaments, whereas monophosphorylated myosin II tends to form smaller filaments suggesting that it is under the equilibrium between monomeric myosin and filamentous myosin (Ikebe and Hartshorne, 1985b; Sellers et al., 1985; Ikebe et al., 1986; Ikebe and Reardon, 1990). It is plausible that diphosphorylated myosin filaments at the peripheral tail portion might be involved in maintenance of a cell morphology during migration, whereas monophosphorylated myosin filaments at the leading edge generates the force necessary for cell migration.

Protein kinases responsible for the phosphorylation of myosin II in migrating cells

A critical question to understand the regulation of myosin II phosphorylation in migrating cells is the identity of the protein kinases phosphorylating MLC_{20}. MLCK has been thought to be a predominant kinase responsible for myosin II phosphorylation in mammalian nonmuscle cells. This is partly because the possibility of other kinases to be physiologically important myosin II kinase has been overlooked. Recently, it was shown that ZIP kinase can phosphorylate MLC_{20} at Ser19 and Thr18. A critical finding is that ZIP kinase unlike MLCK phosphorylates Ser19 and Thr18 with same rate constant yielding diphosphorylated MLC_{20} effectively. Successful production of the antibody recognizing diphosphorylated MLC_{20} enables us to study the nature of critical myosin II kinase in nonmuscle cells. Our results indicate that the protein kinases responsible for the phosphorylation of myosin II are the kinases that phosphorylate MLC_{20} at Ser19 and Thr18 with similar rate constant because (a) a significant level of the diphosphorylated MLC_{20} was present in the cells; (b) the kinases in the cell extract produced diphosphorylated MLC_{20} at the time when a significant portion of unphosphorylated MLC_{20} remained unlike MLCK; and (c) SM-1 as well as wortmannin, MLCK inhibitors, had no detectable effect on myosin II kinase activity in the cell extract. It has been shown in vitro that Rho-kinase can phosphorylate myosin II at Ser19 (Amano et al., 1996). The incubation of the cells with Rho-kinase inhibitor, Y27632, a specific inhibitor of Rho-kinase, significantly inhibited MLC_{20} phosphorylation in cells. However, Y27632 did not inhibit myosin phosphorylation activity in the cell extracts. We think that the inhibition of MLC_{20} phosphorylation in cells by Y27632 is not due to the inhibition of myosin II kinase but due to the activation of MLCP. Supporting this view, we found that MLCP activity obtained from the cells treated with Y27632 was 1.8 times higher than that obtained from the untreated cells (unpublished data). It has been reported that the inhibition of MLCP by phosphatase inhibitor or microinjection of MBS Ab into mammalian cultured cells increases MLC_{20} phosphorylation (Chartier et al., 1991; Totsukawa et al., 2000). Together with our present paper, we think that Rho-kinase mainly contributes to myosin phosphorylation through the regulation of MLCP but not direct myosin II phosphorylation in vivo.

Interestingly, ML-7 diminished the myosin II kinase activity in the cell extracts. Consistently, ML-7 also attenuated the MLC_{20} phosphorylation in cells. However, the kinase activity in the cell extracts was neither inhibited by EGTA nor SM1 peptide, suggesting that ML-7 inhibit the kinases other than MLCK in the cell extract. Supporting this idea, ML-7 inhibited the purified ZIP kinase with similar concentration dependence against the inhibition of the kinases in the cell extracts. Recently, it was reported that ZIP kinase like kinase purified in smooth muscle is inhibited by ML-9 that is similar to ML-7 and the present result is consistent with this observation (Borman et al., 2002). The present result indicates that ML-7 is not specific to MLCK but also inhibits ZIP kinase, therefore, earlier results using ML-7 as a MLCK–specific inhibitor may need to be reevaluated.

To further ensure the importance of ZIP kinase for myosin phosphorylation in mammalian cultured cells, we have used several approaches. First, identity of the major myosin II kinase in the cell extracts as ZIP kinase was demonstrated by the immunodepletion experiment. The depletion of ZIP kinase by the specific antibodies markedly reduced the myosin II kinase activity in the cell extracts, indicating that ZIP kinase is the major kinase responsible for myosin phosphorylation in the cell extracts. Consistently, the immunoprecipitation of the extracts using the ZIP kinase Ab recovered the myosin II kinase activity. Second, the microinjection of ZIP kinase into serum-starved NIH3T3 cells induced myosin phosphorylation (unpublished data). Supporting the idea that ZIP kinase participates in myosin phosphorylation, the overexpression of ZIP kinase in HeLa cells induced myosin phosphorylation (Murata-Hori et al., 2001). It was also reported that ZIP kinase increases myosin phosphorylation of smooth muscle strips and induces contraction (Niiro and Ikebe, 2001). These previous results support the idea that ZIP kinase can increase myosin II phosphorylation and activate the contractile activity of actomyosin.

Further evidence that ZIP kinase is critical for myosin phosphorylation in mammalian cells was obtained using a recently developed siRNA technique (Fire et al., 1998). The

depletion of endogenous ZIP kinase in NIH3T3 fibroblasts by the specific siRNA decreased mono- and diphosphorylation of MLC_{20} without changing myosin expression level. Furthermore, immunocytochemical analysis revealed that diphosphorylated myosin filaments at the central region were remarkably diminished by transfection of the ZIP kinase siRNA. Interestingly, myosin phosphorylation at the cortical region was not completely abolished in the ZIP kinase–depleted cells, suggesting that other kinases may be involved in myosin phosphorylation at this region. Recently, it was shown that MLCK contributes to myosin phosphorylation at the cortical region but not in the center (Totsukawa et al., 2000, 2004), therefore, myosin phosphorylation at the cell cortical region may be in part mediated by MLCK.

ZIP kinase is important for NIH3T3 cell polarity and migration

We found that the disruption of ZIP kinase causes the change in cell morphology and migratory behavior of NIH3T3 fibroblasts. It is widely believed that the reorganization of actomyosin is an essential process for progress of cell migration and that myosin phosphorylation is involved in this process. In the present paper, we found that the interference of ZIP kinase inhibits cell migration activity. Interestingly, attenuation of ZIP kinase induced elongated cell morphology. We think that the decrease in myosin phosphorylation via the depletion of ZIP kinases causes failure of stable myosin filament formation in stress fiber structure and thus changing cytoskeletal structure and cell morphology. This might allow cells to become elongated and lose motility.

In summary, based upon the present paper, we propose that ZIP kinase promotes dynamic rearrangement of myosin structure through the myosin phosphorylation in motile fibroblast cells and contributes to the cell motile processes involving in spreading and migration.

Materials and methods

Materials

Smooth muscle myosin (Ikebe and Hartshorne, 1985a) and MLCK (Ikebe et al., 1987a) were prepared from turkey gizzards. Ca^{2+}/CaM-independent 61-kD MLCK and *Xenopus* oocyte CaM were prepared as described previously (Chien and Dawid, 1984; Ikebe et al., 1987b). Rat MBS cDNA and ROKα cDNA were gifts from P. Cohen (University of Dundee, Dundee, Scotland, UK) and T. Leung (National University of Singapore, Singapore), respectively, and cloned into pFASTBAC HT plasmid. Rho-kinase and GST tagged ZIP kinase were purified from Sf9 cells with Ni^{2+}-nitrilotriacetic acid-agarose (QIAGEN) or glutathione-Sepharose 4B as described previously (Niiro and Ikebe, 2001). SM-1 peptide was synthesized as described previously (Ikebe et al., 1987b). Y27632 was provided by Yoshitomi Pharmaceutical Industries, Ltd., and ML-7 was purchased from Calbiochem.

Antibodies

A phosphopeptide KKRPQRAphosphoTSNVFAMC was coupled to keyhole limpet hemocyanin at COOH-terminal cysteine residue. A pTS Ab was affinity purified using the phosphopeptide and then absorbed with unphosphopeptide. A pSer19 Ab, ZIP kinase Ab, and phosphorylation-specific Ab against MBS at Thr 641 or Ser799 were described previously (Komatsu et al., 2000; Niiro and Ikebe, 2001; Takizawa et al., 2002). A rabbit Ab against heavy chain of myosin IIB, MLC_{20}, and MLCK were provided by R. Adelstein (National Institutes of Health, Bethesda, MD), J. Stull (University of Texas Southwestern Medical Center, Dallas, TX), and P. de Lanerolle (University of Illinois, Chicago, IL), respectively. Anti-MLC_{20}, MBS, ROKα, β-actin, and paxillin Abs were purchased from Sigma-Aldrich, Covance Research Products Inc., and Transduction Laboratories, respectively.

Cell culture, microinjection, and transfection

REF-2A cells (a gift from F. Matsumura, Rutgers University, Piscataway, NJ) and NIH3T3 fibroblast cells were maintained in DME containing 10% newborn calf serum. NRK cells (NRK52E; a gift from Y.-L. Wang, University of Massachusetts, Worcester, MA) and COS 7 cells were cultured in F12 medium (Sigma-Aldrich) containing 10% FBS (GIBCO BRL), 2 mM L-glutamine or DME containing 10% FBS, respectively. Microinjection was performed using a micromanipulator (Transjector 5246; Eppendorf). 0.1mg/ml of ZIP kinase was coinjected with FITC-dextran.

For RNAi, the selected sequences were submitted to a BLAST search to ensure that only ZIP kinase gene was targeted. The targeting sequence of mouse ZIP kinase (AB007143), AAGACAGATGTGGTGCTGATC, corresponding to the coding region 256–276 of ZIP kinase was used for siRNA and synthesized by Dharmacon Research. Double strand siRNA was prepared according to the manufacturer's protocol (Dharmacon), and transfected using Lipofectamine 2000 (Invitrogen). As a negative control (nonspecific siRNA), human ZIP kinase (AB022341) siRNA (AAGACGGACGTGGTCCTCATC) was used. siRNA-transfected cells were cultured on the fibronectin (10 μg/ml)-coated glass coverslips.

Preparation of cell extracts

REF-2A cells were washed and then lysed in buffer I (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EGTA, 5 mM DTT, 5% glycerol, 0.2 mM *N*α-*p*-tosyl-L-lysine chloromethl ketone, 0.2 mM *N*-tosyl-L-phenylal-anine chloromethl ketone, 2 mM PMSF, and 0.05% NP-40). After added 0.4 M NaCl, cell lysates were sonicated and centrifuged at 10,000 *g* for 15 min. Protein concentration was determined by the method of Bradford (1976) by using BSA as a standard. For NIH3T3 cells, nuclear and cytosol fractions were prepared from cells treated with siRNA using Nuclear/Cytosol Fractionation Kit (BioVision, Inc.).

Immunoprecipitation and immunodepletion

The cell extracts were incubated with either nonspecific rabbit IgGs or anti-ZIP kinase Ab at 4°C for 3 h and then protein A-Support (Bio-Rad Laboratories) was added. The immunocomplex was centrifuged, washed three times with wash buffer (0.1 M KCl and Tris-HCl, pH 8.8), and two times with buffer B and used for myosin phosphorylation assay.

Biochemical procedures

Urea/glycerol PAGE (Perrie and Perry, 1970) and SDS-PAGE (Laemmli, 1970) were performed as described previously. MLC_{20} was phosphorylated by MLCK and PKC (Ikebe and Hartshorne, 1985a; Ikebe et al., 1987a). Immunoblotting was done as described previously using nitrocellulose membranes (Yano et al., 1993; Komatsu et al., 2000). In vitro phosphorylation was performed using buffer containing 30 mM NaCl, 5 mM $MgCl_2$, 1 μM microcystin-LR, 0.2 mM ATP, and 30 mM Tris-HCl, pH 7.5, and 0.2 mM $CaCl_2$ for buffer A and 5 mM EGTA for buffer B. Myosin (0.4 mg/ml) or MBS was phosphorylated in the presence of kinase inhibitors (Y27632, ML-7 in buffer A or SM-1 peptide in buffer B) by 0.2mg/ml of cell extracts or exogenous kinases (1 μg/ml Rho-kinase, 1 μg/ml CaM, and 1 μg/ml MLCK in buffer A, Ca^{2+}/CaM-independent 1 μg/ml MLCK or 1 μg/ml GST-ZIP kinase in buffer B) in the presence of kinase inhibitors. The reaction was done for 15 min at 30°C, and then phosphorylated MLC_{20} or MBS was detected by Western blotting analysis.

Immunofluorescence staining and image processing

Immunocytochemistry was performed as described previously (Komatsu et al., 2000). Cells were stained with Texas red–conjugated phalloidin (Molecular Probes) for F-actin. For double staining with pSer19 Ab and pTS Ab, the cells were first stained with pTS Ab for 6 h at 4°C and then followed by pSer19 Ab overnight. DIC and fluorescence images were viewed using a DM IRB laser scanning confocal microscope (Leica) controlled by TCS SP II systems (Leica). All images were taken with same laser out put to directly compare the fluorescence signal intensities. Images were processed using Adobe® Photoshop® 5.5 software.

Migration assays

Cell migration was studied using transwell migration chambers (6.5-mm diam; 8-μm pore size; COSTAR Corp.) coated on both sides of the membrane with 10 μg/ml fibronectin in PBS for 16 h at 4°C. Mock- or siRNA-transfected NIH3T3 cells were cultured for 24 h in DME supplemented with 0.1% newborn calf serum and then detached by trypsinization. Assays were performed by the addition of the cells (5×10^4 cells/well) to the upper compartment of the transwell chamber and allowed to migrate to the membrane in the bottom chambers containing medium supplemented with

20 ng/ml PDGF for 3 h. Migrated cells attached to the bottom surface of the membrane were fixed in methanol, stained with Giemsa, and counted.

Online supplemental material

Fig. S1 shows that immunodepletion of ZIP kinase had no effect on the expression level of both ROKα and MLCK (A) and that ZIP kinase was responsible for myosin II phosphorylation in the cell extracts from different cell types (B). Fig. S2 shows that the decrease in myosin II phosphorylation by mouse ZIP kinase siRNA was rescued by expression of human ZIP kinase. Fig. S3 shows the wound healing assay indicating that the migration of ZIP kinase siRNA-transfected cells is slower than that of mock-transfected cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200309056/DC1.

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References

Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, and K. Kaibuchi. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.* 271:20246–20249.

Borman, M.A., J.A. MacDonald, A. Muranyi, D.J. Hartshorne, and T.A. Haystead. 2002. Smooth muscle myosin phosphatase-associated kinase induces Ca^{2+} sensitization via myosin phosphatase inhibition. *J. Biol. Chem.* 277: 23441–23446.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Chartier, L., L.L. Rankin, R.E. Allen, Y. Kato, N. Fusetani, H. Karaki, S. Watabe, and D.J. Hartshorne. 1991. Calyculin-A increases the level of protein phosphorylation and changes the shape of 3T3 fibroblasts. *Cell Motil. Cytoskeleton.* 18:26–40.

Chien, Y.H., and I.B. Dawid. 1984. Isolation and characterization of calmodulin genes from *Xenopus laevis*. *Mol. Cell. Biol.* 4:507–513.

Choi, O.H., R.S. Adelstein, and M.A. Beaven. 1994. Secretion from rat basophilic RBL-2H3 cells is associated with diphosphorylation of myosin light chains by myosin light chain kinase as well as phosphorylation by protein kinase C. *J. Biol. Chem.* 269:536–541.

Colburn, J.C., C.H. Michnoff, L.C. Hsu, C.A. Slaughter, K.E. Kamm, and J.T. Stull. 1988. Sites phosphorylated in myosin light chain in contracting smooth muscle. *J. Biol. Chem.* 263:19166–19173.

Deng, J.T., J.E. Van Lierop, C. Sutherland, and M.P. Walsh. 2001. Ca^{2+}-independent smooth muscle contraction. A novel function for integrin-linked kinase. *J. Biol. Chem.* 276:16365–16373.

Feng, J., M. Ito, K. Ichikawa, N. Isaka, M. Nishikawa, D.J. Hartshorne, and T. Nakano. 1999. Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J. Biol. Chem.* 274:37385–37390.

Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature.* 391:806–811.

Hartshorne, D.J. 1987. Biochemistry of the contractile process in smooth muscle. *In* Physiology of the Gastrointestinal Tract. L.R. Johnson, editor. Raven Press, New York. 423–482.

Ikebe, M., and D.J. Hartshorne. 1985a. Effects of Ca^{2+} on the conformation and enzymatic activity of smooth muscle myosin. *J. Biol. Chem.* 260:13146–13153.

Ikebe, M., and D.J. Hartshorne. 1985b. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J. Biol. Chem.* 260: 10027–10031.

Ikebe, M., and S. Reardon. 1990. Phosphorylation of bovine platelet myosin by protein kinase C. *Biochemistry.* 29:2713–2720.

Ikebe, M., D.J. Hartshorne, and M. Elzinga. 1986. Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. *J. Biol. Chem.* 261:36–39.

Ikebe, M., D.J. Hartshorne, and M. Elzinga. 1987a. Phosphorylation of the 20,000-dalton light chain of smooth muscle myosin by the calcium-activated, phospholipid-dependent protein kinase. Phosphorylation sites and effects of phosphorylation. *J. Biol. Chem.* 262:9569–9573.

Ikebe, M., M. Stepinska, B.E. Kemp, A.R. Means, and D.J. Hartshorne. 1987b. Proteolysis of smooth muscle myosin light chain kinase. Formation of inactive and calmodulin-independent fragments. *J. Biol. Chem.* 262:13828–13834.

Ikebe, M., J. Koretz, and D.J. Hartshorne. 1988. Effects of phosphorylation of light chain residues threonine 18 and serine 19 on the properties and conformation of smooth muscle myosin. *J. Biol. Chem.* 263:6432–6437.

Itoh, K., T. Hara, F. Yamada, and N. Shibata. 1992. Diphosphorylation of platelet myosin ex vivo in the initial phase of activation by thrombin. *Biochim. Biophys. Acta.* 1136:52–56.

Kamisoyama, H., Y. Araki, and M. Ikebe. 1994. Mutagenesis of the phosphorylation site (serine 19) of smooth muscle myosin regulatory light chain and its effects on the properties of myosin. *Biochemistry.* 33:840–847.

Kamm, K.E., and J.T. Stull. 1989. Regulation of smooth muscle contractile elements by second messengers. *Annu. Rev. Physiol.* 51:299–313.

Kawai, T., M. Matsumoto, K. Takeda, H. Sanjo, and S. Akira. 1998. ZIP kinase, a novel serine/threonine kinase which mediates apoptosis. *Mol. Cell. Biol.* 18: 1642–1651.

Kawano, Y., Y. Fukata, N. Oshiro, M. Amano, T. Nakamura, M. Ito, F. Matsumura, M. Inagaki, and K. Kaibuchi. 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J. Cell Biol.* 147:1023–1038.

Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, et al. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science.* 273:245–248.

Kiss, E., A. Muranyi, C. Csortos, P. Gergely, M. Ito, D.J. Hartshorne, and F. Erdodi. 2002. Integrin-linked kinase phosphorylates the myosin phosphatase target subunit at the inhibitory site in platelet cytoskeleton. *Biochem. J.* 365: 79–87.

Kogel, D., O. Plottner, G. Landsberg, S. Christian, and K.H. Scheidtmann. 1998. Cloning and characterization of Dlk, a novel serine/threonine kinase that is tightly associated with chromatin and phosphorylates core histones. *Oncogene.* 17:2645–2654.

Komatsu, S., T. Yano, M. Shibata, R.A. Tuft, and M. Ikebe. 2000. Effects of the regulatory light chain phosphorylation of myosin II on mitosis and cytokinesis of mammalian cells. *J. Biol. Chem.* 275:34512–34520.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

MacDonald, J.A., M.A. Borman, A. Muranyi, A.V. Somlyo, D.J. Hartshorne, and T.A. Haystead. 2001. Identification of the endogenous smooth muscle myosin phosphatase-associated kinase. *Proc. Natl. Acad. Sci. USA.* 98:2419–2424.

Matsumura, F., S. Ono, Y. Yamakita, G. Totsukawa, and S. Yamashiro. 1998. Specific localization of serine 19 phosphorylated myosin II during cell locomotion and mitosis of cultured cells. *J. Cell Biol.* 140:119–129.

Muranyi, A., J.A. MacDonald, J.T. Deng, D.P. Wilson, T.A. Haystead, M.P. Walsh, F. Erdodi, E. Kiss, Y. Wu, and D.J. Hartshorne. 2002. Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. *Biochem. J.* 366:211–216.

Murata-Hori, M., F. Suizu, T. Iwasaki, A. Kikuchi, and H. Hosoya. 1999. ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. *FEBS Lett.* 451:81–84.

Murata-Hori, M., Y. Fukuta, K. Ueda, T. Iwasaki, and H. Hosoya. 2001. HeLa ZIP kinase induces diphosphorylation of myosin II regulatory light chain and reorganization of actin filaments in nonmuscle cells. *Oncogene.* 20: 8175–8183.

Niiro, N., and M. Ikebe. 2001. Zipper-interacting protein kinase induces $Ca^{(2+)}$-free smooth muscle contraction via myosin light chain phosphorylation. *J. Biol. Chem.* 276:29567–29574.

Nishikawa, M., J.R. Sellers, R.S. Adelstein, and H. Hidaka. 1984. Protein kinase C modulates in vitro phosphorylation of the smooth muscle heavy meromyosin by myosin light chain kinase. *J. Biol. Chem.* 259:8808–8814.

Perrie, W.T., and S.V. Perry. 1970. An electrophoretic study of the low-molecular-weight components of myosin. *Biochem. J.* 119:31–38.

Sellers, J.R., J.A. Spudich, and M.P. Sheetz. 1985. Light chain phosphorylation regulates the movement of smooth muscle myosin on actin filaments. *J. Cell Biol.* 101:1897–1902.

Singer, H.A. 1990. Protein kinase C activation and myosin light chain phosphorylation in 32P-labeled arterial smooth muscle. *Am. J. Physiol.* 259:C631–C639.

Takizawa, N., N. Niiro, and M. Ikebe. 2002. Dephosphorylation of the two regulatory components of myosin phosphatase, MBS and CPI17. *FEBS Lett.* 515:127–132.

Tan, J.L., S. Ravid, and J.A. Spudich. 1992. Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* 61:721–759.

Totsukawa, G., Y. Yamakita, S. Yamashiro, D.J. Hartshorne, Y. Sasaki, and F. Matsumura. 2000. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* 150:797–806.

Totsukawa, G., Y. Wu, Y. Sasaki, D.J. Hartshorne, Y. Yamakita, S. Yamashiro, and F. Matsumura. 2004. Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. *J. Cell Biol.* 164:427–439.

Uehata, M., T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, and S. Narumiya. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature.* 389:990–994.

Yano, K., Y. Araki, S.J. Hales, M. Tanaka, and M. Ikebe. 1993. Boundary of the autoinhibitory region of smooth muscle myosin light-chain kinase. *Biochemistry.* 32:12054–12061.