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

Satoshi Komatsu and Mitsuo Ikebe

Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655

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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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). Kimmura 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 diphasphorylation of MLC_{20} significantly facilitates the formation of stable myosin filaments (Ikebe et al., 1988; Kamiyama 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 dipho-

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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}.

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**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.
Myosin phosphorylation by ZIP kinase

Phosphorylated 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\textsubscript{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\textsubscript{20} and diphosphorylated MLC\textsubscript{20} at both Ser19 and Thr18, although the antibodies recognize the single phosphorylated MLC\textsubscript{20} at Ser19 stronger than the diphosphorylated MLC\textsubscript{20}. To overcome this problem, we developed the antibodies that recognize diphosphorylated MLC\textsubscript{20} but not MLC\textsubscript{20} phosphorylated at only Ser19.

The specificity of pTS Ab was examined by immunoblot analysis. MLC\textsubscript{20} was phosphorylated by MLCK, PKC, and ZIP kinase. The unphosphorylated, mono-, and diphosphorylated MLC\textsubscript{20} 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\textsubscript{20} by MLCK and ZIP kinase, but did not recognize unphosphorylated, monophosphorylated by MLCK and ZIP kinase, or monophosphorylated (Thr9) MLC\textsubscript{20} by PKC. It was shown previously that ZIP kinase phosphorylates Ser19 and Thr18 of MLC\textsubscript{20} with same rate constant thus yielding the same amount of Ser19 phosphorylated MLC\textsubscript{20} and Thr18 phosphorylated MLC\textsubscript{20} (Niiro and Ikebe, 2001). Therefore, the results shown in Fig. 1 indicate that pTS Ab specifically recognizes diphosphorylated MLC\textsubscript{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\textsubscript{20} but not other proteins. pTS Ab and pSer19 Ab recognizing the Ser19 phosphorylated MLC\textsubscript{20} were used as probes to determine the distribution of di- and monophosphorylated myosin II at MLC\textsubscript{20} in motile fibroblasts.

**Distribution of di- and monophosphorylated MLC\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{20} is enriched more than diphosphorylated MLC\textsubscript{20} in this area. It should be noted...
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, a) 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 \(\sim 60\%\) for monophosphorylated...
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 MLC20 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 is consistent with this earlier result. Therefore, the results suggest that the decrease in MLC20 phosphorylation by Y27632 is due to the activation of MLCP by Rho-kinase-induced MBS dephosphorylation, whereas ML-7 decreases MLC20 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 MLC20 phosphorylation by ML-7 is due to the inhibition of MLCK, we examined the effect of wortmannin on MLCP activity in cell extracts. 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-MLC20 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 cell 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 CaCl2 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.

Myosin phosphorylation activity in cell extracts

To identify the candidate kinases for MLC20 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 blotting using pSer19 Ab or pTS Ab. As shown in Fig. 5 A, the cell extracts produced significant level of diphosphorylated MLC20. The extents of mono- and diphosphorylated MLC20 were estimated by using urea/glycerol gel electrophoresis. The diphosphorylated MLC20 appeared whereas a significant portion of MLC20 remained unphosphorylated (Fig. 5 A). The results suggest that the protein kinases responsible for MLC20 phosphorylation phosphorylate Ser19 and Thr18 with similar potency.

Because it is known that MLCK and Rho-kinase phosphorylate MLC20 in vitro, we examined whether these kinases are responsible for MLC20 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 Ca2+ 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-
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\(^{2+}\)/CaM (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 Ser19 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 6](image-url)

**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.

![Figure 7](image-url)

**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.
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 MLC20 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 MLC20 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 siRNA-transfected cells 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 attenuated...
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.

Discussion
Specific localization of mono- and diphosphorylated myosin II during cell migration
Biochemical studies have revealed that myosin II phosphorylation at both Ser19 and Thr18 of MLC20 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 MLC20 was predominantly found at the anterior region, whereas monophosphorylated MLC20 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 MLC20 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 at the 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 MLC20 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 MLC20. 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 MLC20 at Ser19 and Thr18. A critical finding is that ZIP kinase can phosphorylate MLC20, at Ser19 and Thr18 with same rate constant yielding diphosphorylated MLC20 effectively. Successful production of the antibody recognizing diphosphorylated MLC20 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 MLC20 at Ser19 and Thr18 with similar rate constant because (a) a significant level of the diphosphorylated MLC20 was present in the cells; (b) the kinases in the cell extract produced diphosphorylated MLC20 at the time when a significant portion of unphosphorylated MLC20 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 MLC20 phosphorylation in cells. However, Y27632 did not inhibit myosin phosphorylation activity in the cell extracts. We think that the inhibition of MLC20 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 MLC20 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 MLC20 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 (Niir 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\textsubscript{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 (Tosukawa 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 progression 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\textsuperscript{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 ROK\textalpha\, 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-ROK\alpha\, were described previously (Chien and Dawid, 1984; Ikebe et al., 1987b). Anti-MLC\textsubscript{20}, MBS, ROK\alpha\, Adelstein (National Institutes of Health, Bethesda, MD), J. Stull (University of Illinois, Chicago, IL), respectively. Anti-MLC\textsubscript{20}, MBS, CaMKII, MLCK, ZIP, ML-7, and ML-20 were purchased from Sigma–Aldrich, Covance Research Products Inc., and Transduction Laboratories, respectively.

**Antibodies**

A phosphopeptide KKKPRQRPhosphoTSNVFAMC was coupled to keyhole limpet hemocyanin at COOH-terminal cysteine residue. A pT\,SAb 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; Niño 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.

**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\textsubscript{2}, 0.1 mM EGTA, 5 mM DTT, 5% glycerol, 0.2 mM Na-p-tosyl-l-lysine chloromethyl ketone, 0.2 mM N-tosyl-l-phenylalanyl-ane chloromethyl 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 then with buffer B and used for myosin phosphorylation assay.

**Biochemical procedures**

Unlabeled or radioactive Ca\textsuperscript{2+}/CaM-independent 61-kD MLCK and PKC (Ikebe and Hartshorne, 1985a) were used as described previously. MLCK\, was phosphorylated by MLCK and PKC (Ikebe and Hartshorne, 1985a) 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 (AAGACG-GACCTGGTCTCCTGATC) was used. siRNA-transfected cells were cultured on the fibronectin (10 μg/ml)-coated glass coverslips.

We found that the decrease in myosin phosphorylation 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 progression 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.

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**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 DMEM supplemented with 0.1% newborn calf serum and then detached by trypsinization. Assays were performed by the addition of the cells (5 × 10\textsuperscript{3} 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...
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