In Vivo Phosphorylation of Regulatory Light Chain of Myosin II during Mitosis of Cultured Cells

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Abstract. Phosphorylation of the regulatory light chain of myosin II (MLC) controls the contractility of actomyosin in nonmuscle and muscle cells. It has been reported that cdc2 phosphorylates MLC in vitro at Ser-1 or Ser-2 and Thr-9 which protein kinase C phosphorylates (Satterwhite, L. L., M. J. Lohka, K. L. Wilson, T. Y. Scherson, L. K. Cisek, J. L. Corden, and T. D. Pollard. 1992. J. Cell Biol. 118:595-605). We have examined in vivo phosphorylation of MLC during mitosis and after the release of mitotic arrest. Phosphate incorporation of MLC in mitotic cells is found to be 6-12 times greater than that in nonmitotic cells. Phosphopeptide maps have revealed that the MLC from mitotic cells is phosphorylated at Ser-1 and/or Ser-2 (Ser-1/2), but not at Thr-9. MLC is also phosphorylated to a much lesser extent at Ser-19 which myosin light chain kinase phosphorylates. On the other hand, MLC of nonmitotic cells is phosphorylated at Ser-19 but not at Ser-1/2. The extent of phosphate incorporation is doubled at 30 min after the release of mitotic arrest when some cells start cytokinesis. Phosphopeptide analyses have revealed that the phosphorylation at Ser-19 is increased 20 times, while the phosphorylation at Ser-1/2 is decreased by half. This high extent of MLC phosphorylation at Ser-19 is maintained for another 30 min and gradually decreased to near the level of interphase cells as cells complete spreading at 180 min. On the other hand, phosphorylation at Ser-1/2 is decreased to 18% at 60 min, and is practically undetectable at 180 min after the release of mitotic arrest. The stoichiometry of MLC phosphorylation has been determined by quantitation of phosphorylated and unphosphorylated forms of MLC separated on 2D gels. The molar ratio of phosphorylated MLC to total MLC is found to be 0.16 ± 0.06 and 0.31 ± 0.05 in interphase and mitotic cells, respectively. The ratio is increased to 0.49 ± 0.05 at 30 min after the release of mitotic arrest. These results suggest that the change in the phosphorylation site from Ser-1/2 to Ser-19 plays an important role in signaling cytokinesis.

The microfilament cytoskeleton of cultured cells undergoes massive reorganization during mitosis (29, 32). Microfilament bundles or stress fibers are disassembled into a dispersed state during prophase concomitant with cell rounding. Subsequently, a contractile ring is transiently formed between two poles. After chromosome segregation, the contractile ring is activated to separate the cytoplasm into two daughter cells. Stress fibers are re-assembled as cells start to spread. The molecular mechanisms underlying these changes in microfilament organization, however, are largely unknown (for review see references 11 and 24). In particular, what controls the formation and activation of the contractile rings has been a primary objective for investigation.

Myosin II is accumulated in the contractile rings (11, 30), and known to function as the motor for the cytokinesis (8, 20, 25). It is thus quite reasonable to assume that the system controlling cytokinesis should involve the regulation of myosin II activity in contractile rings. Recently, Satterwhite et al. (31) have made a very interesting discovery: that the regulatory light chain of myosin II (MLC) is phosphorylated in vitro by cdc2 kinase at the sites which protein kinase C phosphorylates (Ser-1 or Ser-2 and Thr-9; C-kinase sites). The phosphorylation of MLC at the C-kinase sites is known to inhibit actin-activated myosin II ATPase and to reduce the stability of myosin II filaments (2, 15, 28). As the activity of cdc2 kinase drops abruptly during metaphase/anaphase transition, they have proposed an attractive model, namely that the phosphorylation of MLC at the C-kinase sites keeps myosin II from activating until the onset of anaphase. The dephosphorylation of MLC at the C-kinase sites caused by the drop in the cdc2 activity would thus be a signal to activate contractile rings during the anaphase/telophase transition.

In vivo phosphorylation of MLC during mitosis has not been determined to verify this hypothesis. Using a cultured


1. Abbreviations used in this paper: MLC, light chain of myosin II; 2D, two dimensional.
cell system, we have found that MLC of mitotically arrested cells is indeed phosphorylated mainly at Ser-1/2. Although protein kinase C phosphorylates Thr-9 in addition to Ser-1/2, phosphorylation at Thr-9 has not been observed in vivo. On the other hand, MLC of interphase cells is primarily phosphorylated at Ser-19 which MLCK phosphorylates (MLCK site). After the release of mitotic arrest, the phosphorylation at Ser-1/2 is decreased while the phosphorylation at the MLCK site is greatly increased as cells go into cytokinesis followed by cell spreading. These results suggest that the changes in the MLCK phosphorylation sites may be a signal to activate contractile rings during cell division.

We have also examined in vitro phosphorylation of MLC by cdc2 kinase purified from mitotic HeLa cells. We have confirmed the previous results by Sattherwhite et al., (31) that cdc2 kinase phosphorylates MLC in vitro at the C-kinase sites (Ser-1/2 and Thr-9). However, our cdc2 kinase shows a much lower MLC-kinase activity than does protein kinase C, suggesting that additional factors or other kinase(s) may be involved in the mitosis-specific phosphorylation of MLC.

Materials and Methods

Cell Culture and Preparation of Mitotic Cells

SV-40 transformed rat embryo (REF-4A) and HeLa cells were used in the present study. Both cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% newborn calf serum (NCS) in an atmosphere of 5% CO₂ and 95% air at 37°C.

REF4A cells at metaphase and later stages during cell division were prepared as described previously (13, 38, 39). Briefly, cells were first treated for 3 h with 0.25 µg/ml nocodazole, and mitotic cells were collected as described previously (38). After washed with DMEM at 0°C to remove nocodazole, cells were plated in fresh culture dishes, and incubated at 37°C in DMEM containing 10% newborn calf serum to allow cell cycle progression. Cell morphologies were checked by phase-contrast light microscopy as described previously (13).

Proteins

Smooth muscle and nonmuscle myosin II were prepared from chick gizzard and bovine adrenal medulla, respectively, as described (34). Myosin light chain kinase was a generous gift from Dr. J. Sellers. Myosin light chain kinase was prepared from chick gizzard as described (1). Protein kinase C and Histone HI were purchased from Lipidex and Boehringer, respectively, p13 was provided by Dr. E Nurse) as described (5) and conjugated to Sephacryse 4B (vol/vol). The phosphoaminoacid markers were detected by staining with ninhydrin.

In Vivo Phosphorylation of Myosin Light Chain during Cell Division

REF-4A cells at mitosis or later stages were labeled with [32P] orthophosphoric acid as described previously (13, 38, 39). Briefly, cells were incubated for 3 h in phosphate-free DMEM containing 10% dialyzed newborn calf serum, 0.25 µg/ml nocodazole, and 0.125 mM/µl [32P] orthophosphoric acid. Mitotic and nonmitotic cells were then collected as described (38). In some experiments, cells were continuously labeled with [32P] orthophosphoric acid after the release of mitotic arrest in the manner described below. Mitotic cells were washed quickly with phosphate-free DMEM at 0°C to remove nocodazole, and incubated at 37°C in phosphate-free DMEM containing the same radioactivity of [32P]. Cells at different stages of mitosis were lysed in immunoprecipitation buffer containing 25 mM Tris-HCl, pH 8.0, 100 mM NaF, 250 mM NaCl, 10 mM EGTA, 5 mM EDTA, 100 mM Na₃PO₄, 1 mM PMSF, and 1% NP-40, and stored in a −80°C freezer. After being thawed quickly, the lysates were homogenized by several passages through a 25-gauge needle, and centrifuged in an Eppendorf centrifuge for 30 min. The supernatants were immediately used for immunoprecipitation using anti-myosin II heavy chain polyclonal antibody (kindly provided by Dr. J. Sellers, NIH, Bethesda, MD). The immunoprecipitates were then analyzed on an SDS gel, followed by autoradiography. As myosin heavy chain bands in the immunoprecipitates were visible on Coomassie blue-stained gels, their intensities determined by densitometry were used for normalization of the amounts of myosin.

The stoichiometry of the phosphorylation has been determined by densitometry of two-dimensional (2D) gels which separate phosphorylated forms of MLC from unphosphorylated MLC (19, 23). REF4A cells were labeled for 3.5 h with 40 µCi/ml of TRAN33S-LABEL (ICN, Irvine, CA) in methionine-free DMEM or 2.5 µCi of [3H]-leucine in leucine-free DMEM, followed by 2.5 h of incubation in the presence of nocodazole. Mitotic, nonmitotic cells and cells at 30 min after the release of mitotic arrest were collected as described above. Myosin immunoprecipitated from these cells was analyzed on 2D gels using Investigator 2-D electrophoresis system from Millipore (Millipore Corp., Bedford, MA). The radioactivities of phosphorylated and unphosphorylated forms of MLC were quantitated using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA).

Phosphopeptide Mapping and Phosphoamino Acid Analyses

MLC bands labeled with [32P] either in vivo or in vitro were excised from SDS gels. Tryptic phosphopeptides were prepared with TPCK-trypsin (Worthington Biochem. Corp., Freehold, NJ) and mapped by one dimensional isoelectric focusing as described (27). Briefly, gel slices were lyophilized with 25% 2-isopropl alcohol three times, 10% methanol three times, and dried completely. After addition of 950 µl of 100 mM NH₄HCO₃, and 50 µl of 1 mg/ml of TPCK-trypsin to dried gel slices, the samples were shaken for 16-24 h at 37°C. Another 50 µl of trypsin was added halfway through digestion. Trypsin digests were analyzed on isoelectric focusing gels containing 70% acrylamide, 0.2% bisacrylamide, ampholytes (pH 2.5-4, pH 3.5/5 and pH 5/8, each at 0.8%) and 6 M urea. Focusing was run at 25 watts for 60 min with 0.1 M NaOH as cathode buffer and 0.1 M H₃PO₄ as anode buffer.

Phosphoamino acids of each of the phosphopeptides separated on one-dimensional isoelectric focusing gels were determined in the following way: tissues 6 M urea disturbed phosphoamino acid analyses, phosphopeptides of MLC were first separated on silica gels by two-dimensional isoelectric focusing as described (18). The phosphopeptides thus separated were eluted with water and divided into two aliquots. One half of the eluent was analyzed by one-dimensional isoelectric focusing as described above, and the other half was used for the phosphoaminoacid analyses (14). For acid hydrolysis, the lypophilized samples were directly dissolved in 6 M HCl and hydrolyzed for 2 h at 110°C. After removing HCl under reduced pressure, the hydrolysates were dissolved in a marker mixture containing phosphoserine, phosphothreonine, and phosphotyrosine, each at 1 mg/ml. Acid hydrolysates were analyzed on cellulose thin-layer plates (Kodak chromatogram sheet, 13255 cellulose) (Eastman Kodak Co., Rochester, NY) by electrophoresis at pH 3.5 for 1 h at 1 kV in glacial acetic acid:pyridine:H₂O = 50:9:45 (vol/vol). The phosphoaminoacid markers were detected by staining with ninhydrin.

Fractionation of MLC Kinase and cdc2 Kinase

To prepare MLC kinase and cdc2 kinase from mitotic cells, HeLa cells were grown to subconfluency on 56 large culture dishes (245 × 245 mm, Nunc) in DMEM containing 6% calf serum, and were treated with 0.25 µg/ml of nocodazole for 16 h. Mitotic cells were collected and treated with Triton/xylol/glycerol solution (0.1 M Pipes-NaOH, 5 mM MgCl₂, 0.2 mM EGTA, 4 M glycerol and 0.05% Triton X-100, pH 6.9), and homogenized with an equal volume of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.3) containing 0.2 mM EGTA, 5 mM MgCl₂, 1 mM PMSF, and 1 mM DTT. The supernatants obtained after centrifugation at 200,000 g for 1 h, were fractionated by Sephacryl S-300 gel filtration. Both MLC and histone HI were used as substrates to detect MLC and histone HI kinase activities. Nonmitotic cells after collection of mitotic cells were fractionated in the same way. For the purification of cdc2 kinase, Sephacryl S-300 fractions with a peak histone HI kinase activity were further fractionated by p13 affinity column chromatography as described (21). After loading, the p13 column was sequentially washed with the following three solutions; solution A (20 mM imidazole buffer of pH 7.0, 1 mM DTT, 2 mM MgCl₂, 1 mM EGTA, 0.25 mM PMSF), solution A containing 0.5 M NaCl, solution A containing 50% ethylene glycol. cdc2 kinase was then eluted with solution A containing both 0.5 M NaCl and 50% ethylene glycol. The concentrated, purified cdc2 kinase was found to phosphorylate histone HI at a rate of 16-17 pmol/min/µl. In some experi-

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Analyses of Phosphorylation Sites During Mitosis

In Vivo Phosphorylation of MLC during Mitosis

Results

In Vivo Phosphorylation of MLC during Mitosis

We have first examined whether MLC is phosphorylated in a mitosis-specific way. Mitotic and nonmitotic REF-4A cells were labeled in vivo with \(^{32}\)P-orthophosphate for 3 h in the presence of nocodazole, and mitotic cells were collected as described in the section of Materials and Methods. Both myosin heavy and light chains were immunoprecipitated as a complex from total cell lysates of mitotic and nonmitotic cells using an anti-heavy chain antibody (kindly provided by Dr. J. Sellers). As Fig. 1A shows, MLC is phosphorylated to a much greater extent in mitotic cells than in nonmitotic cells. Quantitative analyses using scintillation counting or densitometry of autoradiographs were analyzed with a densitometer (Chromoscan 3; Joyce-Loebl) as described (17). Protein concentrations were determined by the method of Bradford (4) using bovine serum albumin as a standard.

Analyses of Phosphorylation Sites During Mitosis

The sites of in vivo phosphorylation have been analyzed by one-dimensional phosphopeptide mapping. For comparison, MLC of smooth muscle myosin II was phosphorylated in vitro by either MLCK or protein kinase C, and the phosphorylated MLC bands were analyzed in the same way.

The phosphopeptide pattern of mitotic MLC is similar, but not identical, to that of MLC phosphorylated by protein kinase C. As Fig. 1B shows, the phosphopeptide pattern of mitotic MLC yields two major and one minor spots. Of these, the two major spots comigrate with the phosphopeptide spots generated by the phosphorylation of MLC with protein kinase C. The minor spot, on the other hand, corresponds with the spot produced by the phosphorylation of MLC with MLCK. Phosphoaminoacid analyses have revealed that all mitotic spots contain phosphoserine (data not shown), indicating that mitotic MLC is mainly phosphorylated in vivo at Ser-1 and/or Ser-2 while it is also phosphorylated, though to a much lesser extent, at Ser-19.

On the contrary, little phosphorylation at Ser-1/2 is observed with nonmitotic MLC. Instead, phosphorylation at Ser-19 is recognizable to the extent similar to that observed with mitotic MLC. Several minor phosphopeptide bands are also recognized with nonmitotic MLC. These bands may come from phosphorylated phosphoproteins contaminated in the preparation of nonmitotic MLC because a large amount of nonmitotic MLC had to be loaded, due to the low radioactivity of nonmitotic MLC.

Mitotic MLC lacks two phosphopeptide spots which are
observed in the phosphopeptide pattern generated by protein kinase C. Phosphoamino acid analyses have revealed that one spot close to the origin contains phosphothreonine and the other spot contains phosphoserine (data not shown). This suggests that mitotic phosphorylation does not occur at Thr-9 in contrast to the phosphorylation by protein kinase C. The inclusion of phosphatase inhibitors such as NaF during immunoprecipitation did not change the phosphopeptide patterns of mitotic MLC, suggesting that the absence of the Thr-9 site is not due to contaminating phosphatase activities.

**Changes in the Sites and Extents of Phosphorylation After the Release of Mitotic Arrest**

The above results demonstrate that MLC is phosphorylated in vivo at Ser-1 and/or Ser-2 during prometaphase. Cytokinesis involves activation of myosin II, therefore it is of interest to determine whether the phosphorylation states of MLC change during cell division. Mitotic cells were allowed to proceed through division following the removal of nocodazole from media. Myosin II was immunoprecipitated at various times after the release of mitotic arrest, and the extents of MLC phosphorylation were quantitated by densitometry. At the same time cell morphology was checked by light microscopy as reported previously (13).

The total level of MLC phosphorylation is increased as cells are allowed to divide, and decreased when cells finish spreading (shown by open bars in Fig. 2). At 10 min after the release of mitotic arrest, virtually no cells are dividing and the total level of MLC phosphorylation is unchanged. At 30 min when <10% of the cells exhibit the early stages of cytokinesis, the total level of phosphorylation is increased by more than twofold. By 60 min >60% of the cells complete division, and the level of MLC phosphorylation is slightly lowered by 18%. At 180 min when cells complete spreading and assembly of stress fibers, the total level of phosphorylation is decreased to 23% of the level at 30 min.

Phosphopeptide analyses have been performed to elucidate whether the sites of phosphorylation are changed as cells undergo cell division. Fig. 3 shows the peptide maps of MLC at 30 and 60 min after the release of mitotic arrest. The phosphorylation at Ser-1/2 is decreased as cells are incubated longer, while the phosphorylation at Ser-19 is greatly increased (compare Fig. 3, lanes 1 and 2 with Fig. 1 B, lane 2).

In addition to the phosphorylation at Ser-19, a band (shown by an arrowhead with asterisk) becomes recognizable with the maps obtained at 30 min and 60 min. As the band co-migrates with the minor band generated by the phosphorylation of MLC by MLCK (see Fig. 1 B, lane 4) this minor band, together with the major band corresponding to Ser-19, are assigned as the MLCK sites. This spot may be the di-phosphorylated Ser-19/Thr-18, which is known to be phosphorylated by MLCK.

We have analyzed the changes in the levels of phosphorylation at each site by densitometry of the phosphopeptide maps obtained at 0, 10, 30, 60, and 180 min (Fig. 2). At 10 min when the total phosphorylation level (open bars in Fig. 2) is not altered, the phosphorylation at Ser-1/2 (cross-hatched bars) is decreased by half whereas the phosphorylation at the MLCK sites (filled bars) is increased six times. At 30 min the phosphorylation at the MLCK sites is increased greatly, becoming a major site while phosphorylation at Ser-1/2 seems to be decreased slightly. At 60 min, the level of phosphorylation at the MLCK sites is still high while phosphorylation at Ser-1/2 is greatly reduced to 18% of the level found in mitotic MLC. At 180 min, the level of phosphorylation...
Figure 4. 2D gel analyses of MLCK from nonmitotic cells (A and B), mitotically arrested cells (C and D), and cells 30 min after the release of mitotic arrest (E and F). REF-4A cells were labeled with either a mixture of [35S]methionine or [35S]cysteine (A, C, and E) or 32p-orthophosphate (B, D, and F) as described in Materials and Methods, and immunoprecipitated myosin II were analyzed by 2D gel electrophoresis. Only parts of 2D gels including regulatory and essential light chains are shown. Approximately equal amounts of myosin II were loaded.

The phosphorylation of MLC at the MLCK sites is decreased to 25% of the level at 30 min, whereas the phosphorylation at Ser-1/2 becomes almost undetectable. These results suggest that switching of phosphorylation sites from Ser-1/2 to the MLCK sites seems to occur during cell division.

Stoichiometry of MLC Phosphorylation during Mitosis

To examine whether the increase in phosphate incorporation during mitosis and subsequent cell division reflects real changes in the phosphorylation states of MLC, we have determined the molar ratio of phosphorylated forms to the unphosphorylated form of MLC. Myosin II was immunoprecipitated from cells that had been labeled with either a [35S]-methionine/cysteine mixture or [34C]-leucine. Then unphosphorylated and phosphorylated forms of MLC were separated on 2D gels. Similar results were obtained with either 35S- or 34C-labeled cells.

First, we have identified unphosphorylated and phosphorylated forms of rat cell MLC on 2D gels. Fig. 4A shows the part of the 2D gel which includes both regulatory and essential light chains of 35S-labeled nonmitotic cells. Fig. 4B shows the corresponding part of the 2D gel with 32P-labeled nonmitotic myosin. Spots a and b are assigned as unphosphorylated and phosphorylated forms of MLC, respectively, because only spot b is labeled with 32p-phosphate as shown in Fig. 4B. Spot c in Fig. 4B is likely to be a di-phosphorylated form of MLC, the level of which is too low in nonmitotic cells to be detected by 35S-labeling.

The identifications of the spots a and b were confirmed by the following two ways: First, myosin II partially purified from cultured rat cells was phosphorylated in vitro with MLCK or PKC, and 2D gel patterns of MLC were analyzed. Before phosphorylation, rat cell myosin yielded a 2D pattern of MLC similar to that of Fig. 4A. Upon phosphorylation by either kinase, some mass of spot a shifted to spot b, and at the same time, spot b was radiolabeled with 32P. This suggests that monophosphorylated MLC migrates at the same position on our 2D gel system despite the fact that these two kinases phosphorylate different sites. Second, the isoelectric point of spot a did not change after treatment with phosphatase, suggesting that spot a represents an unphosphorylated form of MLC (data not shown).

Next, we have analyzed the 2D patterns of 35S-labeled MLC in mitotically arrested cells (Fig. 4C) and in cells at 30 min after the release of mitotic arrest (Fig. 4E). We have found that the level of phosphorylated forms of MLC in mitotically arrested cells is higher than that in nonmitotic cells, and that the level of phosphorylation becomes even higher in cells after the release of mitotic arrest than in mitotically arrested cells. As Fig. 4C and E show, the amount of spot b is increased both in mitotically arrested cells and cells at 30 min, and spot c becomes recognizable in cells at 30 min. The 2D gel patterns (Fig. 4D and F) of 32P-labeled MLCs have confirmed that spots b and c are heavily labeled with 32P. It also shows that a multiply phosphorylated form of MLC (spot d) appears in cells 30 min after the release of mitotic arrest (Fig. 4F).

We have determined the molar ratio of the phosphorylated forms (spots b and c) to total MLC (spots a, b, and c) using a PhosphorImager. We did not quantitate intensities of minor spots just below the spots a and b as the intensities of these spots are very low in comparison with the major spots a, b, and c (these minor spots are likely to be a minor isof orm of MLC). The ratios are 0.16 ± 0.06 in nonmitotic cells, 0.31 ± 0.05 in mitotically arrested cells, and 0.49 ± 0.05 in cells 30 min after the release of mitotic arrest.

In Vitro Phosphorylation of MLC by Mitotic and Nonmitotic Extracts

The in vivo phosphorylation of MLC suggests that there are mitosis-specific kinase activities which phosphorylate MLC at Ser-1/2. We have thus examined whether cell extracts show MLC kinase activities in a mitosis-specific way.

We have first fractionated mitotic and nonmitotic HeLa cell extracts by gel filtration on a Sephacryl S-300 column, and kinase activities have been examined using both histone H1 and MLC as substrates. As Fig. 5 shows, MLC kinase activities of both mitotic and nonmitotic extracts are eluted as three peaks from the column although the activity of each
Figure 5. Fractionation of MLC kinase activities in mitotic and nonmitotic extracts by Sephacryl S-300 gel filtration. Mitotic and nonmitotic HeLa cell extracts were loaded on Sephacryl S-300 column, and each fraction was tested for its activity to phosphorylate histone H1 and MLC. A and C are the elution profiles of mitotic and nonmitotic cell extracts, respectively; B and D show Histone H1 and MLC kinase activities of mitotic and nonmitotic eluents, respectively. Histone H1 kinase activity of mitotic cell extracts is eluted as a large single peak while nonmitotic cell extracts show a very low activity of histone H1 kinase activity. Both mitotic and nonmitotic cell extracts appear to have three peaks of MLC kinase activity.

Figure 6. Analyses of phosphorylation sites of MLC phosphorylated with sephacryl S-300 fractions of mitotic and nonmitotic eluents (see Fig. 5). The peak fractions of MLC kinase activity from mitotic and nonmitotic eluents were used to phosphorylate MLC, and the sites were analyzed by peptide mapping. Only the middle peak (fraction M60) of the mitotic eluent contains an activity which phosphorylates MLC at Ser-1/2 while all the other peak activities mainly phosphorylate MLC at Ser-19. Note that fraction M60 does not phosphorylate Thr-9. Lanes 1–3 and lanes 4–6 are from mitotic and nonmitotic eluents, respectively. Lane 1, fraction 51; lane 2, fraction 60; lane 3, fraction 69; lane 4, fraction 48; lane 5, fraction 60; lane 6, fraction 69.

peak is higher in mitotic extracts than in nonmitotic extracts (Fig. 5, B and D). Analyses of phosphorylation sites (Fig. 6) have revealed that only the middle peak activity of mitotic eluents phosphorylates MLC at Ser-1/2 (Fig. 6, lane 2) while all other activities including the three peak activities of nonmitotic eluents phosphorylate MLC at the MLCK sites. It is worthy of note that phosphorylation by the middle peak of mitotic eluents is similar to that observed in vivo in mitotic cells; the phosphorylation sites are at Ser-1/2 but not at Thr-9.

The middle peak of MLC kinase activity from mitotic eluents appears to correspond to the peak of histone H1 kinase activity of mitotic extracts (Fig. 5 B). To examine whether cdc2 kinase phosphorylates MLC at the C-kinase sites as reported (31), cdc2 kinase was purified from the middle peak of the S-300 fractions by pl3 affinity column chromatography. Two methods were used for the specific elution of cdc2 kinase from a pl3 column. One was to elute the column with pl3 protein (5) and the other was to elute with a solution containing both 0.5M NaCl and 50% ethylene glycol (21). Either elution method yielded essentially the same results, and we will describe the results obtained with the latter method.
As Fig. 7A shows, cdc2 kinase bound to the column is eluted by solution A containing both 0.5 M NaCl and 50% ethylene glycol. As expected, the eluted cdc2 kinase shows a very high histone H1 kinase activity. The kinase is also able to phosphorylate MLC when MLC isolated from myosin heavy chain was used as a substrate. Phosphopeptide analyses (Fig. 7B) have revealed that the sites of phosphorylation are Ser-1/2, confirming the results reported by Satterwhite et al. (31). It should be noted that the phosphorylation at Thr-9 becomes much more noticeable with cdc2 kinase than that observed with the kinase of the S-300 fractions (compare Fig. 7B, lane 5 with Fig. 6, lane 2).

MLC is a poor substrate for purified cdc2 kinase when it is compared with other substrates including histone H1 and chick smooth muscle caldesmon. As the time course experiment of Fig. 8 shows, purified cdc2 kinase exhibits a very slow rate of MLC phosphorylation when MLC isolated from heavy chain of smooth muscle myosin II is used as a substrate. On the contrary, both histone H1 and chick smooth muscle caldesmon quickly incorporate phosphate under the same conditions.

Heavy chain-associated MLC from chick smooth muscle is an even poorer substrate for cdc2 kinase as virtually no phosphorylation of MLC is detected in 2 h of incubation (Fig. 8). This situation is slightly improved when nonmuscle myosin II purified from bovine adrenal medulla is used instead of smooth muscle myosin II. Phosphate incorporation is increased from 0 to 0.015 mol per mol protein after 2 h of incubation with purified cdc2 kinase.

Stoichiometry of MLC phosphorylation by cdc2 kinase is very low. A value of 0.076 mol phosphate per mol of MLC is calculated at 2 hr incubation with cdc2 kinase (Table I). On the other hand, cdc2 kinase phosphorylates chick smooth muscle caldesmon at a ratio of 2 mol per mol of protein under the same conditions, the value of which is consistent with the previous report by Mak et al. (16). MLCK and PKC phosphorylate MLC at a ratio of 0.73 and 1.78, respectively.

Discussion

This paper has shown that the in vivo phosphorylation of MLC is drastically changed during mitosis. We have found that (a) the basic level of MLC phosphorylation is very low
in nonmitotic cells; (b) the extent of phosphorylation becomes higher during mitosis; (c) MLC in mitotically arrested cells is mainly phosphorylated at Ser-1/2; (d) the total level of phosphorylation is further increased at 30 min after the release of mitotic arrest when some cells start cytokinesis; and (e) during this process, the sites of phosphorylation are switched from Ser-1/2 to Ser-19.

Quantitation of the phosphorylation state of MLC has revealed that the molar ratio of the phosphorylated forms to total MLC is 0.16 ± 0.06 in nonmitotic cells, 0.31 ± 0.05 in mitotically arrested cells, and 0.49 ± 0.05 in cells 30 min after the release of mitotic arrest. When these values are compared with the incorporation of 32P-radioactivities during mitosis (Fig. 2), the following trends may be noted. First, the rate of phosphate turnover is likely to be very slow during interphase. The extent of 32P-phosphate incorporation in nonmitotic cells is much too low when the values of incorporation are compared with the stoichiometry (the molar ratio) of phosphorylation between mitotic and nonmitotic cells. Second, ≈15% of MLC (the difference in the molar ratio between mitotic and nonmitotic cells) appears to become phosphorylated at Ser-1/2. Third, this 15% of MLC seems to be quickly dephosphorylated and instead, ≈33% of MLC appears to be phosphorylated mainly at Ser-19 after the release of mitotic arrest. Fourth, dephosphorylation of MLC after cytokinesis occurs slowly, suggesting that phosphorylation of MLC at the MLCK sites may be required for cell spreading after cell division (Fig. 2).

These notions further suggest that the phosphorylation of one third of myosin II would be sufficient to complete cell division. The low stoichiometry of phosphorylation during cell division may be related to the report that, in smooth muscle, phosphorylation of only 5 or 20% of myosin (depending on the sources of smooth muscle) is sufficient for tension development (6, 12). It remains to be investigated whether the phosphorylated myosin is specifically localized in the cleavage furrow or uniformly distributed throughout the cytoplasm of dividing cells.

The switching of sites of phosphorylation from Ser-1/2 to Ser-19 is likely to be a signal for the induction of cytokinesis, since it is well established that phosphorylation at Ser-19, the MLCK site, induces contraction of actomyosin in smooth muscle and nonmuscle cells (for reviews see 33, 36). Which kinase phosphorylates MLC at the MLCK sites? A natural candidate is MLCK. Inhibitors of MLCK were reported to block cytokinesis (26). More recently, antisense expression of MLCK has been reported to inhibit cell proliferation of fibroblasts (35). Fishkind et al., however, presented an interesting result suggesting a function for MLCK during mitosis (10). They have microinjected unregulated, constitutively active catalytic fragments of MLCK into mitotic cells, and found two effects. First, there is a significant delay in the transit time from nuclear envelope breakdown to anaphase onset. Second, the microinjection induces motile surface activity during and after metaphase. However, the microinjection neither affects the formation of cleavage furrows nor changes the rate of contraction of cleavage furrows. If MLCK regulates contraction of cleavage furrows, then microinjection of unregulated MLCK would affect the kinetics of cytokinesis and the formation of cleavage furrow. It is thus an open question, at present, whether MLCK or other kinases are involved in the regulation of cytokinesis.

### Table 1. Phosphate Incorporation of Chick Smooth Muscle MIC by cdc2 Kinase, MLCK, and Protein Kinase C (mol phosphate/mol protein)*

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<th>cdc2 kinase</th>
<th>MLCK</th>
<th>PKC</th>
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<tr>
<td>Isolated MLC</td>
<td>0.076</td>
<td>0.73</td>
<td>1.78</td>
</tr>
<tr>
<td>Heavy chain-associated MLC</td>
<td>0</td>
<td>0.94</td>
<td>0.42</td>
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<tr>
<td>Chick smooth muscle caldesmon</td>
<td>2.0</td>
<td>ND</td>
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* The stoichiometry is determined at 2 h of incubation.

ND, not determined.

Satterwhite et al., (31) have proposed that the phosphorylation at the C-kinase sites during prometaphase keeps myosin II from being activated prematurely. This idea is not only very attractive but also possible because phosphorylation of MLC by protein kinase C is known to inhibit both actomyosin ATPase and myosin filament assembly (2, 28). It should be noted, however, that only 15% of myosin appears to be phosphorylated at Ser-1/2 during mitosis. It is possible that such a low fraction of phosphorylated myosin cooperatively inhibits the rest of myosin. Alternatively, the phosphorylated myosin may be specifically localized in the cleavage furrow, thereby keeping the myosin in the cleavage furrow from activating. Another point to be noted is that the sites of phosphorylation during mitosis are not exactly the same as the sites used by protein kinase C. Phosphopeptide analyses have revealed that phosphorylation at Thr-9, one of the major phosphorylation sites by protein kinase C, is not observed in vivo. In addition, the peptide patterns made by the phosphorylation at Ser-1/2 during mitosis are considerably different from the in vitro phosphorylation by protein kinase C. It is thus very important to elucidate whether the mitotic phosphorylation at Ser-1/2 has effects on the in vitro phosphorylation by protein kinase C. Since the mitotic phosphorylation indeed has similar inhibitory effects on myosin assembly, it may also play a role in the disassembly of stress fibers during prophase, which leads to rounding of cell shape.

Which kinase is responsible for the mitosis specific phosphorylation of MLC at Ser-1/2? Protein kinase C may not be involved in this mitotic phosphorylation because the phosphopeptide map was considerably different from that of in vitro phosphorylation by protein kinase C. We have confirmed the results by Satterwhite et al., (31) that purified cdc2 kinase is able to phosphorylate MLC in vitro at Ser-1/2. There remain, however, several questions to be answered before it would be concluded that cdc2 is involved. First, the activity is quite low, particularly when heavy chain-associated MLC is used as a substrate. Second, the phosphorylation is not stoichiometric. Third, the peptide map of in vivo phosphorylation differs from that of in vitro phosphorylation by cdc2 kinase. While the in vivo phosphorylation at Thr-9 is not observed, cdc2 kinase phosphorylates Thr-9 in addition to Ser-1 and/or Ser-2.

These problems could be explained in several ways. For
example, the assay conditions for the phosphorylation of MLC with cdc2 kinase may not be appropriate. Additional factors may be required for effective phosphorylation of MLC with cdc2 kinase. Further, contaminating phosphatases could explain the difference in the peptide maps between in vivo and in vitro phosphorylation; the phosphorylation at Thr-9 may be specifically unphosphorylated during immunoprecipitation in spite of the presence of the phosphatase inhibitor. Indeed, dephosphorylation at Thr-9 was reported to occur more easily (9). Nevertheless, it is equally possible that kinases other than cdc2 kinase are responsible for the phosphorylation of MLC at Ser-1/2. This possibility may be supported by the fact that the crude kinase from the S-300 fraction phosphorylates MLC at Thr-9 to a much lesser extent than does purified cdc2 kinase. However, this could be again explained by contaminating phosphatases, which may be removed during purification with pI3 affinity chromatography. Apparently more studies are needed to solve these problems.

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