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Cell Cycle-Dependent Phosphorylation of Smooth Muscle Myosin Light Chain in Sea Urchin Egg Extracts[1]

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We studied enzymatic activities in sea urchin egg extracts that phosphorylate myosin regulatory light chain (MRLC) from chicken gizzard smooth muscle. The activity in the presence of EGTA showed cell cycle-dependent changes similar to that of histone H1 kinase, namely, it peaked shortly before cleavage, while that in the presence of Ca^{2+} ions did not show significant change during division cycle. Phosphopeptide mapping revealed that both the sites phosphorylatable by smooth muscle myosin light chain kinase (MLCK sites) and the sites phosphorylatable by protein kinase C (PKC sites) were phosphorylated in the presence or absence of Ca^{2+} ions. By analyses using an inhibitor of cdc2 kinase, butyrolactone-I, and ion exchange column chromatography, at least three kinases were detected as kinases that phosphorylate MRLC *in vitro*. These kinases phosphorylated distinct sites on MRLC. The first one, which phosphorylated the PKC sites, was identified as cdc2 kinase. The second one phosphorylated the MLCK sites in the absence of Ca^{2+} ions. The third one phosphorylated unknown sites. Possible implication of these activities in regulation of cytokinesis is discussed.

Key words: cell division, histone H1 kinase, myosin light chain, phosphorylation, sea urchin.

Myosin (Myosin-II) plays a key role in cytokinesis of eukaryotic cells (*1, 2*). Microinjection of antibodies against myosin inhibits cytokinesis of starfish oocytes (*3, 4*). Antisense expression or gene targeting of myosin-II heavy chain in a cellular slime mold, *Dictyostelium discoideum*, caused a defect of cytokinesis in suspension culture (*5, 6*). A mutation in nonmuscle myosin regulatory light chain (MRLC) gene (*spaghetti-squash*) of *Drosophila melanogaster* results in a defect of cytokinesis (*7*).

Smooth muscle and nonmuscle myosins are both positively and negatively regulated by phosphorylation of regulatory light chain (*8, 9*). The actin-activated ATPase activity of these myosins is enhanced by phosphorylation of MRLC by Ca^{2+}-calmodulin (CaM)-dependent light chain kinase (MLCK). Amino acid residues phosphorylated by MLCK (MLCK sites) are mainly Ser19 and, under some conditions, Thr18. This phosphorylation also promotes myosin filament assembly, at least *in vitro*. On the other hand, MRLC is phosphorylated by protein kinase C (PKC) at Ser1, Ser2, and Thr9 (PKC sites) (*10-12*). Phosphorylation at the PKC sites inhibits the myosin ATPase activity in two ways. If the MLCK sites are not phosphorylated, it reduces the subsequent phosphorylation at the MLCK sites by MLCK (*10, 12*). If the MLCK sites are already phosphorylated, it inhibits the actin-activated ATPase activity by reducing the affinity of myosin for actin filaments (*10-13*).

Many cell cycle events are regulated by protein phosphorylation. The entry into M-phase is triggered by M-phase promoting factor (MPF), which consists of at least two components, $p34^{cdc2}$ kinase and mitotic cyclin (*14*). MPF is thought to phosphorylate various specific substrates and thereby regulate microtubule dynamics, intermediate filament arrays and actin filament organizations (*15-18*). The G1/S transition is thought to be regulated by cdk2 or other cell cycle-dependent protein kinases. Cytokinesis could also be regulated by protein phosphorylation. Indeed, ML-9, an inhibitor of MLCK, inhibits formation of the contractile ring in fertilized sea urchin eggs, suggesting the possibility that phosphorylation of MRLC might be a key event in regulation of cytokinesis (*19*).

Recently, Satterwhite *et al.* (*20*) have discovered that cdc2 kinase phosphorylates MRLC *in vitro* at the PKC sites. Yamakita *et al.* (*21*) reported that in nocodazole-arrested mammalian cultured cells MRLC is phosphorylated mainly at the PKC sites (Ser1 or Ser2). This phosphorylation is decreased after release from the arrest. Simultaneously, phosphorylation at the MLCK sites is increased.

Here we report that an MRLC kinase activity under

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Abbreviations: BL-I, butyrolactone-I; CaM, calmodulin; IC_{50}, 50% inhibitory concentration; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MLCK, myosin light chain kinase; MRLC, myosin regulatory light chain; PKC, protein kinase C; MPF, M-phase promoting factor; TAME, $N\alpha$-*p*-tosyl-L-arginine methyl ester; PMSF, phenylmethylsulfonyl fluoride; TFP, trifluoperazine; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

Ca^{2+}-chelating conditions in sea urchin egg extracts is increased at prometa/metaphase (mitotic phase) and decreased at ana/telophase (division phase). Both the MLCK sites and the PKC sites are phosphorylated and at least three kinases are involved in these phosphorylations. Though one of these kinases is likely to be cdc2 kinase, its contribution in the MRLC phosphorylation seems to be smaller than that of other kinases.

MATERIALS AND METHODS

Materials—Eggs of sea urchins *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus*, and *Clypeaster japonicus* were used, among which data obtained for *H. pulcherrimus* eggs are mainly shown in this study. Chicken gizzard myosin light chains (mixture of regulatory and essential light chains) were purified according to the methods of Adelstein and Klee (*22*). Chicken gizzard MLCK (*22*) was provided by Dr. H. Hosoya (Hiroshima University). Rat brain PKCα (*23*) was provided by Drs. K. Mizuno and S. Ohno (Yokohama City University). Starfish oocyte MPF was provided by Drs. E. Okumura and T. Kishimoto (Tokyo Institute of Technology). Butyrolactone-I (BL-I) was provided by Dr. A. Okuyama (Banyu Tsukuba Res. Inst., Tsukuba). Histone Type IIIS was purchased from Sigma Chem. (St. Louis, MO). Bovine testis CaM was from Biomedical Technologies (Stoughton, MA). Leupeptin was from Peptide Laboratory (Osaka).

Preparation of Egg Extracts—Gametes from sea urchins were obtained by intracoelomic injection of 100 mM acetylcholine chloride. The eggs were passed through a nylon cloth (77 μm mesh) to remove the jelly layer, washed three times with natural sea water and inseminated. One minute after insemination, 9 volumes of 1 M urea-10 mM sodium bicarbonate were added to the egg suspension to soften the fertilization membranes, which were then removed by passing the eggs through the nylon cloth. The eggs were washed three times with a Ca^{2+}-free artificial sea water and cultured as 1% (v/v) suspension at 19°C.

The eggs were harvested at desired times after fertilization by hand-driven centrifugation. To 100 μl of packed eggs, 300 μl of chilled homogenizing buffer (0.9 M glycerol, 50 mM KCl, 30 mM β-glycerophosphate, 10 mM NaF, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM sodium orthovanadate, 10 mM TAME, 10 mM MOPS-NaOH, pH 7.2, 1 mM DTT, 4 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF) was added and the eggs were ruptured by 10 strokes with a Teflon-glass homogenizer. The homogenates were immediately centrifuged at 100,000×g for 10 min at 4°C. The supernatants were quickly frozen in liquid nitrogen and stored at −80°C in aliquots. An aliquot was assayed for protein concentration using bovine serum albumin as a standard (*24*).

Kinase Assays—Kinase reaction mixture (25 or 12 μl) consisted of 50 mM KCl, 10 mM β-glycerophosphate, 5 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM MOPS-NaOH, pH 7.2, 50 μM ATP, 50 μCi/ml [γ-^{32}P]ATP, 1 mM DTT, 4 μg/ml leupeptin, 1 μg/ml aprotinin, 0.25 mg/ml chicken gizzard myosin light chains or 0.2 mg/ml histone type III-S, and 0.2 to 0.3 mg/ml egg extract proteins. For assay of Ca^{2+}/CaM-dependent activity, EGTA was omitted and $CaCl_2$ at a concentration of 0.2 mM in excess of that of EGTA from egg extract and 1 μM CaM were included. All preincubations were carried out at 0°C, and reactions were started by addition of labeled ATP. After incubation at 25°C for 5–10 min (for extracts) or 15–20 min (for column fractions), reactions were stopped by addition of 4×SDS-PAGE sample buffer (*25*) followed by boiling for 4 min. Proteins were analyzed by SDS-PAGE (15% acrylamide for histone H1 as substrate and 18% for MRLC), and phosphate incorporation into the substrate proteins was measured by counting the radioactivity of excised bands with a scintillation counter (Beckman LS 1800, Beckman, Fullerton, CA), by densitometry of autoradiographs, or by use of a Fujix BAS 1000 radioimaging analyzer (Fuji Film, Tokyo). The phosphorylation of MRLC by PKCα was done according to Saido *et al.* (*23*). For the experiment on BL-I sensitivity of purified kinases, 0.1 μg/ml MPF or MLCK was included in the reaction mixture.

Tryptic Peptide Mapping—Tryptic phosphopeptide mapping was performed according to Kawamoto and Adelstein (*26*) with some modifications, and tryptic peptides were assigned according to Kawamoto *et al.* (*27*). Protein bands stained with Coomassie Brilliant Blue were excised from dried polyacrylamide gels, washed in 25% (v/v) 2-propanol three times for 15 min each and then in 10% (v/v) methanol three times for 15 min each. The gels were crushed, dried, and resuspended in 0.5 ml of 50 mM ammonium bicarbonate containing TPCK-trypsin (50 μg/ml). After incubation at 37°C for 12 h TPCK-trypsin (20 μg/ml) was further added. After incubation for an additional 12 h the supernatant was transferred to a fresh tube, incubated for 6 h with additional TPCK-trypsin (10 μg/ml) and lyophilized six times, each in 1.0 ml of MilliQ (Nihon Millipore, Yonezawa) water. Phosphopeptides were analyzed on 20×20 cm cellulose plates (Funacel SF, Funakoshi, Tokyo) by electrophoresis at 1,000 V for 35 min in 15 : 5 : 80 acetic acid/formic acid/water as the first dimension and chromatography in 97.5 : 75 : 15 : 60 *n*-butanol/pyridine/acetic acid/water for 4 h as the second dimension, followed by autoradiography with a Fujix BAS 1000 radioimaging analyzer. Phosphorylation at the specific sites on MRLC was roughly estimated by densitometry of the phosphopeptide spots.

MonoQ Column Chromatography—An aliquot of frozen egg extract was thawed and diluted with 10 volumes of column buffer (10 mM Tris-HCl, pH 7.6, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 4 μg/ml leupeptin, and 1 μg/ml aprotinin). The supernatant obtained after centrifugation at 100,000×g for 10 min at 2°C was applied to a MonoQ HR 5/5 column (5×50 mm) connected to an FPLC system (Pharmacia LKB, Uppsala, Sweden) at a flow rate of 1 ml/min. Proteins were eluted with 20 ml of a linear gradient of NaCl (0–0.5 M) dissolved in the column buffer at the same flow rate. Collected fractions (500 μl each) were assayed for protein kinase activities.

RESULTS

Cyclic Activation of MRLC Kinase Activity in the Presence of EGTA in Sea Urchin Egg Extracts—First, we studied changes in MRLC-phosphorylating activities in soluble extracts of sea urchin (*Hemicentrotus pulcherrimus*) eggs during the first two cell division cycles using

chicken gizzard MRLC. The activity in the presence of EGTA underwent cyclic activation during the division cycles (Fig. 1); it peaked around metaphase. This pattern was similar to that of histone H1 kinase (HH1K) activity, which is believed to represent the activity of MPF/cdc2 kinase. The pattern was reproducible in five independent experiments, though there were slight differences in the activity and the time course of cell division among experiments.

The activity in the presence of Ca^{2+} ions (Fig. 1) was almost the same level as that in the presence of Ca^{2+} ions plus added CaM (not shown), probably due to the presence of endogenous CaM in the extracts, and it was roughly two times higher than that in the presence of EGTA (Fig. 1). It was highest in unfertilized eggs and gradually decreased to about a half by 30 min post-fertilization (not shown). Then, it was roughly constant and did not show obvious change during the division cycles. The activity in the presence of both Ca^{2+} ions and trifluoperazine (TFP), a CaM antagonist, was almost constant during the division cycles (Fig. 1).

The cyclic activation of MRLC-phosphorylating activity in the presence of EGTA was also clearly observed with eggs of another sea urchin, *Pseudocentrotus depressus*, but was not so remarkable with those of *Anthocidaris crassispina* and the sand dollar *Clypeaster japonicus* (data not shown). The eggs of *H. pulcherrimus* were used in the following experiments.

Phosphorylation Sites on MRLC Phosphorylated in Egg Extracts—We determined the sites on gizzard MRLC phosphorylated by kinases in egg extracts by two-dimensional mapping of tryptic phosphopeptides (Fig. 2A). We compared the maps with those of MRLC phosphorylated by chicken gizzard MLCK or rat brain PKC (Fig. 2B).

Phosphorylation by egg extracts at both the MLCK and PKC sites was observed throughout the division cycles irrespective of the presence of Ca^{2+}-CaM. Percentage phosphorylations at the MLCK sites and the PKC sites at the period other than metaphase in the presence of EGTA were around 65 and 30%, respectively. The relative level at the PKC sites was slightly elevated around metaphase to about 45%. No obvious change of the pattern of phosphorylation during the cell cycles was observed in the presence of Ca^{2+}-CaM, although Ca^{2+}-CaM slightly elevated the relative level of phosphorylation at the MLCK sites to about 80% from 50 to 65% in the presence of EGTA. In addition, phosphorylations of unidentified sites were observed in the presence of EGTA. The level of these phosphorylations was estimated to be less than 5% of the total phosphorylation (Fig. 2).

Effects of a Cdc2 Kinase Inhibitor on MRLC Kinase Activities—Since we were interested in the cell cycle-dependent changes, the MRLC phosphorylating activity in the presence of EGTA was further investigated. Our results shown in Fig. 1 led us to investigate whether the activity is due to MPF or not. BL-I is a highly specific inhibitor of cdc2 kinase (*28*). The concentration causing 50% inhibition (IC_{50}) of cdc2 kinase from mammalian cells is about 1 μM, though that for other kinases is higher than 90 μM. We first examined the effect of BL-I on chicken gizzard MLCK. IC_{50} of BL-I for chicken gizzard MLCK was found to be 100 μM (Fig. 3A). On the other hand, IC_{50} for histone H1 phosphorylating activity of starfish oocyte MPF was 3 μM. The activity of the MPF toward MRLC showed a similar sensitivity.

Sensitivity of egg extract kinases to BL-I, that is, the contribution of cdc2 kinase to the MRLC phosphorylating activity in the egg extracts was investigated (Fig. 3B). HH1K activity consisted of two components: a BL-I-sensitive one, which may represent cdc2 kinase activity; and a BL-I-insensitive one. The BL-I sensitive portion comprised about 70% of the HH1K activity in the extract prepared at 75 min after fertilization (metaphase), and IC_{50} for this portion was 3 μM. Furthermore, this portion disappeared in the extract prepared 95 min after fertilization (telophase), as was expected for the cdc2 kinase activity. The MRLC-phosphorylating activity also consisted of BL-I-sensitive and -insensitive components. However, BL-I-sensitive activity was relatively small (less than 15% of the total activity), indicating that cdc2 kinase is not the major kinase that phosphorylates MRLC in the egg extracts.

Column Chromatography of MRLC Kinases—To further characterize the MRLC kinase activities in the egg extracts, we fractionated these activities by MonoQ anion exchange column chromatography. We also determined the sites on MRLC phosphorylated by the peak fractions. A metaphase extract or a telophase extract was applied to the column and eluted with a linear gradient of 0-0.5 M NaCl (Fig. 4). Three activity peaks were resolved: a peak eluted at 0.22 M NaCl (peak 1), a broad minor peak (peak 2) following peak 1, and a major peak eluted at 0.4 M (peak 3).

Peak 1 MRLC kinase activity was detected in the

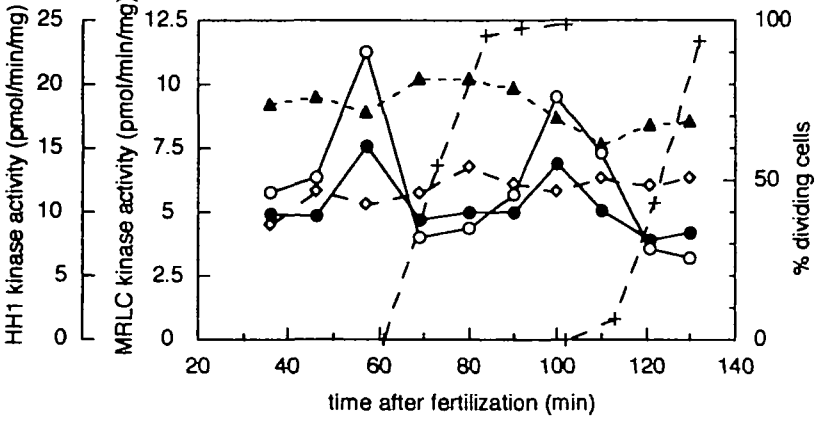

Fig. 1. **Time course of the changes in the activities that phosphorylate MRLC following fertilization of sea urchin eggs.** Protein kinase activities of *H. pulcherrimus* egg extracts toward MRLC in the presence of 5 mM EGTA (●), 0.2 mM $CaCl_2$ (▲), or 0.2 mM $CaCl_2$ plus 100 μM trifluoperazine (◇). ○, histone H1 kinase activity. For assay conditions, see "MATERIALS AND METHODS." +, percentage of cells that had entered division phase.

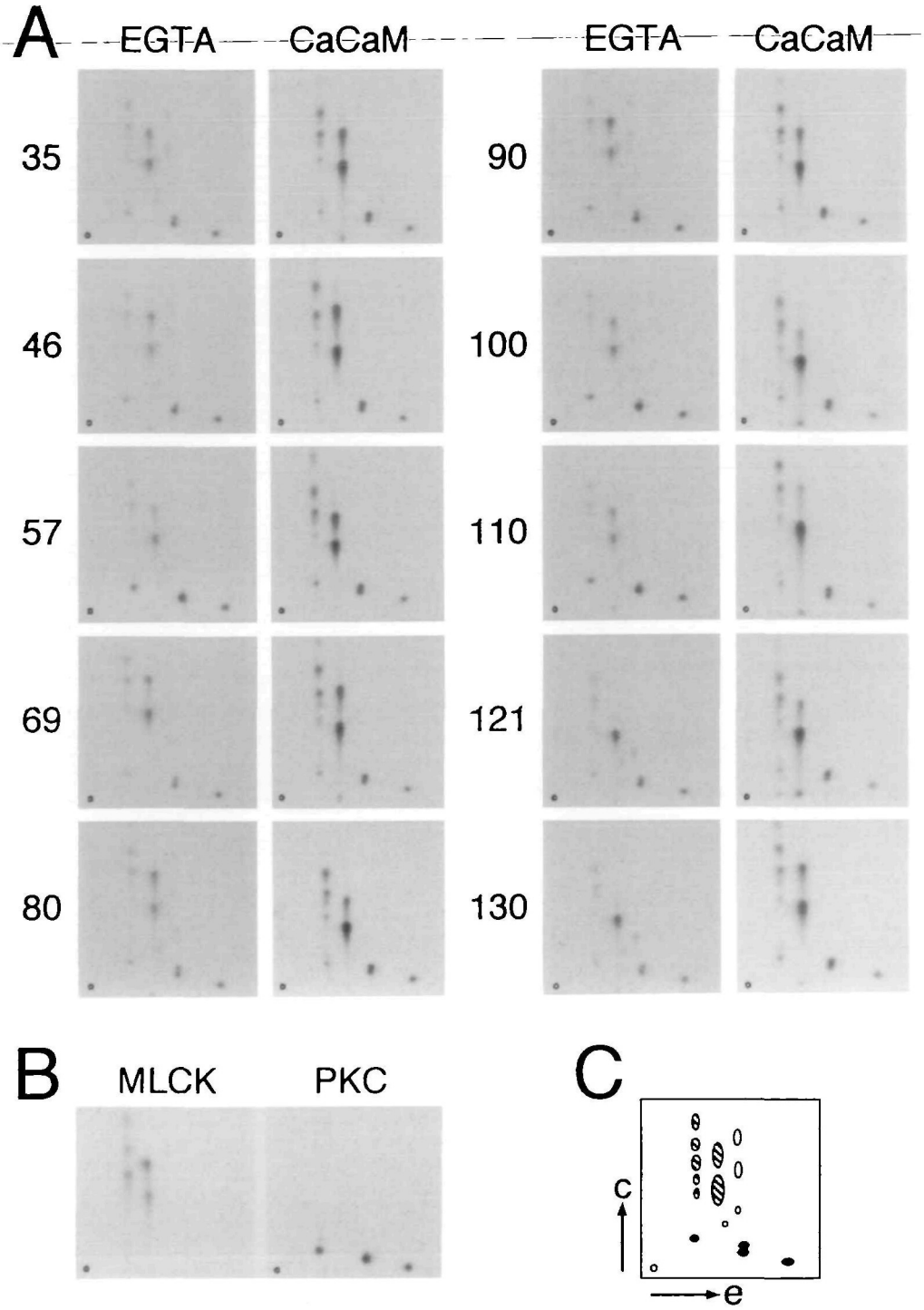

Fig. 2. **Analyses of phosphorylation sites of MRLC phosphorylated by egg extracts.** (A) Phosphorylation sites of MRLC by egg extracts were analyzed by 2D tryptic phosphopeptide mapping. Autoradiograms of maps derived from MRLC phosphorylated in the presence of EGTA (EGTA) or Ca^{2+} ions plus calmodulin (CaCaM) by the extracts prepared at the indicated times (min) after fertilization are shown. This experiment employed the same extracts as in Fig. 1. Applied radioactivities were the same for each series of mappings (EGTA or CaCaM). (B) Phosphopeptide maps of MRLC phosphorylated by purified MLCK or PKC are shown as standards. (C) A cartoon of the phosphopeptide map. Hatched spots are assigned to MLCK sites and closed spots are assigned to PKC sites. Open spots represent peptides having unidentified phosphorylation sites. O, origin; c, direction of chromatography; e, direction of electrophoresis.

metaphase extract, but not in the telophase extract. The ionic strength for elution of this peak was equal to that of the histone H1 kinase activity. Furthermore, peak 1 kinase phosphorylated the PKC sites on MRLC (Fig. 5), which cdc2 kinase phosphorylates *in vitro* (*20*). In addition, cdc2 kinase in starfish egg extracts is eluted at the corresponding position under similar conditions (*29*). Therefore, it is likely that peak 1 corresponds to cdc2 kinase.

Peak 2 activity was also detected at metaphase but not at telophase (Fig. 4). Though this fraction could include the tail of peak 1, peak 2 seemed to contain a kinase distinct from the peak 1 kinase, because the activity phosphorylated the MLCK sites in addition to the PKC sites (Fig. 5). Kinases that phosphorylated respectively the MLCK sites and the PKC sites could not be separated by this chromatography.

Peak 3 showed the strongest activity among the three peaks of the column eluates. Peak 3 kinase was active both at metaphase and telophase (Fig. 4). The phosphopeptide map obtained using this fraction was different from that

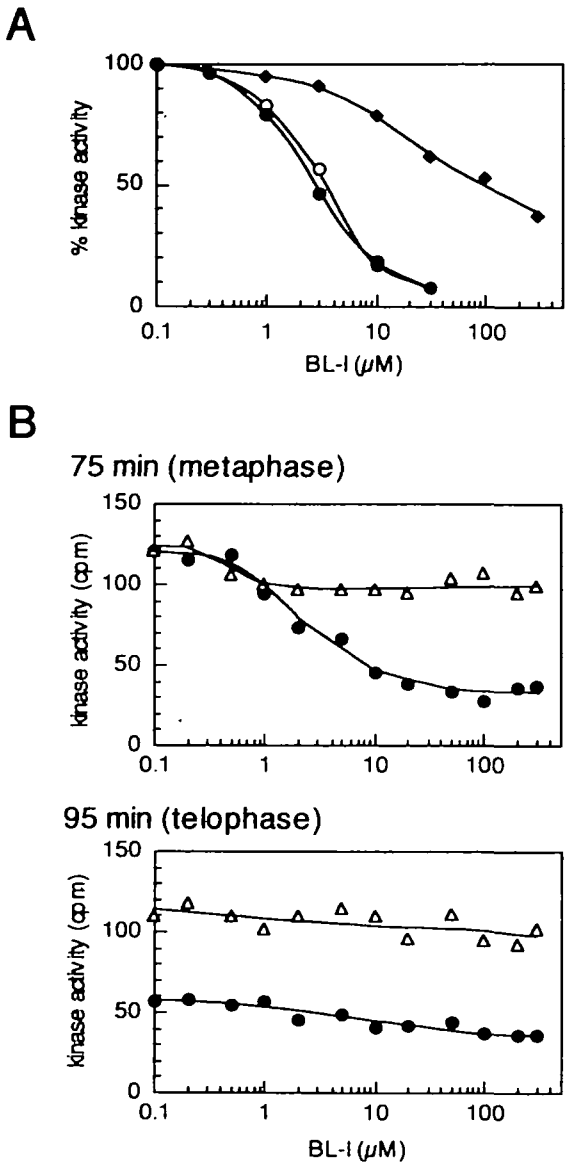

Fig. 3. **Effects of butyrolactone-I on MRLC-phosphorylating activities.** (A) Effect of BL-I on the activity of purified kinases. Phosphate incorporations into HH1 by starfish MPF (●), MRLC by MPF (○), and MRLC by MLCK (◆) are shown as percentages of those without BL-I. (B) Effect of BL-I on MRLC kinase activity in the presence of EGTA (△) or histone H1 kinase activity (●) of egg extracts at 75 min (metaphase) or 95 min (telophase) after fertilization. BL-I was included in the reaction mixtures at the indicated concentrations.

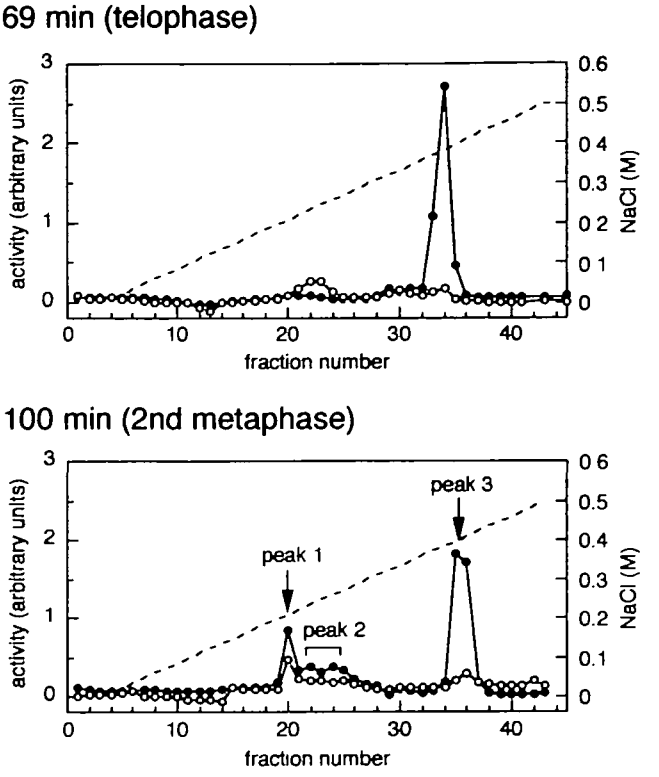

Fig. 4. **MonoQ column chromatography of MRLC kinases.** Metaphase or telophase egg extracts were fractionated by FPLC using a MonoQ column. The column eluates by 0-0.5 M NaCl linear gradient were assayed for phosphorylating activity towards histone H1 (○) or MRLC (●) in the presence of EGTA.

obtained using MLCK or that obtained using PKC (Fig. 5), indicating that the peak 3 kinase phosphorylated unknown sites different from Ser1, Ser2, Thr9, Thr18, or Ser19. Phosphorylation of these sites was detected to a small extent with the egg crude extract as mentioned above.

DISCUSSION

We have found for the first time a cyclic activation of MRLC-phosphorylating activity during the division cycle. The cycle was observed when the activity was assayed in the absence of Ca^{2+} ions. The activation peak was observed at metaphase, in parallel to the activity change of HH1 kinase. Both MLCK sites and PKC sites on MRLC, whose phosphorylation could affect myosin ATPase activity, were phosphorylated. A cortical actin reorganization initiates at this stage toward cytokinesis in the sea urchin eggs (*30*). It is reasonable to consider from these results that the cyclic activation of MRLC phosphorylation would lead to actin-myosin reorganization to form the contractile ring or lead to contraction of the contractile ring during cytokinesis. While the present experiments were done using isolated myosin light chains, we have observed similar changes in the MRLC-phosphorylating activity in the absence of Ca^{2+} ions using intact myosin molecules (unpublished data).

The phosphorylation of MLCK sites on MRLC in the egg extracts was distinct from that by MLCK from vertebrate tissues, which requires Ca^{2+}-CaM for its activity, in that it was observed in the absence of Ca^{2+} ions. It was found that Ca^{2+} ions plus added CaM elevated the level of phosphorylation at the MLCK sites, but the activation was at most only 2- to 3-fold, as estimated from the total activity obtained from Fig. 1 and the relative activity obtained from Fig. 2. By contrast, purified smooth muscle MLCK is activated by Ca^{2+}-CaM in the order of at least 100-fold (*22*). These results suggest that the egg protein kinase that phosphorylated the MLCK sites is different from smooth muscle or vertebrate nonmuscle MLCK. The Ca^{2+}-CaM-activated MLCK has not been identified in sea urchin eggs. Purification of Ca^{2+}-CaM-dependent light chain kinase activity from a crude actomyosin fraction of fertilized sea

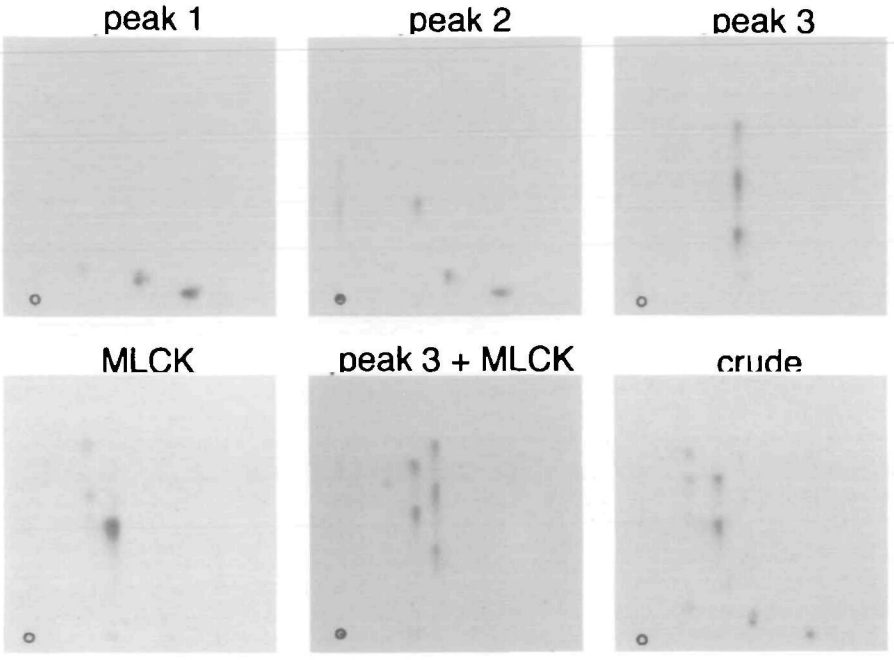

Fig. 5. **Analyses of phosphorylation sites of MRLC phosphorylated by MonoQ column fractions.** Phosphorylation sites of MRLC by MonoQ column eluates were analyzed by 2D tryptic phosphopeptide mapping. Autoradiograms of maps derived from MRLC phosphorylated in the presence of EGTA by column eluates (peak 1, peak 2, and peak 3, see Fig. 4). MLCK, a map derived from MRLC phosphorylated by MLCK. Peak 3 + MLCK, the phosphopeptides were separately prepared and mixed prior to application to the plate. Crude, MRLC phosphorylated by crude extracts before chromatography.

urchin eggs revealed that this kinase was multifunctional Ca^{2+}/CaM-dependent protein kinase II (*31*). Interestingly, Ca^{2+}/CaM-dependent protein kinase II from vertebrate brain shows Ca^{2+}-CaM-independent activity when it is partially autophosphorylated (*32*). It has also been shown that this kinase phosphorylates the MLCK sites on MRLC (*8*). On the other hand, MLCK from *Dictyostelium discoideum* has a catalytic domain homologous to that of conventional MLCKs and a substrate specificity similar to conventional MLCKs, but it is independent of Ca^{2+} ions or CaM for its activity (*33, 34*). Therefore, there are several candidates for the MRLC-phosphorylating enzyme that phosphorylates the MLCK sites in the presence of EGTA. First, it could be the autophosphorylated Ca^{2+}/CaM-dependent kinase II. Second, it could be a Ca^{2+}-CaM-independent MLCK like the *Dictyostelium* enzyme. Third, it could be derived from partial degradation products of the conventional MLCK, although this possibility is unlikely since we prepared the extracts in the presence of protease inhibitors and froze them quickly. We found three MRLC-phosphorylating activities by means of ion exchange column chromatography. The peak 2 kinase phosphorylates the MLCK sites in the absence of Ca^{2+} ions. Therefore, it is likely that this kinase corresponds to the one discussed above that phosphorylated the MLCK sites on MRLC in the crude egg extracts in the Ca^{2+}-independent manner.

The PKC sites on MRLC were also phosphorylated in the crude extracts. Therefore, it is possible that this phosphorylation is due to MPF/cdc2 kinase and/or PKC. The peak 1 kinase of the MonoQ chromatography seems to be cdc2 kinase, judging from the timing of its activation, its chromatographic behavior and its phosphorylation sites on MRLC. However, contribution of cdc2 kinase to the MRLC phosphorylation seems to be relatively small, judging from the limited sensitivity to BL-I of the MRLC kinase activity in the egg extracts. The peak 2 fraction also contained an activity that can phosphorylate the PKC sites besides the one that can phosphorylate the MLCK sites. This activity might correspond to sea urchin egg PKC itself.

In addition to the above known sites for phosphorylation on MRLC, unidentified sites were also phosphorylated in the crude extracts in the absence of Ca^{2+} ions. The phosphorylation of these sites seems to be due to the peak 3 kinase of the MonoQ chromatography. It is curious that this activity was not prominent in the crude extracts but gave a large peak in the ion-exchange column chromatography. Several explanations are possible. First, this kinase activity might have been inhibited in the crude extract and released from the inhibition by the chromatography. Second, there may have been a phosphatase in extracts that preferentially targeted the sites phosphorylated by the peak 3 kinase and that was removed by the chromatography. Casein kinase II has been reported to phosphorylate MRLC at Thr134 *in vitro*, though this phosphorylation does not affect the myosin ATPase activity (*35, 36*). The elution position of the peak 3 kinase seems to correspond to that of one of the casein kinases in starfish oocytes (*29*). Thus, peak 3 may be a casein kinase. Whatever the case, the phosphorylation by this kinase does not seem to be physiologically important since it was very weak in the crude extracts.

What kinase(s) is mainly responsible for the MRLC phosphorylating activity in the presence of EGTA that is activated at metaphase? Results shown in Fig. 2A indicate that activity toward PKC sites was relatively elevated at metaphase. However, a simple estimation of absolute level of phosphorylation by multiplying total activity in Fig. 1 and relative activities obtained from Fig. 2A shows that the activities toward the MLCK sites and the PKC sites were both elevated at metaphase, about 1.4- and 2.1-fold, respectively. From the sensitivity to BL-I, these activities are likely to be different from cdc2 kinase. Peak 2, which was distinct from the peak of histone H1 kinase, contained kinases that phosphorylate both the MLCK sites and the PKC sites. This peak was detected at metaphase but not at telophase. Therefore, kinases in peak 2 are likely to be

mainly responsible for the MRLC phosphorylating activity in the presence of EGTA that is activated at metaphase. Further analysis is required to identify the nature of these kinases.

Simultaneous activation of kinase activities with opposite effects on myosin ATPase activity, that is, one that phosphorylates the MLCK sites and activates the ATPase activity and another that phosphorylates the PKC sites and inactivates the ATPase activity, is apparently curious. Several explanations are conceivable. First, the time intervals at which egg extracts were obtained may have been too long. Experiments with shorter time intervals during the division cycle yielding extracts from highly synchronized eggs might reveal differences in the activation time courses. Second, each kinase might be spatially segregated in the cell such that the MLCK site-phosphorylating activity resides in a future cleavage furrow region and the PKC site-phosphorylating activity resides in another region. Third, if the phosphorylations of MLCK sites and PKC sites are mutually exclusive (*10, 12*), a small difference in the kinase activities could result in phosphorylation at only MLCK sites or only PKC sites. It is important to know the *in vivo* phosphorylation state of MRLC in dividing sea urchin eggs.

It would be interesting to investigate whether similar cyclic activation of the MRLC-phosphorylating activity is observed in other cells, such as *Xenopus* oocytes or mammalian cultured cells. Yamakita *et al.* (*21*) reported that phosphorylation of endogenous MRLC in mammalian cultured cells arrested at prometaphase by nocodazole is increased by up to 6- to 12-fold over that in nonmitotic cells, and that phosphorylating activity toward Ser1 and Ser2 is elevated in mitotic cells. Therefore, the cyclic activation may also occur in mammalian cells.

The proposal by Satterwhite *et al.* (*20*) that cdc2 kinase could inactivate myosin by phosphorylating MRLC at the PKC sites and thereby regulate cytokinesis is attractive, since it directly links regulation of the entry into M-phase and induction of cytokinesis by changes in the activity of a single enzyme. However, our data indicate that the contribution of cdc2 kinase to the MRLC phosphorylating activity is relatively small. This is compatible with the report by Yamakita *et al.* (*21*) that MRLC is a poor substrate for cdc2 kinase, compared with other substrates such as histone H1 or caldesmon. They also reported that Ser1 or Ser2 is phosphorylated *in vivo* but phosphorylation at Thr9 is hardly observed, while cdc2 kinase phosphorylates Thr9 *in vitro* to the same extent as Ser1 or Ser2. In addition, MRLC does not contain a consensus sequence for phosphorylation by cdc2 kinase, that is, S/TPXK (*37*), in its primary sequence. Therefore, it is premature to consider that cdc2 kinase regulates cytokinesis by phosphorylating the PKC sites of MRLC.

It is not known whether the MRLC phosphatase activity shows cyclic change during the division cycle. This should be investigated in order to fully understand the regulation of myosin ATPase activity in the cell cycle through MRLC phosphorylation. Moreover, it has recently been reported that heavy chain of sea urchin egg myosin is phosphorylated *in vivo* and this phosphorylation is decreased during cytokinesis especially around the furrow region (*38*). It is also important, therefore, to investigate the role of the heavy chain phosphorylation in the cleavage.

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