Phosphorylation of Myosin-II Regulatory Light Chain by Cyclin-p34^{cdc2}: A Mechanism for the Timing of Cytokinesis

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Abstract. To understand how cytokinesis is regulated during mitosis, we tested cyclin-p34^{cdc2} for myosin-II kinase activity, and investigated the mitotic-specific phosphorylation of myosin-II in lysates of *Xenopus* eggs. Purified cyclin-p34^{cdc2} phosphorylated the regulatory light chain of cytoplasmic and smooth muscle myosin-II in vitro on serine-1 or serine-2 and threonine-9, sites known to inhibit the actin-activated myosin ATPase activity of smooth muscle and nonmuscle myosin (Nishikawa, M., J. R. Sellers, R. S. Adelstein, and H. Hidaka. 1984. *J. Biol. Chem.* 259:8808–8814; Bengur, A. R., A. E. Robinson, E. Appella, and J. R. Sellers. 1987. *J. Biol. Chem.* 262:7613–7617; Ikebe, M., and S. Reardon. 1990. *Biochemistry.* 29:2713–2720). Serine-1 or -2 of the regulatory light chain of *Xenopus* cytoplasmic myosin-II was also phosphorylated in *Xenopus* egg lysates stabilized in metaphase, but not in interphase. Inhibition of myosin-II by cyclin-p34^{cdc2} during prophase and metaphase could delay cytokinesis until chromosome segregation is initiated and thus determine the timing of cytokinesis relative to earlier events in mitosis.

Most eukaryotic cells undergo cytoplasmic division late in mitosis, yet little is known about the molecular control of cytokinesis. Myosin-II and actin filaments accumulate to form a contractile ring around the cell during anaphase (Schroeder, 1973; Fujiwara and Pollard, 1976; Mittal et al., 1987; Kitanishi-Yumura and Fukui, 1989) at a position specified by the mitotic spindle (Rappaport, 1986), and myosin-II is the motor essential for contraction of the cleavage ring (Mabuchi and Okuno, 1977; Knecht and Loomis, 1987; DeLozanne and Spudich, 1987). The signals that direct the formation and contraction of the cleavage furrow and the mechanisms that link cytokinesis with the nuclear mitotic cycle are unknown (reviewed by Satterwhite and Pollard, 1992).

Given the hyperphosphorylation that occurs upon entry into mitosis (Davis et al., 1983; Karsenti et al., 1987; Lohka et al., 1987), we focused our attention on myosin-II because its activity is controlled by phosphorylation. The myosin-II molecule is a hexamer of two heavy chains, two "essential" light chains, and two "regulatory" light chains. We will refer to the 20K regulatory light chain as LC-20. The light chains associate with the NH_2-terminal half of the heavy chains to form the two enzymatically active heads, while the remainder of the heavy chains form an alpha-helical coiled tail. The tail regions of myosin-II monomers polymerize to form bipolar filaments.

Both LC-20 and the myosin-II heavy chains are known to be phosphorylated in vitro and in vivo (reviewed by Sellers, 1991). The actin-dependent ATPase of cytoplasmic and smooth muscle myosin is activated by phosphorylation of LC-20 by calcium–calmodulin-dependent myosin light chain kinase (MLCK)[1]. MLCK phosphorylates both serine 19 (S19) and threonine 18 (T18) of the cytoplasmic and smooth muscle myosin-II LC-20. These myosins show little detectable ATPase activity in the absence of phosphorylation (Fig. 1). In addition, LC-20 of cytoplasmic and smooth muscle myosin are phosphorylated by calcium and phospholipid-dependent protein kinase C (PKC) at serine and threonine residues located NH_2-terminal to the activating site. Phosphorylation of serine-1 (S1) or serine-2 (S2) and threonine-9 (T9) inhibits myosin ATPase activity by two mechanisms (Nishikawa et al., 1984; Bengur et al., 1987). First, if myosin is not phosphorylated, phosphorylation at S1 or S2 and T9 reduces the rate of subsequent phosphorylation at S19 by MLCK, thus making myosin less likely to be activated. Second, if myosin is phosphorylated at the activating site, addi-

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1. *Abbreviations used in this paper*: LC-20, 20K regulatory light chain; HMM, heavy meromyosin; MMR, Marc's minimal ringers; MLCK, myosin light chain; MPF, maturation promoting factor; PKC, protein kinase C; SCW, surface contraction waves.

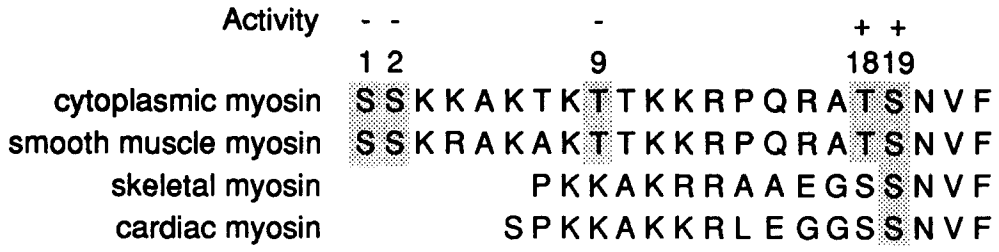

Figure 1. The NH_2-terminal sequences of the LC-20s of rat cytoplasmic myosin-II LC-20 (Grant et al., 1991), chicken smooth muscle myosin-II LC-20 (Messer and Kendrick-Jones, 1988), chicken skeletal myosin regulatory light chain (Matsuda et al., 1977), and rat cardiac myosin regulatory light chain (Kumar et al., 1986) are aligned at phosphorylation sites shared between regulatory light chains (*shading*).

tional phosphorylation at S1 or S2 and T9 inhibits the actin-activated myosin ATPase by reducing the affinity of myosin for actin filaments. Phosphorylation of LC-20 at S19 also promotes filament assembly of smooth muscle and nonmuscle myosin-II in vitro (Scholey et al., 1980; Craig et al., 1983), and LC-20 phosphorylation alone is sufficient for activation of the myosin ATPase (Trybus, 1989). Heavy chain phosphorylation appears to regulate filament disassembly in *Dictyostelium* (Kuczmarski and Spudich, 1980), and platelet myosin-II heavy chain is phosphorylated by PKC in vitro and in vivo (Kawamoto et al., 1989; Conti et al., 1991).

Maturation promoting factor (MPF) is a key cell cycle–regulated kinase that catalyzes entry into meiosis and mitosis in eukaryotes (Masui and Markert, 1971; Smith and Ecker, 1971; Nuse, 1990). MPF consists of at least two components, a regulatory cyclin (Draetta et al., 1989; Labbe et al., 1989) and the $p34^{cdc2}$ kinase (Dunphy et al., 1988; Gautier et al., 1988). MPF activity peaks during prophase and metaphase and drops abruptly at the metaphase–anaphase transition due to proteolysis of cyclin (Minshull et al., 1989; Murray and Kirschner, 1989; Murray et al., 1989; Glotzer et al., 1991).

Here we report that MPF phosphorylates LC-20 of myosin-II at sites known to inhibit the myosin-II ATPase activity. One of these same sites is phosphorylated on LC-20 of *Xenopus* myosin-II purified from stable mitotic lysates of *Xenopus* eggs. We propose that phosphorylation of LC-20 by cyclin-$p34^{cdc2}$ during mitosis plays a role in delaying cytokinesis until chromosome segregation has initiated at anaphase.

Materials and Methods

Materials

Reagents were purchased from the following sources: 1,2-dioleoyl glycerol # 800811, phosphatidylserine # 840032 (10 mg/ml^{-1} stocks in chloroform) from Avanti Polar Lipids (Alabaster, AL); A15M agarose resin from Biorad (Melville, NY); histone H1 # 223549 from Boehringer-Mannheim Biochemicals (Indianapolis, IN); calmodulin # 208690, rat brain protein kinase C # 539494 and antibiotic A23187 # 100105 from Calbiochem-Behring Corp. (San Diego, CA); rabbit non-specific IgG # 6012-0800 from Organon Teknika-Cappel (West Chester, PA); γ-^{32}P-ATP # NEG-002 and NEG-002A from Dupont-NEN (Boston, MA); DTT and cellulose plates without fluorescent indicators # 1366061 from Eastman Kodak Co. (Rochester, NY); protein A-Sepharose CL4B # 170780 and Sephacryl S200 SF resin # 170871 from LKB-Pharmacia (Piscataway, NY); Cibacron Blue F3G1 affinity matrix # 20280 from Pierce Chemical Co. (Rockford, IL); Na β-glycerophosphate, chymostatin, leupeptin, antipain, pepstatin and adenosine 5'-triphosphate from Sigma Chemical Co. (St. Louis, MO); DE81 paper # 3658915 and P81 paper # 369815 from Whatman (Hillsboro, OR); TPCK-trypsin # 3740 from Worthington (Freehold, NJ).

Buffers

Urea buffer: 8 M urea, 50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 5 mM DTT.

S200 column buffer: 50 mM NaCl, 20 mM MOPS, pH 7.0, 1 mM DTT, 0.1 mM PMSF.

CB column buffer: 20 mM MOPS, pH 7.0, 1 mM DTT, 0.1 mM PMSF.

LC-20 storage buffer: CB column buffer containing 25 mM NaCl and 20% sucrose.

Low salt buffer: 60 mM KCl, 15 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF.

High salt buffer: low salt buffer made 0.6 M KCl.

KI-ATP: 0.6 M KI, 20 mM MOPS, pH 7.0, 5 mM ATP, 5 mM DTT, 1 mM $MgCl_2$, 0.1 mM PMSF.

Myosin-II storage buffer: low salt buffer made 45% sucrose.

Bead buffer: 50 mM Tris-HCl, pH 7.8, 250 mM NaCl, 5 mM NaF, 5 mM EDTA, 0.1% NP-40, 10 μg ml^{-1} leupeptin, 0.1 mM PMSF.

Binding buffer: 80 mM Na β-glycerophosphate, pH 7.5, 20 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 10 μg ml^{-1} chymostatin, leupeptin, aprotinin, pepstatin.

Immunoprecipitation buffer: 10 mM Tris-HCl, pH 7.8, 1% NP-40, 250 mM NaCl, 100 mM $Na_4P_2O_7$-$10H_2O$, pH 7.0, 5 mM NaF, 5 mM EDTA, 0.1 mM PMSF, 10 μg ml^{-1} leupeptin.

Protein Purification

Substrates. Smooth muscle myosin-II and smooth muscle myosin light chain kinase were purified from turkey gizzard (Sellers et al., 1981). LC-20 was purified from smooth muscle myosin-II by a modification of Sellers et al. (1981) and Mittal et al. (1987) as follows: Tropomyosin-free gizzard myosin was diluted 1:1 in urea buffer, stirred at room temperature for 1 hour, diluted 1:10 in ice cold dH_2O, and centrifuged at 10,000 *g* for 40 min, at 4°C. Light chains in the supernatant were precipitated in 3% TCA for 30 min on ice, collected at 10,000 *g* for 20 min, fractionated by S200 column chromatography, and applied to a Cibacron Blue F3GA affinity matrix. LC-20 was eluted with 1.5 M NaCl, dialyzed into LC-20 storage buffer, and frozen in liquid nitrogen. Skeletal muscle myosin was purified from rabbit muscle (Pollard, 1982) and cytoplasmic myosin-II from human platelets (Pollard et al., 1974). Smooth muscle muscle heavy meromyosin was prepared by limited chymotryptic digestion (Sellers and Harvey, 1988).

Xenopus egg myosin-II was purified from a high speed stable interphase egg lysate by a modification of Ezzell et al. (1983) as follows: *Xenopus* eggs were collected in 1× Marc's minimal ringers (MMR) (Karsenti et al., 1987), de-jellied using 2% cysteine, pH 8.0, and activated in 0.2× MMR by brief addition of A23187 in DMSO to a final concentration of 10 μM. Activation was observed by a contraction and relaxation of the pigmented animal pole. Eggs whose animal poles did not relax were not processed further. Activated eggs were washed three times in 0.2× MMR, three times in egg lysis buffer (Wilson and Newport, 1988), packed by centrifugation at 300 *g* for 5 min, and lysed by centrifugation at 12,000 *g* for 15 min, at 4°C. The middle viscous layer was centrifuged at 150,000 *g* for 1 h, at 4°C, and the supernatant made 30 μg/ml^{-1} in aprotinin, leupeptin, chymostatin, and pepstatin. This

high speed stable interphase lysate was dialyzed overnight against low salt buffer at 4°C. The precipitate was collected at 11,000 *g*, washed once in low salt buffer, extracted on ice for 2 h in high salt buffer with occasional mixing, and clarified by centrifugation. The supernatant was dialyzed overnight against low salt buffer, the precipitate collected by centrifugation, dissolved in KI-ATP, and fractionated by A15M column chromatography. Pure myosin-II was concentrated and dialyzed into low salt buffer for use in kinase assays, or dialyzed into myosin-II storage buffer, and frozen in liquid nitrogen. All kinase substrates were unphosphorylated when evaluated by glycerol-urea gel electrophoresis (Perrie and Perry, 1971) and free of contaminating kinase activities by the gel electrophoretic kinase assay described below.

Kinases. MPF (cyclin B-p34^{cdc2} kinase) was purified from unfertilized *Xenopus* eggs according to Lohka et al. (1988). A mixture of cyclin A-p34 and cyclin B-p34 kinases was also purified from *Xenopus* egg mitotic lysates by binding to p13-Sepharose (Brizuela et al., 1987; Dunphy et al., 1988). p13-Sepharose (6–8 μl packed resin, ~12 μg p13) was washed briefly in 1.0 ml bead buffer (Meijer et al., 1989), resuspended in 1.0 ml binding buffer, and incubated with 120 μg *Xenopus* mitotic lysate protein (30 mg/ml^{-1}) prepared according to Newport and Spann (1987) for 30 min at 4°C. Enzyme bound to p13-Sepharose beads was washed four times in 1.0 ml bead buffer and once in 1.0 ml kinase reaction buffer. The murine homologue of cyclin B-p34^{cdc2} kinase was purified from mouse Erlich ascites cells according to Cisek and Corden (1991) and further purified by binding to p13-Sepharose for some experiments.

Biochemical Assays

Cyclin-p34 Kinase Assays. Kinase reactions (30 μl) contained 20 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 10 μg/ml^{-1} chymostatin, leupeptin, antipain, and pepstatin, 200 μM γ-^{32}P-ATP at a specific activity of 2.0 Ci mmol^{-1}, and 0.2 mg/ml^{-1} LC-20 (10 μM) or 1.5 mg/ml^{-1} intact myosin-II (3 μM). Kinase reactions were started by the addition of either 1–10 ng purified MPF, 90 ng murine cyclin B-p34^{cdc2}, or p13-bound mitotic kinase purified from 120 μg *Xenopus* mitotic lysate protein, and incubated 30 min at 25°C for *Xenopus* enzyme or 37°C for murine enzyme. Negative controls for kinase activity present in the substrate were started by addition of ATP. In competition experiments, substrates were mixed before addition of enzyme. Reactions were stopped by addition of 10 μl 5× Laemmli sample buffer and heating to 90°C for 10 min. Reaction products were separated on 12.5 or 15% SDS–polyacrylamide gels (Anderson et al., 1972), stained with Coomassie brilliant blue, dried onto Whatman DE81 paper, and autoradiographed.

Myosin-II Light Chain Kinase Assay. Kinase reactions (30 μl) contained 20 mM Tris-HCl, pH 7.8, 0.2 mM $CaCl_2$, 0.1 mM EGTA, 10^{-7} M calmodulin, 200 μM γ-^{32}P-ATP at a specific activity of 2.0 Ci/mmol^{-1}, 0.2 mg/ml^{-1} LC-20 (10 μM), and 10^{-8} to 10^{-9} M MLCK.

Protein Kinase C Assay. Kinase reactions (30 μl) contained 20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 20 μM 1,2-dioleoyl glycerol, 50 μg/ml^{-1} phosphatidylserine, 200 μM γ-^{32}P-ATP at a specific activity of 2.0 Ci/mmol^{-1}, 0.2 mg/ml^{-1} LC-20 (10 μM) or 0.5–1.5 mg/ml^{-1} *Xenopus* egg myosin-II (1–3 μM), and 10^{-8} M rat brain PKC. Lipids in chloroform were dried under a nitrogen stream, resuspended in buffer components, and sonicated in a bath sonicator for 5 min before substrates, ATP, and kinase were added. Reactions that contained *Xenopus* egg myosin-II were incubated for 60 min at 25°C.

Quantitative Analysis of Phosphorylation. The rate and extent of phosphorylation in the kinase reactions were analyzed quantitatively by the method of Glass et al. (1978) as follows: Aliquots from kinase reactions were transferred to 2 cm × 2 cm P81 filters, air dried, washed four times in 250 ml 75 mM phosphoric acid to remove unincorporated γ-^{32}P-ATP, partially dried, and counted by liquid scintillation. Counts per minute were converted to pmoles phosphate by comparison to a standard amount of γ-^{32}P-ATP of known specific activity. The stoichiometry of phosphorylation of *Xenopus* LC-20 purified by immunoprecipitation from the mitotic lysate was estimated by converting counts per minute in a gel slice that contained LC-20 to pmoles phosphate, assuming the concentration of ATP in the mitotic lysate to be 1 mM. The concentration of immunoprecipitated *Xenopus* myosin heavy chain was estimated by comparison to a known concentration of skeletal myosin heavy chain, and the concentration of LC-20 calculated from the concentration of immunoprecipitated *Xenopus* myosin heavy chain, assuming 1 mole LC-20 per mole heavy chain.

Immunoprecipitation of Myosin-II from Xenopus Egg Extracts. Phosphoproteins from 45 μl mitotic or interphase lysate (~1,350 μg) (Newport and Spann, 1987; Wilson and Newport, 1988) of unfertilized *Xenopus* eggs were labeled by incubation with 100 μCi γ-^{32}P-ATP at a specific activity of 3.3 Ci/mmol^{-1} (assuming the concentration of endogenous ATP to be 1 mM) for 30–45 min at 25°C. An aliquot (28 μg) was removed to monitor phosphate incorporation. The remaining lysate was diluted in 1.0 ml nondenaturing immunoprecipitation buffer (Kawamoto et al., 1989) that contained either 150 μg rabbit non-specific IgG (Cappel) or 150 μg crude antiserum to *Xenopus* cytoplasmic myosin-II heavy chain, and incubated 4 h at 4°C. Immune complexes were collected by incubation with protein A-Sepharose (25 μl packed gel) for an additional 30 min at 4°C and centrifugation, washed four times in 1.0 ml of nondenaturing immunoprecipitation buffer, washed once in 20 mM Tris-HCl, pH 7.8, 20 mM NaF, resuspended in 5× SDS-PAGE sample buffer, and analyzed by SDS-PAGE.

Tryptic Peptide Mapping. We used the mapping method of Kawamoto and Adelstein (1987) and tryptic peptide assignments of Kawamoto et al. (1989). Coomassie brilliant blue stained protein bands were excised from dried polyacrylamide gels, destained in 25% isopropanol for 3 h, and in 10% methanol overnight, lyophilyzed, and resuspended in 0.5–1.0 ml TPCK-trypsin (0.15 mg/ml^{-1}) in 50 mM ammonium bicarbonate, pH 8.0, for 16 h at 37°C. The solution of trypsin and phosphopeptides was transferred to a fresh tube, dried, resuspended, and lyophilized four times in 1.0 ml dH_20. Phosphopeptides were analyzed on 20 × 20 cm cellulose plates by electrophoresis at 1,000 V for 90 min in 15:5:80 acetic acid/formic acid/water, chromatography in 97.5:75:15:60 *n*-butanol/pyridine/acetic acid/water for 5 h, and autoradiography. Phosphoamino acid analysis was according to Kawamoto and Adelstein (1988).

Results

Phosphorylation of Purified Myosin-II and LC-20 by p34 Kinases

In an in vitro kinase assay, purified MPF phosphorylated isolated avian smooth muscle myosin LC-20 (Fig. 2). Cyclin-p34 kinase complexes purified from mitotic lysates of *Xenopus* eggs using p13-Sepharose and cyclin B-p34 kinase purified from log phase mouse Erlich ascites cells also phosphorylated avian smooth muscle LC-20 (Fig. 2). LC-20 was also phosphorylated in vitro by *Spisula* cyclin B-p34 (L. Satterwhite, J. Westendorf, and J. Ruderman, unpublished data), starfish cyclin-p34c^{dc2} kinase (L. Meijer, unpublished data), and *Xenopus* cyclin A-p34 kinase (J. Minshull, unpublished data). Preliminary reports of LC-20 phosphorylation by two murine p34^{cdc2} homologues appeared previously (Satterwhite et al., 1990; Pollard et al., 1990).

Pure *Xenopus* MPF phosphorylated LC-20 of intact smooth muscle myosin, but did not phosphorylate the LC-20 of skeletal myosin (Fig. 3). This was the first evidence that MPF might phosphorylate LC-20 on the NH_2-terminal S1 or S2 and T9 because skeletal myosin LC-20 lacks these sites (Fig. 1).

Purified *Xenopus* MPF phosphorylated smooth muscle LC-20 and heavy meromyosin (HMM) at the same rates that depended on the concentration of the substrates. The concentration of MPF was too low to calculate a reliable V_{max}, but the K_m of MPF for both LC-20 and HMM were in the micromolar range judging from the rates at 0.57 μM substrate of 3.3 nmoles phosphate min^{-1}, and at 5.7 μM substrate of 17.5 nmoles phosphate min^{-1}.

Identification of the LC-20 Residues Phosphorylated by Cyclin-p34 Kinases

We determined the sites on avian LC-20 phosphorylated by MPF directly by two dimensional mapping of tryptic phosphopeptides, and by comparison with known standards, LC-20 phosphorylated by MLCK or PKC. The mobility of

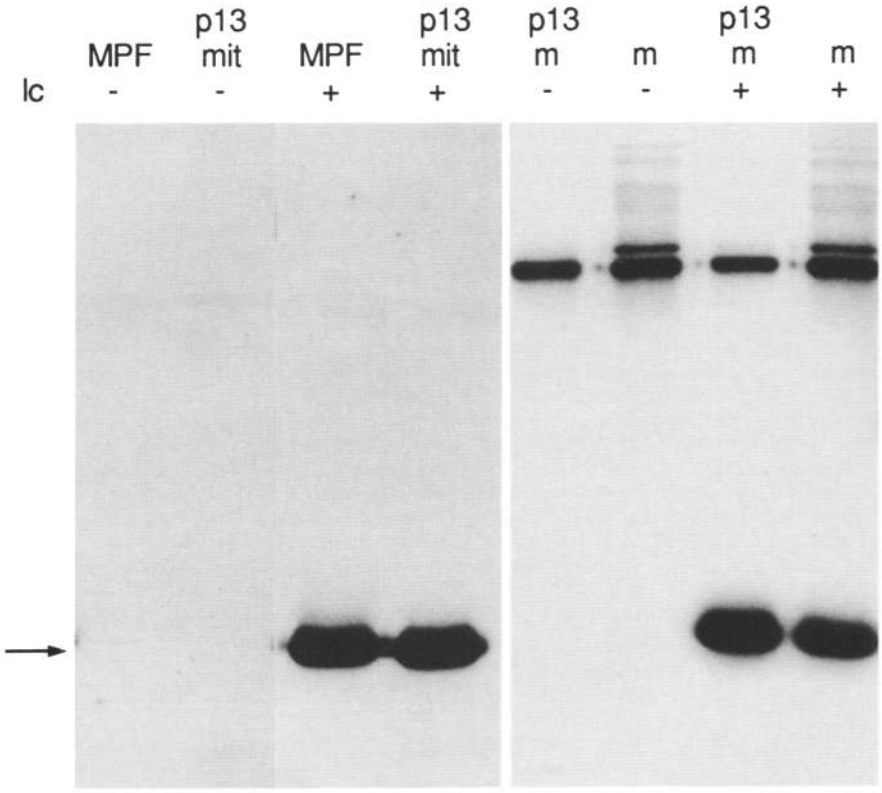

Figure 2. Phosphorylation of purified avian smooth muscle myosin-II LC-20 by three different preparations of cyclin-p34^{cdc2} kinase. The products of the kinase reactions are shown on autoradiographs of SDS–polyacrylamide gels. Components in these reactions are indicated as follows: (*MPF*) purified *Xenopus* maturation promoting factor which contains cyclin B and p34^{cdc2}; (*p13 mit*) kinase purified from mitotic extracts of *Xenopus* eggs by binding to p13-Sepharose; (*m*) cyclin B-p34^{cdc2} isolated from mouse Erlich ascites cells; (*p13 m*) murine cyclin-p34^{cdc2} further purified by binding to p13-Sepharose; (*arrow*) purified smooth muscle LC-20. The minor 45K phosphoproteins in both MPF and p13-bound *Xenopus* mitotic enzyme, and the 60K phosphoproteins common to both murine p34 kinase and p13-bound murine p34 kinase, are cyclin B.

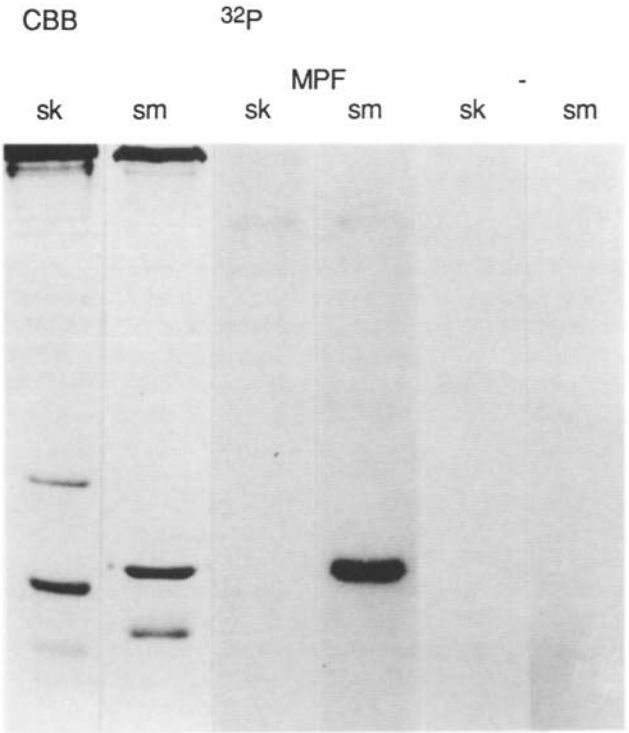

Figure 3. MPF phosphorylates the LC-20 of intact smooth muscle myosin-II (sm), but not the regulatory light chain of skeletal muscle myosin (*sk*). The proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue (*CBB*). Phosphoproteins were detected by autoradiography (^{32}P). (*MPF*) reactions that contained MPF, (−) control reactions lacking exogenous kinase.

tryptic peptides recovered from LC-20 phosphorylated by cyclin-p34^{cdc2} were identical to the phosphopeptides recovered from PKC-phosphorylated LC-20 (Fig. 4). The residues phosphorylated by PKC are known to be S1 or S2 and T9 (Kawamoto et al., 1989). The two peptides labeled "S1 or 2" are alternate tryptic peptides of the same product, a peptide that contains phosphoserine-1 or -2. The relative abundance of these peptides varies with respect to extent of cleavage or batch of trypsin used. "S1 and 2" is a peptide that contains both phosphoserine-1 and phosphoserine-2; "T9" contains phosphothreonine 9. "S19" contains phosphoserine 19, and "T18, S19" contains both phosphothreonine 18 and phosphoserine 19. Murine cyclin-p34^{cdc2} phosphorylated the same LC-20 peptides as *Xenopus* MPF (data not shown). By phosphoamino acid analysis, LC-20 phosphorylated by cyclin-p34^{cdc2} contained only phosphothreonine and phosphoserine (data not shown).

We purified *Xenopus* egg myosin-II, phosphorylated it with PKC in vitro, and compared the distribution of LC-20–derived phosphopeptides to that of a known standard, avian LC-20 phosphorylated by PKC. By peptide mapping, the phosphopeptides derived from the *Xenopus* egg myosin LC-20 are indistinguishable from the phosphopeptides derived from PKC-phosphorylated avian smooth muscle myo-

Skeletal muscle myosin consists of a 200K heavy chain and three light chains: two essential light chains of 24 and 15K mobilities, and a LC-20 of 18K mobility. Smooth muscle myosin-II contains a 200K heavy chain, a 20K regulatory light chain, and a 17K essential light chain.

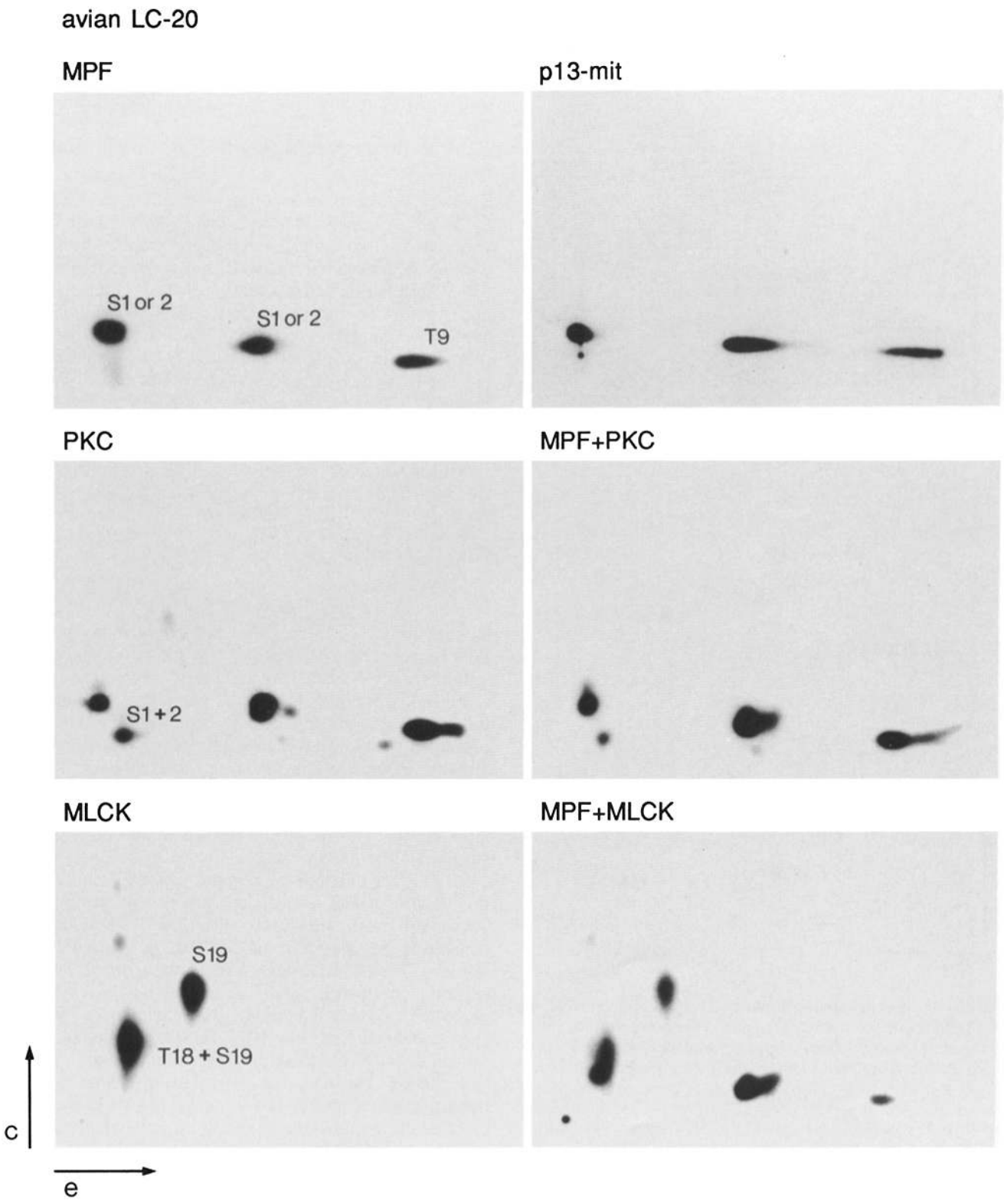

Figure 4. MPF phosphorylates avian smooth muscle myosin-II LC-20 on the same sites as PKC. Autoradiograms of two dimensional maps of tryptic phosphopeptides. Reactions contained the following kinases as indicated: (*MPF*) purified Xenopus; (*p13-mit*) cyclin-p34^{cdc2} purified from *Xenopus* mitotic lysates by binding to p13-Sepharose; (*PKC*) rat brain PKC; or (*MLCK*) smooth muscle myosin light chain kinase. Tryptic phosphopeptides were prepared separately and mixed for analysis (*MPF+PKC* and *MPF+MLCK*). In the MPF+MLCK sample, the phosphopeptide containing T18 and S19 is resolved from that containing S1 or S2 on a shorter exposure. The two peptides labeled "S1 or 2" are alternate tryptic peptides of the same product, a peptide that contains phosphoserine 1 or 2. "S1+2" is a peptide that contains both phosphoserine 1 and 2; "T9" contains phosphothreonine 9; "S19" contains phosphoserine 19, and "T18+S19" contains both phosphoserine 19 and phosphothreonine 18. The arrows indicate the electrophoretic dimension (*e*) and the chromatographic dimension (*c*).

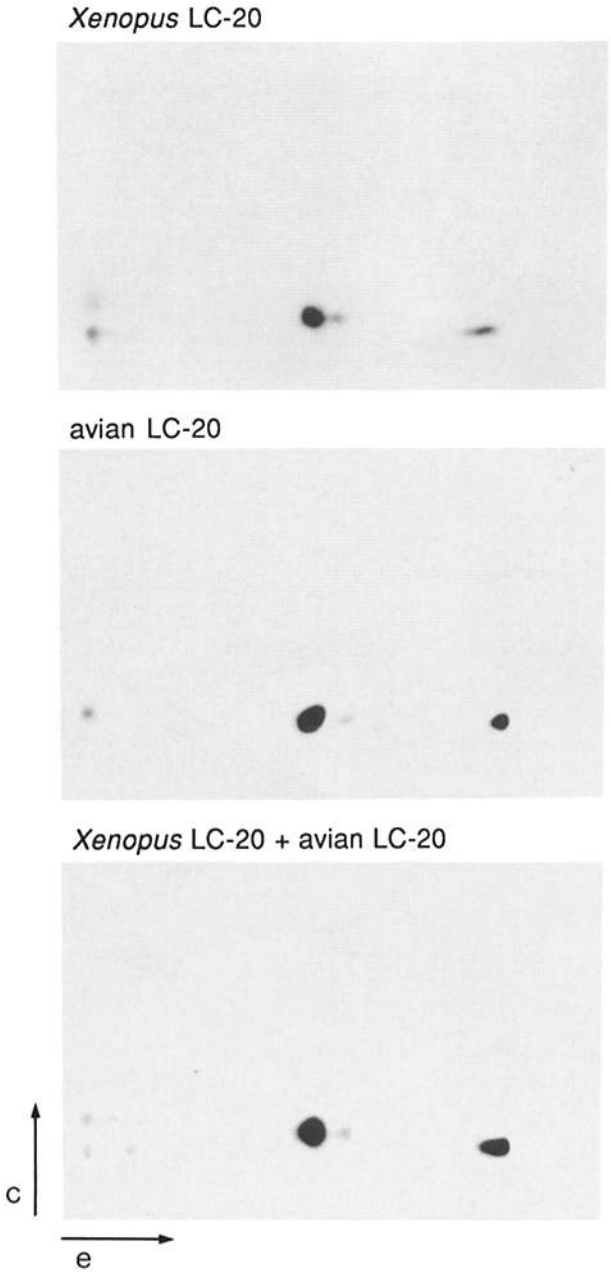

Figure 5. Purified *Xenopus* myosin LC-20 is phosphorylated on S1 or S2 and T9 by PKC. Autoradiograms of two dimensional maps of tryptic phosphopeptides. Tryptic phosphopeptides were prepared separately and mixed for analysis in the lower panel (*Xenopus LC-20 + avian LC-20*). The single peptide containing S1 or 2 in these maps is the same as the avian LC-20 phosphopeptide containing S1 or S2 that displays greater electrophoretic mobility (shown in Fig. 4).

sin LC-20 (Fig. 5). Here, the single peptide that contains S1 or 2 is the same as the second of the two S1- or S2-containing peptides shown in Fig. 4. In addition, *Xenopus* egg myosin phosphorylated by cyclin-p34^{cdc2} in vitro yields phosphopeptides identical to those derived from PKC-phosphorylated *Xenopus* myosin-II (data not shown). The identical mobility of the phosphopeptides on the these maps is explained by the fact that the sequence of LC-20 of *Xenopus* egg myosin-II is 94% identical to avian LC-20 including conservation of all five known phosphorylation sites (A. Bresnick and T. D. Pollard, unpublished data). Thus, all of the phosphorylation sites on LC-20 of *Xenopus* egg myosin-II are conserved, and *Xenopus* egg myosin is a substrate of PKC and cyclin-p34^{cdc2} in vitro.

Controls for the Specificity of LC-20 Phosphorylation by p34 Kinases

Three types of control experiments provided evidence that cyclin-p34^{cdc2}, rather than a contaminating kinase, phosphorylated LC-20 using *Xenopus* kinase prepared by binding to p13-Sepharose. First, two well-characterized substrates of p34^{cdc2}, histone H1 and a peptide (hepta 6) (Cisek and Corden, 1989) that contain multiple p34^{cdc2} kinase consensus motifs (Moreno and Nurse, 1990), each competed for phosphorylation of LC-20 by cyclin-p34^{cdc2} kinase purified by p13-Sepharose (Fig. 6). Second, the extent of phosphorylation of LC-20 by p13-bound mitotic kinase was not affected by adding activators of other light chain kinases, including calcium, calmodulin, phospholipid, and cAMP (data not shown). Third, we could not detect PKC protein in the murine cyclin-p34^{cdc2} preparations using an ELISA assay and antibody to PKC that detects both calcium-sensitive and -insensitive forms of PKC (gift of Dr. K.-P. Huang, National Institutes of Health, Bethesda, MD).

Cell Cycle Regulation of Myosin-II Phosphorylation

To study the cell cycle–dependent phosphorylation of myosin-II, we purified intact *Xenopus* egg myosin by immunoprecipitation from ^{32}P-labeled *Xenopus* egg cytoplasmic lysates stabilized in either metaphase or interphase, and analyzed the LC-20 phosphopeptides. Under conditions known to preserve myosin heavy chain-light chain interactions, antibody to *Xenopus* egg myosin heavy chain specifically precipitated a 200K protein visualized by Coomassie brilliant blue staining (Fig. 7 *A*). The protein of ~75K in the immune lanes is a component of this polyclonal antisera (data not shown). If the lysates are pre-labeled by incubation with γ-^{32}P-ATP, the antibody to *Xenopus* myosin-II heavy chain immunoprecipitates a prominent 20K phosphoprotein from both mitotic and interphase lysates (Fig. 7 *B*). Using nondenaturing immunoprecipitation, the 20K phosphoprotein was always recovered in lanes where myosin heavy chain was present. The myosin heavy chain was not heavily phosphorylated. A second antibody to human platelet myosin-II (Kawamoto and Adelstein, 1987) also precipitated the 200K protein and 20K phosphoprotein (data not shown). We believe the 20K phosphoprotein is LC-20 of *Xenopus* myosin because mAbs to myosin-II heavy chain (Kaiser et al., 1989) detected the immunoprecipitated 200K polypeptides on immunoblots, and mAbs to smooth muscle LC-20 (Trybus and Lowey, 1988) immunoprecipitated a 20K phosphoprotein that comigrated with the 20K phosphoprotein shown in Fig. 7 *B*. The stoichiometry of phosphorylation of LC-20 recovered from the mitotic lysate ranged from 0.3–0.6 moles phosphate per mole LC-20, and was dependent on the specific mitotic lysate used. Because these lysates were stable in mitosis as γ-^{32}P-ATP was introduced, the extent of phosphate incorporation could also be dependent on phosphatase activity.

The phosphorylated *Xenopus* myosin LC-20 recovered by immunoprecipitation was analyzed by tryptic peptide map-

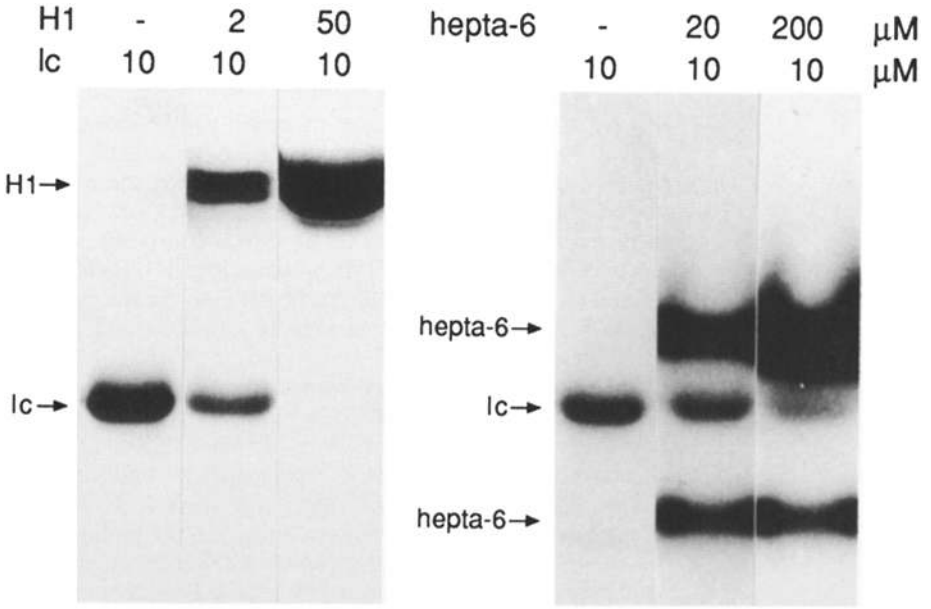

Figure 6. Known substrates of $p34^{cdc2}$ kinase compete for phosphorylation of avian LC-20 by p13-bound *Xenopus* mitotic cyclin-p34 kinase. The products of the kinase reactions are shown on autoradiograms of SDS–polyacrylamide gels. The hepta-6 peptide, $(SPTSPSY)_6$, is homologous to a portion of the COOH-terminal domain of RNA polymerase. Each reaction contained 10 μM avian LC-20 (*lc*). The concentrations of competing substrates, histone H1 (*H1*), and the hepta-6 peptide (*hepta-6*) are indicated on the figure.

ping (Fig. 8). LC-20 was phosphorylated on different sites in the mitotic and interphase lysates. LC-20 recovered from the mitotic lysate was phosphorylated on S1 or 2. We analyzed the phosphopeptides derived from the immunoprecipitated *Xenopus* myosin LC-20 (as in Fig. 8) and avian LC-20 phosphorylated by MPF in vitro (as in Fig. 4) on the same peptide map. The peptide that contains S1 or 2 in the avian LC-20 co-migrates with the phosphopeptide recovered from the *Xenopus* myosin LC-20 in the mitotic lysate (data not shown). In seven out of seven experiments using two different

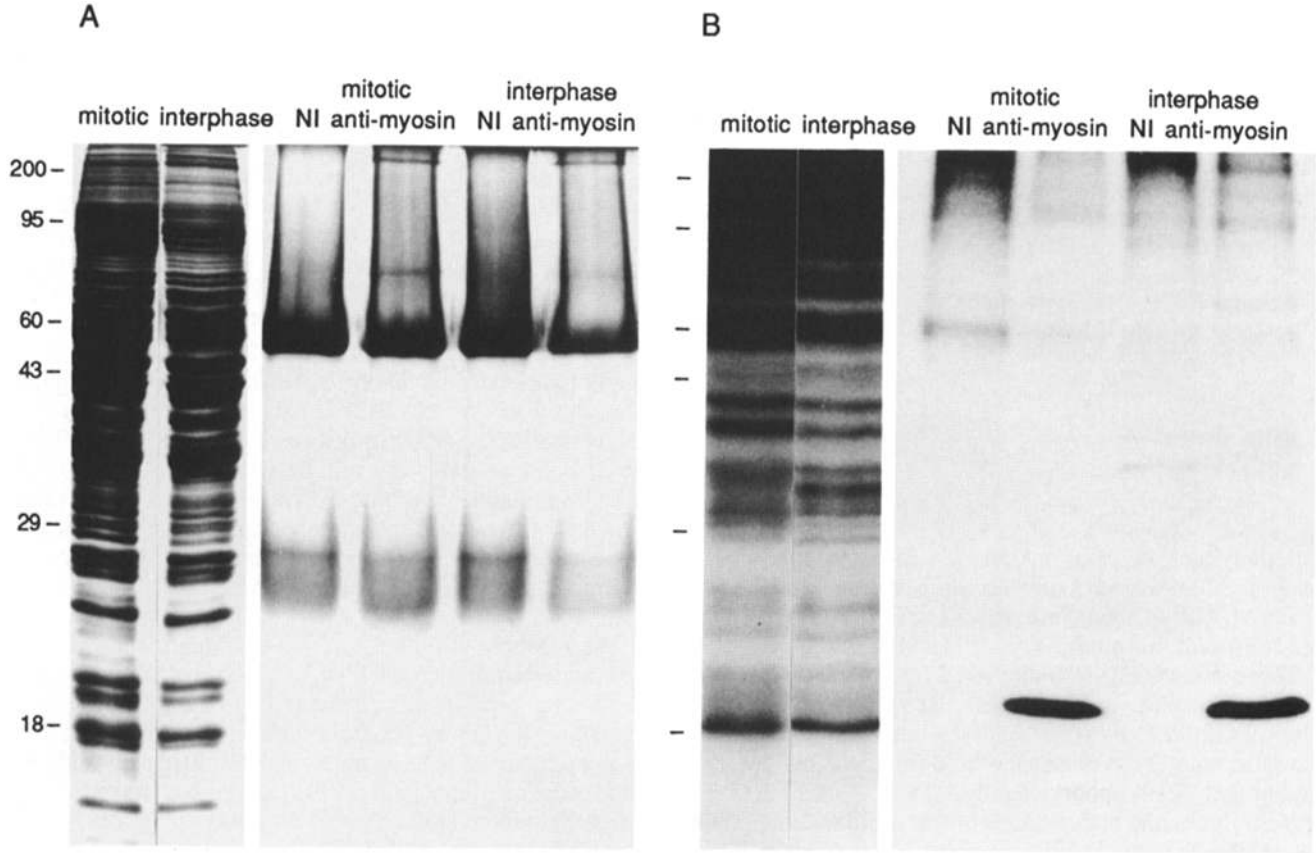

Figure 7. LC-20 of endogenous *Xenopus* myosin-II is phosphorylated in stable mitotic and interphase lysates of Xenopus eggs. (*A*) Coomassie brilliant blue stained SDS–polyacrylamide gel showing total proteins in either mitotic or interphase lysates (*left*) and in protein A-bound immune complexes prepared by immunoprecipitation using nonimmune serum (*NI*) or antiserum specific for *Xenopus* egg myosin-II heavy chain (*anti-myosin*). Mobilities of molecular weight standards are indicated at the left. (*B*) An autoradiogram of an SDS–polyacrylamide gel showing total phosphoproteins in mitotic and interphase extracts (*left*), and immune complexes (*right*).

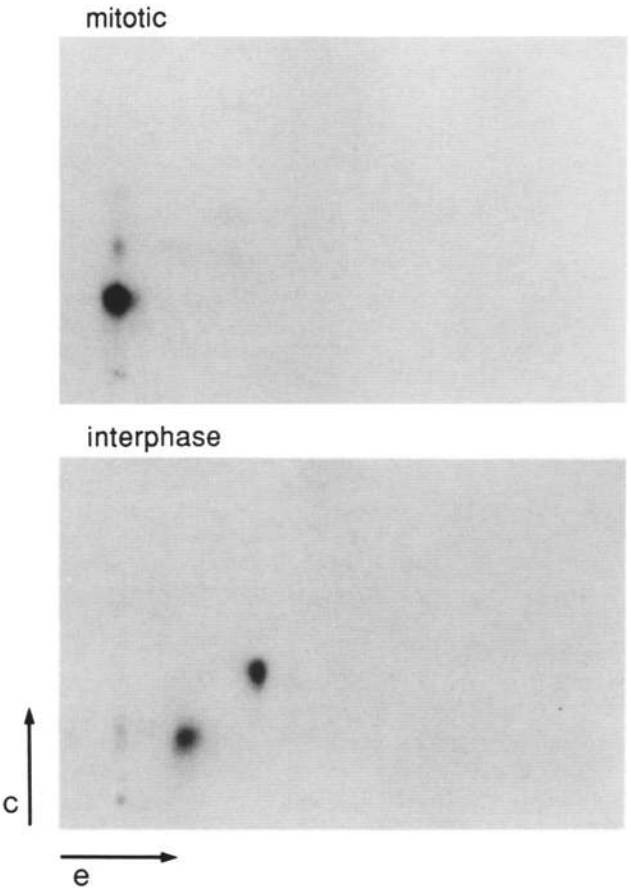

Figure 8. Xenopus myosin-II LC-20 is phosphorylated on S1 or S2 in stable mitotic lysates of eggs, and LC-20 is phosphorylated on T18 and S19 in stable interphase lysates. Autoradiograms of two dimensional maps of tryptic phosphopeptides recovered from the phosphorylated LC-20 shown in Fig. 6. The S1 or 2 containing phosphopeptide shown here co-migrates with the first of the two S1 or S2-containing phosphopeptides recovered from avian LC-20 phosphorylated by MPF in vitro (shown in first panel of Fig. 4). The arrows indicate the electrophoretic dimension (*e*), and the chromatographic dimension (*c*).

mitotic lysates, *Xenopus* myosin LC-20 was phosphorylated on S1 or S2. In six out of seven experiments, phosphorylation at the MLCK sites was minimal (as in Fig. 7), and in none did it exceed phosphorylation of the MPF site.

Xenopus myosin LC-20 recovered from the interphase lysate was phosphorylated on S19 and T18 (Fig. 8). The LC-20 phosphopeptides recovered from the interphase lysate show the same mobility as those recovered from purified *Xenopus* myosin LC-20 phosphorylated by MLCK in vitro (data not shown). In two out of three experiments using one interphase lysate, the *Xenopus* LC-20 was phosphorylated on S19 and T18. The extent of phosphorylation of LC-20 in the interphase lysates was not affected if the interphase lysates were made 80 mM Na-β-glycerophosphate to equal the concentration of this phosphatase inhibitor in the mitotic lysates (data not shown).

Discussion

Phosphorylation of LC-20 by p34 Kinases

Five different preparations of cyclin-p34^{cdc2} kinase from four species phosphorylated the LC-20 of smooth muscle and cytoplasmic myosin-II. In each case tested, these kinases phosphorylated S1 or S2 and T9 of myosin LC-20. The estimated K_m of MPF for LC-20 in vitro is consistent with the concentration of myosin-II in nonmuscle cells of 1–2 μM, as well as with the reported K_m of MPF for a second well-characterized substrate, histone H1 (Erikson and Maller, 1989).

The sequence of LC-20 phosphorylated by cyclin-p34^{cdc2} kinase, SSKRAKTKTTKK, is related to but different from the consensus sequence reported for other substrates of this enzyme (S/T-P-X-Z, where X = polar and Z = basic residues) (Moreno and Nurse, 1990). The competition of LC-20 phosphorylation in vitro by known substrates of cyclin-p34^{cdc2} kinase demonstrated that a proline COOH-terminal to the phosphate acceptor is not strictly required. Furthermore, no contaminating kinase activities were detected in the preparations of purified *Xenopus* MPF. Thus, all substrates of the p34^{cdc2} kinase are not identified simply by homology to the current consensus sequence.

Significance of Phosphorylation of Myosin-II in Xenopus Lysate

Phosphorylation of S19 of myosin-II LC-20 in vivo causes contraction in smooth muscle cells (Sellers, 1991), and correlates with shape change in platelets (Daniel et al., 1984), chemotaxis in *Dictyostelium* (Berlot et al., 1985), and cycles of gelation and relaxation in lysates of *Xenopus* eggs (Ezzell et al., 1983). In contrast, phosphorylation of myosin LC-20 by PKC in vivo causes a slow relaxation of contracted smooth muscle (Kamm et al., 1989), and correlates with secretion in nonmuscle cells (Kawamoto et al., 1989; Ludowyke et al., 1989). However, virtually nothing is known about LC-20 phosphorylation during mitosis. Based on other studies of myosin phosphorylation, one might have predicted that cytokinesis would simply require phosphorylation of LC-20 S19 to initiate the contraction. We found that LC-20 of *Xenopus* myosin-II was phosphorylated on S1 or S2 in stable egg mitotic lysates, and only on S19 and T18 in stable interphase egg lysates. This is the first evidence for the mitosis-specific phosphorylation of cytoplasmic myosin LC-20, and is consistent with the idea that cyclin-p34^{cdc2} could regulate myosin activity early in mitosis.

The recovery of only the serine-containing phosphopeptide from the mitotic lysate, in the absence of the threonine-containing peptide, is consistent with the previous work describing the in vivo phosphorylation of myosin-II by PKC. In smooth muscle cells, platelets and RBL2H3 cells, only S1 or S2 were recovered, and no T9 was detected (Kamm et al., 1989; Kawamoto et al., 1989; Ludowyke et al., 1989). Although the physiological significance of this is unknown, recent evidence suggests that a T9-specific phosphatase is active in vivo (Barany et al., 1989). Additional work at both the biochemical and cellular level will be required to confirm that phosphorylation of S1 or 2, or of S1 or 2 and T9 inhibits contractile activity in vivo in nonmuscle cells. The recent re-

port that LC-20 of myosin-II is essential for cytokinesis in *Drosophila* provides an excellent opportunity to test for the function of the LC-20 phosphorylation sites in vivo (Karess et al., 1991).

The stable mitotic lysate used in this study was prepared from unfertilized eggs, and represents metaphase of meiosis II. Because this mitotic lysate reproduces many of the key reactions of mitosis such as nuclear envelope breakdown, lamin disassembly, and chromosome condensation (Wilson and Newport, 1988; Boman and Wilson, 1992), we expect that our findings will be directly applicable to events in the early embryonic cleavage cycles. It is possible that our findings will be applicable to cytokinesis in somatic cells as well since the cyclin B-p34 kinases are functionally equivalent from yeast to man (Nurse, 1990) and the p34 phosphorylation sites on the myosin-II LC-20 are conserved throughout evolution (Sellers, 1991; Karess et al., 1991; A. Bresnick and T. Pollard, unpublished data).

Possible Biological Significance

The myosin-II light chain kinase activity of cyclin-p34^{cdc2} described here, the evidence that LC-20 of myosin-II is phosphorylated during mitosis, and the known cyclic activation of cyclin-p34^{cdc2}, suggest a biochemical mechanism for the timing of cytokinesis. We propose that cyclin-p34^{cdc2} phosphorylates myosin LC-20 during prophase and metaphase and inhibits the actin-activated myosin ATPase. At the metaphase–anaphase transition, this inhibition is released as cyclin-p34^{cdc2} activity plummets. Inhibition of myosin by cyclin-p34^{cdc2} during prophase and metaphase could delay cytokinesis until chromosome segregation has initiated at anaphase, and thus provide the cell with a mechanism to prevent premature cytokinesis.

Alternatively, phosphorylation of myosin LC-20 by cyclin-p34^{cdc2} could represent a regulatory step in contractile ring assembly. In *Dictyostelium* or somatic cells in culture, myosin undergoes a dramatic redistribution at prophase and becomes diffusely distributed in the cytoplasm during metaphase. At anaphase, myosin reassociates with actin filaments in the cortex and accumulates at the site of the presumptive cleavage furrow. (Mittal et al., 1987; Kitanishi-Yumura and Fukui, 1989). Because phosphorylation of S1 or S2 and T9 of LC-20 lowers the affinity of myosin for actin filaments (Nishikawa et al., 1984; Bengur et al., 1987), the changes in myosin distribution upon entry into mitosis could result from a reduction in the affinity of myosin for actin filaments. Likewise, dephosphorylation of S1 or 2 and T9 at anaphase could favor an initial redistribution to the actin-rich cortex because unphosphorylated myosin has an affinity for actin filaments that is intermediate between the low affinity of myosin that contains S1 or S2 and T9 phosphorylated LC-20 (Nishikawa et al., 1984) and high affinity of myosin containing LC-20 phosphorylated on S19 (Sellers et al., 1982). After an initial redistribution to the cell cortex, subsequent phosphorylation of myosin LC-20 by MLCK could represent an additional regulatory step in contractile ring assembly. Phosphorylation of LC-20 S19 stimulates filament assembly in vitro (Scholey et al., 1980; Craig et al., 1983; Sawin and Scoley, 1991), and if mediated by a spatially restricted calcium transient such as that described recently in medaka eggs (Fluck et al., 1991), could create a sink for myosin-II accumulation (Pollard et al., 1990) in that it might couple myosin polymerization and the further increase in affinity for actin.

The position of the cleavage furrow is specified by the asters of the mitotic spindle in anaphase (Rappaport, 1986), yet the signal from the spindle asters may be present earlier in metaphase, leading Sluder and Miller (1990) to propose that the timing of cytokinesis is determined by a change in cortical responsiveness to this signal. In previous studies, a change in cortical responsiveness termed "furrow inducing capacity" was observed to propagate across the surface of eggs in slight advance of the furrow tip and to be stable to transplantation (Kubota, 1969; Sawaii, 1972, 1974). We suggest that a redistribution of unphosphorylated myosin to the cell cortex following release of inhibition by cyclin-p34^{cdc2} at the metaphase–anaphase transition constitutes a step in the acquisition of cortical responsiveness to the ongoing signal from the spindle asters.

The contractile behavior of the egg cortex during mitosis is quite complex. Cycles of cortical contraction and relaxation have been observed in the fertilized and activated eggs of the sea urchin, *Ciona*, and *Xenopus* that appear to be equivalent to the characteristic rounding response that somatic cells undergo upon entry into mitosis (Danielli, 1952; Bell, 1962; Hara et al., 1980; Yoneda and Schroeder, 1984). The contraction is observed by time lapse photography as a pair of closely spaced surface contraction waves (SCW) that are not inhibited by colchicine and occur in anucleate egg fragments in step with the cycle in control eggs (Hara et al., 1980; Kirschner et al., 1980). In sea urchin eggs, an increase in cortical stiffness can be measured mechanically and is biphasic with a slow rise during prometaphase and metaphase followed by an abrupt increase at anaphase and telophase (Mitchison and Swann, 1955). This increase in cortical stiffness is also insensitive to colchicine in some species (Yoneda and Schroeder, 1984).

Hara et al. (1980) proposed that the SCWs reflected the existence of a cytoplasmic "biological clock" that controlled the timing of the cell cycle. Therefore, we can speculate that MPF-mediated phosphorylation of the contractile machinery might underlie the SCWs and the cycles of cortical stiffness. Although we predict myosin to be inhibited by MPF during metaphase, perhaps phosphorylation of other components of the actomyosin contractile system might lead to the initial increase in stiffness or rounding via reorganization of actin filaments or some other novel mechanism. In addition, relocalization of dephosphorylated myosin to the cortical actin filaments in early anaphase or phosphorylation of the activating site of myosin LC-20 might lead to the second abrupt increase in cortical tension observed in anaphase.

We have attempted to keep our proposal simple by addressing a single component of the contractile ring, myosin-II. However, at least 10 other contractile ring components have been identified (reviewed by Satterwhite and Pollard, 1992) which will ultimately need to be integrated into any model for the regulation of cytokinesis. In particular, the recent genetic identification of ACT2, an intriguing actin-like protein required late in mitosis (Schwob and Miller, 1992; Lees-Miller et al., 1992), and the biochemical studies of the cell cycle regulation of the actin-binding protein caldesmon (Yamashiro et al., 1990, 1991) underscore the possibility that a process as important for cell viability as cytokinesis might

be regulated by multiple mechanisms. Despite the obvious morphological variation of cytokinesis in organisms as diverse as *Xenopus*, *S. cerevisiae*, *S. pombe*, and in human somatic cells, we propose that the fundamental regulatory mechanisms that govern cytokinesis are highly conserved.

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