Microtubule-entrained kinase activities associated with the cortical cytoskeleton during cytokinesis

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SUMMARY

Research over the past few years has demonstrated the central role of protein phosphorylation in regulating mitosis and the cell cycle. However, little is known about how the mechanisms regulating the entry into mitosis contribute to the positional and temporal regulation of the actomyosin-based contractile ring formed during cytokinesis. Recent studies implicate p34\textsuperscript{cdc2} as a negative regulator of myosin II activity, suggesting a link between the mitotic cycle and cytokinesis. In an effort to study the relationship between protein phosphorylation and cytokinesis, we examined the in vivo and in vitro phosphorylation of actin-associated cortical cytoskeletal (CSK) proteins in an isolated model of the sea urchin egg cortex. Examination of cortices derived from eggs or zygotes labeled with \textsuperscript{32}P-orthophosphate reveals a number of cortex-associated phosphorylated proteins, including polypeptides of 20, 43 and 66 kDa. These three major phosphoproteins are also detected when isolated cortices are incubated with \textsuperscript{32}P\textsuperscript{2}ATP in vitro, suggesting that the kinases that phosphorylate these substrates are also specifically associated with the cortex. The kinase activities in vivo and in vitro are stimulated by fertilization and display cell cycle-dependent activities. Gel autophosphorylation assays, kinase assays and immunoblot analysis reveal the presence of p34\textsuperscript{cdc2} as well as members of the mitogen-activated protein kinase family, whose activities in the CSK peak at cell division. Nocodazole, which inhibits microtubule formation and thus blocks cytokinesis, significantly delays the time of peak cortical protein phosphorylation as well as the peak in whole-cell histone H1 kinase activity. These results suggest that a key element regulating cortical contraction during cytokinesis is the timing of protein kinase activities associated with the cortical cytoskeleton that is in turn regulated by the mitotic apparatus.

Key words: Cell cycle, Cytokinesis, Protein phosphorylation, Cytoskeleton
Kirschner, 1990), microtubule-associated proteins such as MAP4 (Ookata et al., 1995), motor proteins such as Eg5 and CENP E (reviewed by Vernos and Karsenti, 1996), and vimentin (Chou et al., 1990). Caldesmon and non-erythroid spectrin have also been shown to be phosphorylated by p34cdc2, and this phosphorylation is accompanied by a concomitant reduction of caldesmon/spectrin association with the actin cytoskeleton (Yamashiro et al., 1991; Fowler and Adam, 1992). It is postulated that phosphorylation of these proteins results in the massive reorganization of the cytoskeleton that accompanies cell division.

With regard to cytokinesis, the regulation of myosin II activity represents one of the most relevant examples of cell cycle regulation of an actin-associated protein during mitosis. Studies of myosin II light chain phosphorylation in higher eukaryotes reveal that p34cdc2 kinase phosphorylates the regulatory light chain (Satterwhite et al., 1992; Yamakita et al., 1994) on residues previously shown to inhibit myosin ATPase activity in vitro (Nishikawa et al., 1984; Bengur et al., 1987). From these findings, it has been proposed that p34cdc2 inhibits myosin activity until anaphase when cyclin B destruction would result in a reduction of p34cdc2 activity (Satterwhite and Pollard, 1992). At this time, MLCK may then stimulate myosin filament formation and ATPase activity. Thus, the regulation of p34cdc2 activity is hypothesized to provide the ‘timer’ that regulates cortical contraction. Recent genetic analysis of the light chain phosphorylation sites in *Dictostelium* (Ostrow et al., 1994), however, suggests that myosin II regulation in lower eukaryotes may be more complicated than that proposed in the Satterwhite and Pollard model (Satterwhite and Pollard, 1992).

There is an increasing body of experimental evidence to suggest that protein kinases coordinate the transition from mitosis to cytokinesis, and may also control the position and organization of the contractile ring. Studies using an antibody against a phosphoepitope of glial fibrillary acidic protein (GFAP) reveal the presence of a protein kinase activity that is not active until the metaphase-anaphase transition (Sekimoto et al., 1996). In *Saccharomyces cerevisiae* the Dbf2/Dbf20 protein kinases are activated upon dephosphorylation following the metaphase-anaphase transition (Toyn and Johnston, 1994). Polo kinase, however, may represent the most attractive candidate for a late mitotic kinase that acts to regulate spindle-cortical dynamics. Originally identified as a gene required for proper spindle assembly in *Drosophila* (Llamazaes et al., 1991), polo is highly conserved throughout the phylogenetic tree (Ohkura et al., 1995; Golsteyn et al., 1995). Polo kinase activity is activated at the G2/M transition, but extends beyond the metaphase-anaphase transition (Fenton and Glover, 1993; Golsteyn et al., 1995). Polo mutants in *S. pombe* result in elongated spindles, and a failure in actin ring assembly and septum formation (Ohkura et al., 1995). Localization of human polo-like kinase (PLK1) in tissue culture cells reveals that upon anaphase onset, PLK1 translocates from the mitotic spindle to the equatorial plane (Golsteyn et al., 1995). While the substrates of this kinase remain unknown, studies in mammalian cells indicate that PLK1 interacts with CHO1/MKLP-1, a kinesin-like molecule (Lee et al., 1995), and the *Xenopus* homolog phosphorylates the p34cdc2-activating phosphatase, cdc25 (Kumagai and Dunphy, 1996). Thus, while polo kinases may be essential to spindle regulation, the extended activity of polo during mitosis (in comparison to p34cdc2), its redistribution to the equatorial zone, and its association with microtubule motors make it an attractive candidate for the microtubule-based regulation of cortical remodeling during the onset of cytokinesis.

Accumulating genetic and biochemical evidence point towards a role for protein kinases not only in regulating cortical remodeling at the G2/M transition, but also in the formation and induction of the contractile ring. However, the identity of the kinases, their substrates within the cortical cytoskeleton, and the functional consequences of this regulation remain elusive. Previous work in this laboratory developed a model of the sea urchin zygote cortical actin cytoskeleton that retains the biochemical and functional characteristics of the intact, cleaving blastomere (Walker et al., 1994). In this study, we employed this detergent-extracted preparation of the sea urchin zygote to ask whether there are elements of the isolated cortex subject to regulation by kinases acting late in mitosis. Results of these studies indicate there are three major polypeptides (p20, p43 and p66) that are phosphorylated by kinases associated with the actin cortex, and 2-dimensional electrophoresis and immunoprecipitation studies reveal that p20 is myosin regulatory light chain. As for the kinases that phosphorylate these substrates, the combined approaches of phosphoamino acid analyses, in-gel kinase assays, western blotting, and phenyl-Sepharose chromatography reveal the presence of multiple kinases associated with the actin cortex. Of these cortical kinases, p34cdc2 and a homolog of mitogen-activated protein kinase (MAP kinase), demonstrate cyclic fluctuations in activities (but not levels) through the cell cycle. The activation of cortical protein phosphorylation is sensitive to nocodazole-, but not to staurosporine-treatment of cleaving embryos, suggesting that the recruitment or activation of cortical kinase activity is dependent upon the integrity of the mitotic apparatus, but not protein kinase C (PKC). The cell cycle- and microtubule-dependent phosphorylation of these cortical substrates suggest that these polypeptides may represent targets of multiple regulatory mechanisms, where p34cdc2 and other cortical kinases regulate the timing and formation of the contractile ring.

**MATERIALS AND METHODS**

**Culture of sea urchin zygotes**

The gametes of the California urchin *Strongylocentrotus purpuratus* were used for all experiments (Marinus, Long Beach, CA). Sheding of gametes was induced by the injection of 0.5 M KCl and eggs were de-jellied by brief exposure to pH 5.0 artificial sea water (ASW) followed by washing 1× in ASW. The vitelline membrane was then removed by treatment with DTT in ASW (pH 9.1). Eggs were allowed to settle and then washed 5× in ASW by settling.

Eggs were fertilized with 10 µl dry sperm for every 1 ml packed eggs in ASW. The zygotes (1 ml) were cultured in 50 mls of ASW at 15-16°C in a spinner flask after briefly washing out excess sperm. In some experiments nocodazole at 10 µM (Sigma Co., St Louis, MO) was added to experimental flasks at 10 minutes after fertilization to promote microtubule disassembly.

**Preparation of sea urchin egg/zygote cytoskeletons**

Cortical CSKs were isolated as detailed by Walker et al. (1994). Briefly, eggs or embryos (1 ml packed cells) were washed once in 10 ml of isolation buffer containing 1 M glycerol, 5 mM EGTA, 5 mM MgCl2, 10 µM Na2VO4 and 20 mM Pipes (pH 7.3 unless specified as
In vivo protein phosphorylation

De-jellied and de-membranated eggs were washed with phosphate-free synthetic sea water (PFSSW). The eggs were then pre-incubated for 1 hour on ice with 2.5 mM/ml 32P-orthophosphate in an egg suspension of 1 ml packed eggs in 2 ml PFSSW. Eggs were then diluted into 50 ml of PFSSW (unfertilized) or 50 ml of PFSSW/sperm suspension (zygote or embryo) and cultured in spinner flasks.

In vitro protein phosphorylation

Cytoskeletal proteins were labeled endogenously by addition of [γ-32P]ATP. The reaction was carried out by addition of 200 μl cortex cytoskeleton suspension (in isolation buffer) to a mixture of 20 μl (0.25 mM) cold ATP, 4 μl [32P]ATP (22.5 μCi) and 1 μl 1 M CaCl₂ (free Ca²⁺= 23.23 μM). For most experiments the reaction was allowed to continue for 10 minutes at room temperature and then stopped by addition of 80 μl hot 4× SDS-sample buffer and boiled for 2 minutes.

Phosphoamino acid analysis

SDS-PAGE gels of 32P-labeled CSK were electropheretically transferred to Immobilon P membranes (Towbin et al., 1979). The bands of interest were excised from the blot, cut into pieces and placed into 1.5 ml Eppendorf microfuge tubes. The proteins were then hydrolyzed by addition of 200 μl 5.7 N HCl and incubated at 110°C for 1 hour. The hydrolysate was removed and lyophilized. The amino acid samples were then dissolved in pH 1.9 buffer containing 2.2% formic acid and 1.3611 M acetic acid supplemented with 0.2 mg/ml each of phosphoserine, phosphothreonine and phosphotyrosine. Electrophoretic separation of individual amino acids was achieved by thin layer electrophoresis on cellulose thin layer chromatography plates (Boyle et al., 1991).

In situ kinase assay

To identify kinases associated with the cortical cytoskeleton, cortices were resolved by SDS-PAGE, the proteins renatured and subjected to in situ phosphorylation (Hutchcroft et al., 1991). Briefly, SDS was removed by washing gels 2× in 20% propanol, 50 mM Tris-HCl, pH 8.3, for 30 minutes each, followed by washing in 50 mM Tris-HCl, pH 8.3, 5 mM β-ME for 1 hour. Proteins were then denatured by incubating gels in 7 M guanidine, 50 mM Tris-HCl, pH 8.3, for 1 hour at room temperature. The proteins were slowly renatured by washing 4× in 300 ml of 50 mM Tris, 5 mM β-ME, 0.04% Tween-40 over 18 hours at 4°C. The gel was then incubated in 100 ml of pre-incubation buffer (40 mM Heps, pH 8.0, 10 mM MgCl₂, 0.5 mM EGTA, 2 mM DTT) for 1 hour at 20°C. The phosphorylation of endogenous renatured kinases was carried out in phosphorylation buffer containing 40 mM Heps, pH 8.0, 0.5 mM EGTA, 10 mM MgCl₂, and 100 μCi [32P]ATP for 2 hours at 20°C. The free 32P was removed by washing gels 4× 500 ml in 5% TCA, 1% sodium pyrophosphate. The gels were then stained with Coomassie Blue R-250, dried and the labeled proteins visualized by autoradiography.

Histone H1 kinase assay

To detect histone H1 kinase activity, resuspended CSK (200 μl in isolation buffer) were incubated with 20 μl of [32P]ATP/ATP mix (final=1.5625x10⁻⁶ M, 50 μCi), 6.4 μl protein kinase inhibitor (0.08 μM) to inhibit PKA activity, and 20 μl (20 μg) histone H1 (Boehringer-Mannheim, Indianapolis, IN) for 10 minutes at 25°C. In some assays myelin basic protein (MBP) was added (40 μg) to simultaneously assay for MAP kinase activity. The reaction was stopped by addition of 1/4 volume 4× SDS-PAGE sample buffer. The samples were run on SDS-PAGE and autoradiographed. Samples for whole egg activity were prepared by lysing eggs with 10 volumes of distilled water followed by freeze thawing.

Immunoblot analysis

SDS-PAGE gels were run and proteins transferred to Immobilon-P membranes at 24 volts for 90 minutes in a Genie blotting apparatus (Idea Scientific; Minneapolis, MN). The blots were blocked with 5% non-fat dry milk, 0.2% Tween-20, TBS for 1 hour at 20°C. The blots were then incubated with either a 1/2,000 dilution of rabbit anti-MAP kinase R1 erk1-k-nein III Upstate Biotechnology, Lake Placid, NY) or a 1/1,000 dilution of rabbit anti-human cdc2 kinase (PSAIR; Upstate Biotechnology, Lake Placid, NY) in 1% non-fat dry milk, 0.04% Tween-20, TBS for 1 hour at 20°C or overnight at 4°C. Tubulin was detected by using a 1/2 dilution of a monoclonal mouse anti-alpha tubulin tissue culture supernatant (generous gift from Dr Charles Walsh, University of Pittsburgh). Blots were washed 3 times with TBS containing 0.2% Tween-20. Bound antibodies were detected by incubation with HRP-conjugated goat anti-rabbit IgGs for 1 hour at room temperature and blots were visualized using Enhanced Chemiluminescence (Amersham; Arlington Heights, IL).

To quantify the relative fraction of kinases associated with the actin cortex, cortices were prepared from unfertilized eggs, interphase or dividing zygotes or, alternatively, whole eggs were placed directly into hot sample buffer. Equal amounts of cortical and whole egg protein were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against p34cdc2, MAP kinase, fyn (Upstate Biochemical, Lake Placid, NY), β subunit CAM kinase II (Zymed, San Francisco, CA), kinesin heavy chain (provided by John Scholey, University of California at Davis), or lamin B (provided by Gary Wessel, Brown University).

MBP kinase preparation

MBP kinase was purified from early sea urchin embryos by the method of Sanghera et al. (1992). Embryos at first cleavage were homogenized in 3 ml homogenization buffer containing 40 mM MOPS, pH 7.2, 120 mM sodium β-D-glycerolphosphate, 10 mM EGTA, 2 mM EDTA, 2 mM sodium orthovanadate with a Potter-Elvehjem homogenizer on ice. The sample was clarified by centrifugation at 115,000 g for 60 minutes. The supernatant was recovered and passed through a 0.45 μm filter. Samples (1 ml) were applied to a phenyl-Sepharose column (Protein Pak Glass HIC phenyl-5PW, Waters/Millipore; Bedford, MA). After washing unbound protein from the column, bound proteins were eluted with a 7 ml linear gradient of 0-60% ethylene glycol, 250-25 mM NaCl at 0.2 ml/minute. Fractions were then assayed for MBP and H1 kinase activities, and probed for the presence of MAP kinases and p34cdc2 by western blotting.

Substrate-blot assay

To identify candidate cortical substrates for MBP kinase and p34cdc2, a substrate blot assay was devised. Briefly, SDS-PAGE gels were run and proteins transferred to Immobilon-P. The blots were dried and then blocked with 5% BSA, 30 mM Tris-HCl, pH 7.5, for 1 hour. The blots were rinsed in reaction buffer (30 mM Tris-HCl, pH 7.35, 10 mM MgCl₂, 2 mM MnCl₂). The blots were labeled by incubation with 25 μl reaction buffer containing 250 μCi (2.2 nM) [γ-32P]ATP and either sea urchin MAP kinase or p34cdc2/cyclin B complex (Upstate Biomedical, Lake Placid, NY). Parallel control blots were prepared and incubated in reaction buffer containing 250 μCi (2.2 nM) [γ-32P]ATP with no exogenously added kinase. The blots were incubated at room temperature for 60 minutes with agitation. The blots are then washed twice with TBS/0.05%NP-40 and then twice with TBS. The blots were then incubated with 1 N KOH for 10 minutes followed by a brief 1 N KOH rinse. The KOH was removed by washing twice with TBS. Phosphorylated proteins were detected by autoradiography of the dried blots.
RESULTS

Cytoskeletal protein phosphorylation in the isolated sea urchin cortex

The cortical cytoskeleton (CSK) prepared in this study is an actin-rich, detergent-insoluble cytoskeleton that retains the basic morphology of the original blastomeres (Fig. 1.), but lacks the overlying plasma membrane. This preparation, whose structural, biochemical, and functional characteristics are described more fully elsewhere (Walker et al., 1994), possesses the known egg cortical cytoskeletal proteins actin, myosin II, spectrin, and fascin. As shown by Coomassie staining, actin is the most predominant protein in the CSK preparation (Fig. 1C, lane 3). Electron microscopy of isolated cortices reveals that the cortex consists of an anastomosing meshwork of filaments that retains microvillar actin cores as well as the hyaline layer (Walker et al., 1994).

To explore the role of kinases in modulating the actin cytoskeleton during cytokinesis, whole eggs or zygotes were labeled with $^{32}$P-orthophosphate and the phosphoproteins associated with the isolated cortices compared with those found in the whole egg (Fig. 1C). Whole cell samples from unfertilized eggs contain numerous phosphorylated polypeptides (Fig. 1C, lane 2), the vast majority of these polypeptides are not associated with the actin cortex (Fig. 1C, lane 4). In contrast, cortices isolated from interphase (Fig. 1C, lane 5) or cleavage-stage zygotes (Fig. 1C, lane 6) following in vivo labeling reveal a distinct subset of whole egg or embryo phosphoproteins present in the isolated actin cortex. While some high molecular mass phosphoproteins are shared by dividing embryos and unfertilized eggs, cortices derived from dividing blastomeres contain phosphoproteins not evident in the unfertilized egg CSK, including phosphoproteins co-migrating with actin (~43 kDa) and serum albumin (66 kDa). Other phosphoproteins associated with the cortex in unfertilized and cleaving eggs include a 20 and 36 kDa species. For all four phosphoproteins, there is a dramatic increase in phosphate incorporation when cortices are isolated from cleavage-stage embryos.

Protein kinase activities are associated with the cortical cytoskeleton

To ask whether kinase activities are themselves associated with the cortical cytoskeleton, cortices were derived from zygotes, incubated in vitro with [$y^{32}$P]ATP, and the phosphoproteins analyzed by SDS-PAGE. Control experiments indicate that peak in vitro $^{32}$P incorporation into cortical protein occurs approximately 10 minutes after initiation of phosphorylation in both unfertilized and cleavage stage cortices (data not shown). Incubations longer than 10 minutes result in reduced $^{32}$P incorporation, possibly due to the actions of phosphatases associated with the cortex. Having determined the optimum conditions for phosphate incorporation, all subsequent in vitro phosphorylation reactions were carried out for 10 minutes.

Examination of in vitro-labeled cortices reveals that p20, p43, and p66 are phosphorylated in cortices derived from cleavage-stage embryos (Fig. 2, lanes 3 and 6). In contrast, the 36 kDa polypeptide observed in in vivo-labeled eggs and zygotes is not observed in the in vitro-labeled cortices. The highest level of protein phosphorylation is observed in cortices isolated from cleavage-stage blastomeres (Fig. 2, lanes 3 and 6) in comparison to those from unfertilized eggs (Fig. 2, lanes 1 and 4) or from interphase zygotes (Fig. 2, lanes 2 and 5). Because pH-dependent protein tyrosine kinase activities have been reported in sea urchin eggs, in vitro phosphorylation
changes were carried out at pH values corresponding to the cytoplasmic pH of unfertilized (pH 6.8; Fig. 2, lanes 1-3) or fertilized (Fig. 2; lanes 4-6) eggs (Jiang et al., 1990). Slight quantitative, but no qualitative differences in protein phosphorylation are observed in cortices phosphorylated in vitro at pH 6.8 versus, pH 7.3, suggesting that the pH-dependent protein tyrosine kinase may not be the kinase phosphorylating cleavage-stage cortical polypeptides. The similar patterns of phosphorylation observed between in vivo and in vitro-labeled cortices suggest that the kinases phosphorylating these substrates during cytokinesis are, in fact, specifically associated with the cortical cytoskeleton.

Changes in cortical protein phosphorylation during the cell cycle
To follow the phosphorylation of p20, p43, and p66 through the cell cycle, cortices were isolated from embryos at different stages through the first two cell cycles, and labeled in vitro. As shown in Fig. 3, the level of p43 and p20 phosphorylation is highest in cortices isolated from cleavage-stage blastomeres (Fig. 3, lane 4), decreases in interphase isolated cortices (Fig. 3, lanes 5 and 6) and is again elevated at the time of the second cleavage (Fig. 3, lane 7). A more variable pattern of in vitro phosphorylation of p66 occurs following 90 minutes post-fertilization (lane 4), with no consistent cycling observed following the first cleavage.

Identification and partial characterization of in vitro-labeled phosphoproteins
Biochemical as well as genetic lines of experimentation have underscored the requirement of myosin II function in cytokinesis (Mabuchi and Okuno, 1977; Kiehart et al., 1982; Delozanne and Spudich, 1987; Knecht and Loomis, 1987; Karess et al., 1991), and we have shown that myosin II is present in isolated cortices that undergo ATP-dependent contraction in vitro (Walker et al., 1994). Additionally, several reports indicate that myosin II regulatory light chain (LC 20) is phosphorylated in dividing cells, and can be phosphorylated in vivo and in vitro by p34cdc2 kinase (Yamakita et al., 1994; Satterwhite et al., 1992). To determine whether the 20 kDa polypeptide phosphorylated in vivo and in vitro is myosin LC 20, cortices were labeled with [32P]ATP in vitro, solubilized, and anti-sea urchin egg myosin antibodies used to immunoprecipitate myosin II and its associated light chains (Fig. 4A).

Autoradiography of these immunoprecipitations revealed that myosin LC 20, and to a lesser extent myosin heavy chain, is phosphorylated in vitro. Confirmation that the phosphorylated 20 kDa protein is LC20 came from two-dimensional IEF/PAGE, where the 20 kDa phosphoprotein migrates with a similar mobility (Fig. 4B, lower arrow) as purified egg myosin II 20 kDa light chain (data not shown).

To partially characterize the other major cortical phosphoproteins, two-dimensional PAGE and non-equilibrium pH gel electrophoresis were used to determine the isoelectric points of p43 and p66. A series of phosphorylated species corresponding to the 66 kDa phosphoprotein can be detected focusing at a slightly more acidic pI (pI ≈ 5) than actin (Fig. 4B). Conventional two-dimensional IEF/PAGE did not reveal any polypeptides corresponding to the 45 kDa species. However, phosphorylated species corresponding to the 43 kDa polypeptide can be detected by non-equilibrium pH gel electrophoresis (NEpHGE) (Fig. 4C), whose pl values were in the region of ≈ 9.

In light of recent evidence indicating that, in addition to serine-threonine kinases such as p34cdc2, there are tyrosine kinases active during mitosis (Roche et al., 1995), phosphoamino acid analysis was performed to determine the relative distribution of phosphoamino acids in LC 20, p43, and p66. As shown in Fig. 5, phosphoamino acid analysis of in vitro-labeled p20, p43, and p66 from cleavage-stage blastomeres reveals that p66 is evenly phosphorylated on all three phosphoamino acids (Fig. 5A). Phosphoserine is the major phosphoamino acid in the 43 kDa polypeptide, which is also phosphorylated to a lesser extent on threonine and tyrosine (Fig. 5B). Phosphoserine is the major phosphoamino acid found in LC20 (Fig. 5C) with phosphothreonine present at a much lower level. Thus, from phosphoamino analysis of in vitro-labeled p20, p43 and
p66, there appears to be both serine-threonine and tyrosine kinase activities associated (and active) with the actin cortex of dividing blastomeres.

**Identification of cortical kinase activities**

As a first measure toward characterizing candidate kinases present in the isolated sea urchin cortex, cortices were resolved by SDS-PAGE, renaturated, and assayed for kinase autophosphorylation (Fig. 6A). Three major autophosphorylating kinases, with approximate molecular masses of 84, 45 and 42 kDa, can be detected by this method in unfertilized and cleavage-stage cortices (Fig. 6A). These kinases appear distinct from the major kinase substrates detected by in vivo and in vitro phosphorylation experiments based on mobility in SDS-PAGE. Phosphoamino acid analysis of the 45 kDa and 84 kDa kinases indicate that both are autophosphorylated on threonine and serine residues (data not shown). No evidence of tyrosine autophosphorylation could be detected by this assay.

When cortical kinases were renaturated in PAGE gels containing immobilized myelin basic protein (MBP) as a substrate, kinase activity is readily detected in CSKs from both unfertilized eggs and cleaving zygotes (Fig. 6B). However, no additional kinase activities are detected by this method. Using this assay system, the activity of these kinases also fluctuate through the cell cycle, with the highest activities found in CSKs isolated from cleavage-stage zygotes (Fig. 6A and B, lanes 4 and 7). The presence of MBP kinase activity was confirmed by inclusion of MBP in CSK in vitro labeling reactions, where peak MBP kinase activity can be detected in cortices isolated immediately prior to cleavage (data not shown).
Histone H1 serves as a substrate for many kinases, including the catalytic component of maturing promoting factor, p34\textsuperscript{cdc2} (Langan et al., 1989). To ask whether an analogous activity is associated with cortices derived from dividing sea urchin blastomeres, histone H1 was mixed with CSKs isolated from zygotes at different stages in the presence of [γ\textsuperscript{32}P]ATP. and the phosphorylated histone H1 resolved by SDS-PAGE and autoradiography (Fig. 6C). As shown in Fig. 6C, histone H1 is phosphorylated with a peak in H1 kinase activity in cortices isolated from cleavage stage embryos (Fig. 6, lane 3). These results indicate that both H1 and MBP kinase activities exhibited by the isolated cortices appear to be cyclic in nature, with both activities peaking at the time of cell division.

Cortical CSK phosphorylation is independent of protein kinase C

Protein kinase C (PKC) is thought to play an important role in egg activation and mitosis (Mabuchi and Takano-Ohmuro, 1990; Bement and Capco, 1991). To determine if PKC plays a role in cytokinesis or the phosphorylation of cortex-associated proteins, zygotes were continuously incubated in staurosporine beginning at different times following fertilization (Fig. 7). Examination of staurosporine-treated zygotes reveals, as expected, that cleavage is sensitive to staurosporine treatment in a time-dependent manner, with incubations in the drug starting any time up to 40 minutes after fertilization blocking cleavage in over 75% of zygotes (Fig. 7A). Progressively later initiation of staurosporine treatment resulted in a progressively lesser impact on cleavage. If drug treatments were begun after about 70 minutes post-fertilization (corresponding to the onset of mitosis), zygotes divide normally. Hoechst dye was used to monitor the state of the mitotic cycle in samples of staurosporine treated zygotes (data not shown). Staurosporine treatment beginning 10 minutes post fertilization resulted in 80% of zygotes remaining in interphase with only 20% having a telophase stage mitotic apparatus or having cleaved. In contrast, 47% of zygotes continuously cultured in staurosporine beginning at 70 minutes post fertilization had cleaved or were in telophase and another 45.5% possessed a mitotic apparatus in some stage of mitosis.

Cortices were prepared from staurosporine-treated and control cultures at the time of first cleavage in control cultures and the in vitro phosphorylation of LC20 and p43 monitored (Fig. 7B). Continuous treatment of zygotes beginning 10 minutes post fertilization with staurosporine inhibits by over 60% the level of in vitro phosphorylation of p43 and LC20 over that in controls. In contrast, initiation of staurosporine treatment at 70 minutes, which delayed entry into mitosis, results in less than a 40% inhibition of p43 or LC20 phosphorylation relative to that in cortices from control cultures. Consistent with these results was the finding that incubating isolated cleavage stage cortices in H7, another potent PKC inhibitor (Hidaka et al., 1984), did not affect the in vitro phosphorylation of p43 or LC20 (data not shown).

Characterization of cell cycle kinases associated with the cortical cytoskeleton

Results of in vitro labeling as well as in-gel phosphorylation assays suggest that kinases associated with the cortex may mediate the phosphorylation of p20, p43 and p66. And since the phosphorylation of cortical proteins by endogenous kinases appears to be cell cycle-regulated, we sought to identify specific kinases known to play roles in cell cycle and growth regulation by western blotting of isolated cortical CSK proteins. Western blotting with anti-MAP kinase antibodies reveal the presence of a 49-50 kDa polypeptide present in cortices throughout the cell cycle (Fig. 8, MAPK). In addition, the MAP kinase R2 (erK1-CT) antibody recognizes two other peptides of about 46 kDa and 44 kDa (data not shown). Antibodies to p34\textsuperscript{cdc2} kinase recognize several species of approximately 34 kDa (Fig. 8, cdc2). Additionally, antibodies specific for the tyrosine kinase c-fyn, recognize a single 60 kDa species constitutively associated with the cortical cytoskeleton (data not shown). In addition, we have recently demonstrated the presence of e-abl, another tyrosine kinase family member within the cortical cytoskeleton (Walker et al., 1996). Western blotting of cortical and whole cell fractions reveals that while the levels of the 49 kDa MAP kinase, p34\textsuperscript{cdc2}, and fyn in the isolated cortex increase following fertilization (Fig. 8), statistical analyses indicate that the small changes in the fraction of kinases associated with the cortex from interphase to cell
cytokinesis (Table 1) are not significantly different (P=0.05 for all).

Although we found that the cortex represents less than 2% of total egg protein, experiments were performed to determine whether the cortex-associated kinase activities were due to non-specific trapping (Fig. 8; Table 1). Tubulin, which is largely soluble in unfertilized eggs cannot be detected in the CSK prior to fertilization, suggesting that there is minimal trapping of soluble proteins in these preparations (Fig. 8, Tub). Tubulin only becomes detectable after fertilization when it becomes specifically associated with the CSK in the form of short microtubules as detected by immunofluorescence (data not shown). Levels of kinesin heavy chain, whose association with the mitotic apparatus has previously been shown to be detergent-sensitive (Wright et al., 1991), do not appreciably change between interphase and dividing cells. Additionally, while the β subunit of calcium-calmodulin dependent kinase II can be easily detected in whole cell lysates, it cannot be detected in any cortical preparations (data not shown). Because nuclei are normally extruded during the detergent extraction and Dounce homogenization of the cortices, lamin B was used as a probe for nuclear contamination. Additionally, lamins are hyperphosphorylated and subsequently solubilised during prophase (Ward and Kirschner, 1990), and therefore represent another control for nonspecific trapping of soluble molecules. Western blotting of cortical and whole cell fractions reveals that while there exists a minor amount of contaminating lamin B present in the cortices (probably due to the occasional nucleus contaminating the preparation), the percentage of lamin present in dividing and interphase cortices does not appreciably change although lamins are solubilised during mitosis, and could be trapped within the cortex (Table 1). Therefore, based on these experiments and controls, it is likely that the kinase activities detected in the cortex are specifically associated and are not artifically trapped.

**Table 1. Percentage of proteins associated with the cortical cytoskeleton**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Unfertilized</th>
<th>Interphase</th>
<th>Mitotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>p34&lt;sup&gt;cdc2&lt;/sup&gt;</td>
<td>1.48</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>1.01</td>
<td>3.3</td>
<td>1.0</td>
</tr>
<tr>
<td>lyn</td>
<td>2.81</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td>CAM kinase</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Kinesin HC</td>
<td>4.7</td>
<td>3.6</td>
<td>3.54</td>
</tr>
<tr>
<td>Lamin B</td>
<td>1.8</td>
<td>1.4</td>
<td>1.2</td>
</tr>
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Equal loads of cortical and whole cell protein derived from unfertilized eggs, interphase or dividing zygotes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed for the presence of several kinases, kinesin heavy chain, and lamin B. The relative fractions of each protein were determined by normalizing the signal detected in the cortex (which represents 1.5% of total cell protein) to the corresponding whole cell signal. n=6. n.d., not detected.
Nocodazole treatment of eggs prior to cortex isolation drastically reduces the amount of tubulin present in the cortex (Fig. 10B), disrupts all zygote and cortical microtubules (Fig. 9A), blocking mitosis and cytokinesis.

Zygotes were treated with nocodazole, processed for whole-cell H1 kinase activity (Fig. 11) and for determination of in vitro/in vivo phosphorylation of cortical CSK proteins (Fig. 12). Histone H1 kinase activity in whole cell homogenates (Fig. 11) made from nocodazole-treated zygotes reveals that nocodazole significantly delays the peak of H1 kinase activity through the time course corresponding to the first two cell cycles (Fig. 11). Examination of in vivo and in vitro labeling of p43 and LC20 reveals that both p43 and LC20 phosphorylation are affected by nocodazole disruption of microtubules (Fig. 12A-D), with nocodazole delaying the peak in p43 and LC20 phosphorylation until after the time of cleavage of control zygotes. Quantitation of p43 phosphorylation from cortices labeled in vivo or in vitro reveals that phosphorylation levels in nocodazole-treated cultures is on average 25% of...
controls prepared at the time of first cell division (Fig. 12E). When a single batch of zygotes was used for both whole cell H1 kinase and in vitro phosphorylation assays, the delayed peak of histone H1 phosphorylation activity determined in whole cell homogenates from nocodazole-treated embryos correlated precisely with the delayed peak of p43 and LC20 phosphorylation seen in vivo and in vitro.

DISCUSSION

While genetic analyses as well as morphological approaches continue to identify components of the contractile ring, the molecular mechanisms that determine the spatial and temporal regulation of cleavage furrow formation remain elusive. In an effort to define the potential targets of cell cycle regulation within the actin cortex, we used a preparation of the sea urchin embryo cortex previously described as retaining the biochemical and contractile characteristics of the intact zygote cortex (Walker et al., 1994). Using in vivo and in vitro labeling techniques, three polypeptides were identified that are the major phosphoproteins associated with the cortex at the time of cleavage. Immunoprecipitation studies reveal that the 20 kDa phosphoprotein is the myosin regulatory light chain (LC 20), which has been previously shown to be a substrate of p34\(^{cdc2}\) and MLCK (Satterwhite et al., 1992; Yamakita et al., 1994). The identity of the other two polypeptides, p43 and p66, remain unknown at this time, although the presence of phosphoserine/threonine as well as phosphotyrosine residues suggests that these molecules are the substrates of at least two different protein kinases. In-gel kinase assays, western blotting, as well as biochemical fractionation of sea urchin lysates reveal that two cell cycle-associated protein kinases, p34\(^{cdc2}\) and a homolog of MAP kinase, the tyrosine kinases fyn and abl (Walker et al., 1996), and several other unidentified kinases are present and active within the cortex. Control experiments indicate that the association of these kinases with the cortex is not due to non-specific trapping of soluble molecules or of differential association of these kinases during the cell cycle. While the actual fractions of p34\(^{cdc2}\) and MAP kinase associated with the cortex does not change during the cycle, their respective activities peak concomitant with the onset of cleavage. The activation of cortical kinase activities may be dependent on microtubules, since nocodazole-treatment of zygotes results in a delay in cortical LC 20/p43 phosphorylation. These results point toward a mechanism whereby the mitotic apparatus directs the position of the cleavage furrow by the local delivery or activation of cortical kinases.

**Cell cycle-regulated kinase activities are associated with the cortical cytoskeleton**

Our results indicate that p34\(^{cdc2}\), a sea urchin homolog of the erk family of kinases, tyrosine kinases, and possibly other kinases are active and specifically associated with the actin-rich cortical cytoskeletons of dividing blastomeres. These results are not surprising since there are many examples of specific cytoskeletal associated kinases, such as FAK (Dash et al., 1995), pp60src (Weemink and Rijksen, 1995), and MLCK (De Lanerolle et al., 1981), which retain activity as part of an isolated CSK complex. While we have identified at least two cell cycle regulated serine/threonine kinases (a MAP kinase and p34\(^{cdc2}\)) that are associated with the cortex of the dividing cell it is likely, on the basis of in-gel kinase assays, that additional serine/threonine kinases are also associated with the CSK. By implication, myosin light chain kinase is also active within these cortices, since we have previously reported cortical contractility (Walker et al., 1994) and now demonstrate phosphorylation of myosin light chain in cortices derived from dividing cells. We have not, however, mapped the phosphorylation sites on LC 20, and therefore cannot specifically attribute the phosphorylation of cortical LC20 to MLCK. Additionally, our findings that multiple kinases can be identified in an in-gel kinase assay (including kinases of 84, 44 and 42 kDa) are consistent with the presence of several as yet unidentified kinases in the cortical CSK. The fact that neither staurosporine nor H-7, when applied to whole eggs immediately prior to cell division or to cortices isolated from dividing cells (Fig. 7), have any effect on the phosphorylation of the cortical phosphoproteins or cytokinesis suggests that protein kinase C is not active or essential in the cortical CSK of dividing cells. It will be of great interest to determine whether the kinases active within the cortical cytoskeleton act to mediate the anaphase-telophase transition.

How the kinases are associated with the cortex is not clear. MAP kinases have been reported to associate with the mitotic apparatus (Verlhac et al., 1993), and as well as with microtubules within hippocampal neurons (Morishima-kawashima and Kosik, 1996). Cyclin B specifically associates with MAP 4, thus providing a docking site for p34\(^{cdc2}\) with the mitotic apparatus (Ookata et al., 1995), and myosin light chain kinase has been shown to redistribute from actin-containing stress fibers in interphase cells (De Lanerolle et al., 1981) to kinetochore spindle fibers during mitosis (Guerrero et al., 1981). Our results suggest that it is unlikely that intact microtubules are involved in the quantitative association of kinases with the cortical CSK since we found disruption of microtubules with nocodazole delayed peak cortex-associated kinase activity but did not inhibit the activity of cortical kinases. It is entirely possible that the cortex-associated kinases are part of large complexes that includes their specific substrates since the p66,
p43 and LC20 phosphoproteins are phosphorylated in vitro in these detergent models of the cell cortex.

Control experiments indicate that the presence of these cortical kinases is not due to non-specific trapping (Fig. 8, Table 1). We found that molecules such as tubulin and nuclear lamina are not retained within the cortex when both molecules are in a soluble, non-filamentous form. Additionally, the abundant cytoplasmic protein kinase calcium-calmodulin dependent kinase II, cannot be detected associating with the cortex. Additional evidence against non-specific trapping comes from the in vivo phosphorylation of a 36 kDa cortical protein that is not phosphorylated under in vitro labeling conditions (Figs 1 and 2). These results indicate that the kinase(s) responsible for phosphorylating p36 is soluble and not retained during CSK preparation. Thus, those kinases identified by in-gel kinase assays, western blotting and biochemical fractionation are, in all likelihood, specifically associated with the cortical cytoskeleton.

We have also identified tyrosine kinase activity associated with the cortex, as detected by phosphoamino acid analysis of p66 (Fig. 5). We have recently shown that an abl-related tyrosine kinase, termed e-abl (Moore and Kinsey, 1994), is active and associated with the cortical CSK from fertilized eggs (Walker et al., 1996). Immunoblot analysis of the isolated cortical cytoskeletons reveals that in addition to e-abl, the src homolog fyn is also present. However, it remains to be seen whether these are, in fact, the kinases that phosphorylate p66 during cytokinesis. Src is known to be associated with the membrane cytoskeleton and is active at the G2/M transition (Chackalaparambil and Shalloway, 1988). Additionally, antibody injection experiments suggest that src family members (in particular, fyn) are required for proper cell

**Fig. 12.** In vitro and in vivo phosphorylation of cortical CSK proteins in nocodazole-treated embryos. (A-D) In vivo (A,B) and in vitro (C,D) phosphorylation reactions were performed on control (solid lines) or nocodazole-treated (hatched lines) embryos, and the levels of p43 (A,C) and LC20 (B,D) phosphorylation were assayed. (E) p43 phosphorylated in vivo or in vitro was assayed at the time of normal cleavage (90 minutes post-fertilization) in control- or nocodazole-treated embryos. n=3.
division (Roche et al., 1995). Indeed, cytokinesis defects have been observed in mice deficient for the fyn gene (Yasunaga et al., 1996). Alternatively, tyrosine phosphorylation of p66 and p43 may be mediated by dual specificity kinases such as the MAP kinase kinase (MEK), which play a role in regulating cell cycle-related events (reviewed by Ahn et al., 1992). Understanding the putative relationship between tyrosine- or dual specificity kinases and p66 may lend considerable insights into the role of tyrosine kinases in cortical remodeling during mitosis, as well as their role in regulating the actin cytoskeleton in general.

The identities of the other CSK phosphoproteins (p43 and p66) are not known at this time. Recently, Bachman and McClay (1995) have shown that the sea urchin egg possesses a 75 kDa member of the ERM family of actin-binding and tyrosine-phosphorylated proteins. Moesin redistributes to the cell cortex upon fertilization, where its localization is dependent on actin filaments (Bachman and McClay, 1995). Preliminary immunoblot analysis indicates that moesin is present in the isolated cortical CSK; and while immunoprecipitation analysis indicates that sea urchin moesin is phosphorylated following fertilization, we find that moesin and p66 are immunologically distinct proteins (data not shown). The identity of the 36 and 43 kDa cortical CSK phosphoproteins are also unknown at this time. It is unlikely that the p43 is a MAP kinase, since phosphoamino acid analysis reveals only a limited amount of phosphotyrosine in p43, and two-dimensional gel analysis indicates that p43 has a very basic isoelectric point.

**Regulation of cortical cytoskeletal kinase activity by the cell cycle and the mitotic apparatus**

Our finding that the kinase activities associated with the cortical cytoskeleton peak at cell division suggests that these kinases may play a key role in the regulation of cytokinesis. Both p34cdc2 and MAPK are well documented as regulators of cell growth and division. There is an extensive literature concerning p34cdc2 and its role in regulating the G2/M transition (Nurse, 1990). In terms of its role in regulating cytokinesis, it has been proposed that p34cdc2 activity acts as a timer for cytokinesis by negatively regulating cortical contractility until anaphase, when pp34cdc2 activity falls off (Satterwhite and Pollard, 1992). This regulation is thought to be accomplished through the phosphorylation of myosin regulatory light chain (Satterwhite et al., 1992; Yamakita et al., 1994). However, aside from the regulatory light chain, caldesmon (Yamashiro et al., 1991), and spectrin (Fowler and Adam, 1992), few other substrates of p34cdc2 have been identified that associate with the actin cytoskeleton. p66 represents a potential novel substrate for p34cdc2, and further characterization will reveal how phosphorylation modulates the activities of this polypeptide and its association with the cortical cytoskeleton.

Our results from in vitro labeling studies have identified a histone H1 kinase activity that is highest in cortices isolated from cleaving blastomeres (Fig. 6C). However, H1 kinase activity has been widely shown to undergo an abrupt decline during anaphase, and it has been proposed that this loss of p34cdc2 activity signals the onset of contractile ring formation and contraction (Satterwhite and Pollard, 1992). Our results suggest that the H1 kinase activity associated with the actin cytoskeleton may be either mediated by a second, possibly novel mitotic kinase or, alternatively, the cortex-associated H1 kinase activity may represent a sequestered pool of active p34cdc2, whose activity lingers on after the cytoplasmic activity has declined due to cyclin degradation. If this is indeed the case, determining the spatial distribution of p34cdc2 and cyclin within the cortex relative to the position of the furrow may yield important insights into the mechanisms by which the position of the furrow is determined.

MAP kinases have been shown to play roles in the spindle assembly checkpoint pathway during mitosis (Minshull et al., 1994), microtubule dynamics (Gotoh et al., 1991b; Karsenti et al., 1984), and are thought to associate with microtubule organizing centers at mitosis (Verlhac et al., 1993). *Xenopus* MAP kinase has been shown to be phosphorylated during meiotic maturation and the embryonic cell cycle (Ferrell et al., 1991), implying that its activity may be modulated directly or indirectly by p34cdc2 (Gotoh et al., 1991a). Similarly, Pelech et al. (1988) reported that sea urchin zygote whole cell MAP kinase activity cycles with histone H1 activity during the cell cycle. Our findings that cortex-associated MAP kinase activity cycles through the embryonic cell cycle, and peaks at cell division confirm and extend the results of Pelech et al. (1988, 1990).

One of the more intriguing findings of our studies is that nocodazole disruption of microtubules, which interferes with the stimulation of cleavage furrow formation by disrupting the mitotic asters, delays the peak of p34cdc2 and other cortex-associated kinase activities (Figs 11 and 12). Both in vivo phosphorylation of specific cortical phosphoproteins and in vitro kinase activity of the cortex-associated kinases are significantly altered by the disruption of microtubules (Fig. 12). The study of surface contraction waves (SCW) in *Xenopus* eggs, which occur in a microtubule-independent manner (Hara et al., 1980), have called into question whether microtubules influence cyclic MPF activity. More recent evidence suggests that the apparent microtubule independence of MPF in early embryos is due to a low ratio of chromatin:cytoplasm, which inhibits the normal MAP kinase-mediated spindle checkpoint pathway (Minshull et al., 1994). Our results resemble the delay in peak H1 kinase activity seen in nocodazole-treated *Xenopus* cycling extracts (Minshull et al., 1994), more than the observations of SCWs that have been shown to be regulated by pathways distinct than those mediating cleavage furrow formation (Asada-Kubota and Kubota, 1991).

Our results suggest that p34cdc2 regulates the activity of the cortex-associated kinases that phosphorylate LC20 and p43, or that the regulatory pathways that control p34cdc2 activity also modulate these cortical kinases. However, since disruption of microtubules did not prevent but only delayed the activation of the cortical kinases coincident with the peak in p34cdc2 activity, it is possible that the kinases are resident in the cortical CSK rather than being transported along microtubules to the cortex. Rappaport and Rappaport (1993) have provided evidence that the cortex is responsive to the signal from the mitotic apparatus over an extended, but finite, part of the cell cycle. If p34cdc2 acts to entrain cortical kinase activities (required as a part of the signaling cascade for cytokinesis) during this period of responsiveness, then a delay in p34cdc2 activity beyond this window would not signal cleavage furrow formation, in spite of a subsequent elevation of activity. Our results are therefore consistent with a model for cytokinesis involving the astral microtubule-dependent activation of protein kinases, including...
some associated with the cortical actin cytoskeleton, which play a role in the signaling cascade for cytokinesis.

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