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Cell cycle-dependent phosphorylation of the 77 kDa echinoderm microtubule-associated protein (EMAP) in vivo and association with the p34^{cdc2} kinase

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SUMMARY

The most abundant microtubule-associated protein in sea urchin eggs and embryos is the 77 kDa echinoderm microtubule-associated protein (EMAP). EMAP localizes to the mitotic spindle as well as the interphase microtubule array and is a likely target for a cell cycle-activated kinase. To determine if EMAP is phosphorylated in vivo, sea urchin eggs and embryos were metabolically labeled with $^{32}PO_4$ and a monospecific antiserum was used to immunoprecipitate EMAP from ^{32}P-labeled eggs and embryos. In this study, we demonstrate that the 77 kDa EMAP is phosphorylated in vivo by two distinct mechanisms. In the unfertilized egg, EMAP is constitutively phosphorylated on at least five serine residues. During the first cleavage division following fertilization, EMAP is phosphorylated with a cell cycle-dependent time course. As the embryo enters mitosis, EMAP phosphorylation increases, and as the embryo exits mitosis, phosphorylation decreases. During mitosis, EMAP is phosphorylated on 10 serine residues and two-dimensional phosphopeptide mapping reveals a mitosis-specific site of phosphorylation. At all stages of the cell cycle, a 33 kDa polypeptide copurifies with the 77 kDa EMAP, regardless of phosphorylation state. Antibodies against the cdc2 kinase were used to demonstrate that the 33 kDa polypeptide is the p34^{cdc2} kinase. The p34^{cdc2} kinase copurifies with the mitotic apparatus and immunostaining indicates that the p34^{cdc2} kinase is concentrated at the spindle poles. Models for the interaction of the p34^{cdc2} kinase and the 77 kDa EMAP are presented.

Key words: Cell cycle, p34^{cdc2} kinase, MPF, Cytoskeleton, Mitosis, Cell division

INTRODUCTION

Microtubules are essential cytoskeletal elements of both interphase and mitotic cells. The transition from a relatively stable interphase microtubule array to the highly dynamic mitotic apparatus requires a dramatic reorganization of the microtubule network and a concomitant increase in microtubule dynamics (reviewed by Inoue and Salmon, 1995). It is likely that the increase in microtubule dynamics results from the modulation of specific parameters of dynamic instability, a non-equilibrium process whereby microtubules exist in growing and shrinking states (Mitchison and Kirschner, 1984).

Interphase to metaphase-like changes in microtubule dynamics can be demonstrated in cell extracts in vitro by the activation of the catalytic subunit of M-phase promoting factor (MPF), the p34^{cdc2} protein kinase (Verde et al., 1990; Belmont et al., 1990). MPF, initially purified from *Xenopus* eggs, is a complex of two proteins, p34 and p45 (Lohka et al., 1988). The catalytic p34 subunit is homologous to the protein product of the key yeast cell cycle gene, CDC2 (Lohka, 1989; Labbe et al., 1989). The regulatory p45 subunit is the homolog of the sea urchin cyclin protein (Evans et al., 1983; Meijer et al., 1989). The p34^{cdc2}-cyclin B complex is a key cell cycle regulator in all eukaryotic organisms examined.

Both catalytic and regulatory subunits of MPF have been identified in the mitotic and meiotic spindles of a variety of cell types (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Pines and Hunter, 1991; Tombes et al., 1991; Ookata et al., 1992; Buendia et al., 1992; Maldonado-Codina and Glover, 1992). More evidence for the association of p34^{cdc2}-cyclin B with microtubules comes from the detection of the regulatory cyclin subunit (Ookata et al., 1995) and the catalytic p34^{cdc2} kinase (this report) with purified microtubules. Further, these microtubule preparations contain high histone H1 kinase activity, indicating that active MPF may associate with microtubules in vivo. In primate tissue culture cells, MPF associates with microtubules through a direct interaction of the cyclin subunit with MAP4, a major non-neuronal MAP (Ookata et al., 1995). The indirect immunofluorescence localization of both MPF subunits to the spindle, and to centrosomes in a number of different organisms, as well as direct biochemical association with microtubules is strong evidence for MPF involvement in microtubule reorganization and spindle function. Potential targets for the p34^{cdc2} kinase include ATP-dependent microtubule-severing proteins (Vale, 1991), proteins that increase microtubule dynamics (Belmont and Mitchison, 1996; Walczak et al., 1996), and microtubule-associated proteins (MAPs) that suppress microtubule dynamics (reviewed by Cassimeris, 1993).

Among proliferating cells, the phosphorylation of MAPs appears to be tightly coupled to the mitotic cycles. The ubiqui-

tous MAP-4 (Vandre et al., 1991; Tombes et al., 1991) and the *Xenopus* XMAP (Gard and Kirschner, 1987), *Xenopus* p220/p230 MAP (Shiina et al., 1992; Andersen et al., 1994), *Xenopus* MAP4-like protein (Faruki and Karsenti, 1991), as well as mammalian E-MAP-115 (Masson and Kreis, 1995), all undergo cyclic phosphorylation and dephosphorylation as these cells enter and exit mitosis. In mammalian cells, MAP phosphorylation during mitosis correlates to a dramatic decrease in the microtubule polymer level (Zhai and Borisy, 1994). Since MAPs are known to stabilize microtubules against disassembly, it is suggested that the introduction of negative charge by phosphorylation reduces the affinity of MAPs for microtubules and thereby increases microtubule instability (reviewed by Maccioni and Cambiazo, 1995). Mitotic hyper-phosphorylation of E-MAP-115, a novel epithelial MAP, diminishes its ability to bind microtubules (Masson and Kreis, 1995), while in vitro phosphorylation of MAP4 by the cdc2 kinase does not alter its binding to microtubules (Ookata et al., 1995). These results indicate that cell cycle-dependent phosphorylation of various MAPs may act through different mechanisms to affect microtubule dynamics, mitotic apparatus assembly and function.

To explore further the mechanisms for regulating microtubule function by phosphorylation, we examined the phosphorylation of a MAP in vivo during the first embryonic cleavage division of the sea urchin, *Strongylocentrotus purpuratus.* Sea urchins provide large quantities of synchronously dividing embryos for the biochemical characterization of microtubule proteins and cell cycle regulatory components. The major sea urchin MAP was identified in isolated mitotic apparatuses as a polypeptide capable of binding to microtubules during several cycles of assembly and disassembly (Keller and Rebhun, 1982). Unfertilized eggs contain a large pool of this protein that can be isolated by cycles of pH- and temperature-dependent assembly (Suprenant and Marsh, 1987) or by taxol-induced assembly (Vallee and Bloom, 1983). Because the protein is abundant in starfish, sand dollars and sea urchins, we refer to it as the echinoderm microtubule-associated protein (EMAP) (Suprenant et al., 1993). The primary amino acid sequence of EMAP is unique among MAPs and the microtubule binding domain has not been identified (Li and Suprenant, 1994). EMAP contains several divergent WD-40 repeats that may be involved in protein-protein interactions, as well as a number of phosphorylation consensus sites for protein kinases, including the $p34^{cdc2}$ protein kinase (Li and Suprenant, 1994). Further, EMAP is localized to microtubules of the mitotic apparatus (Suprenant et al., 1993; Vallee and Bloom, 1983; Keller and Rebhun, 1982). Here we report the in vivo phosphorylation of EMAP from metabolically labeled embryos with a time course of phosphorylation characteristic of a cell cycle kinase. In addition, we characterize the phosphorylation sites by two-dimensional phosphoamino acid analysis and peptide mapping. Finally, evidence is presented that indicates that the $p34^{cdc2}$ kinase may interact with the 77 kDa EMAP in vivo. The implications for the regulation of mitosis, as a result of the pattern of EMAP phosphorylation, are discussed.

MATERIALS AND METHODS

Antibodies

For immunoblotting, the anti-EMAP antiserum (A58) was used at a final concentration of 1:1,000 and has been previously described (Suprenant et al., 1993). Anti-PSTAIRE and anti-cdc2 antibodies were obtained from Oncogene Science (Uniondale, NY) and were used at a final concentration of 1:2,000. Secondary antibodies, alkaline-phosphatase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG, were obtained from Zymed Laboratories, Inc. (San Francisco, CA) and HyClone Laboratories, Inc. (Logan, Utah), respectively, and were used at a final concentration of 1:2,000.

Sea urchin microtubule protein (MTP)

Strongylocentrotus purpuratus were obtained from Marinus, Inc. (Long Beach, CA) and were maintained in environmentally controlled artificial salt water aquaria at 12°C. Microtubule protein (MTP) was isolated from unfertilized eggs by three cycles of pH- and temperature-dependent assembly and disassembly (Suprenant and Marsh, 1987).

Mitotic apparatus isolation

Washed and dejellied sea urchin eggs were suspended to 10% (v/v) in 0.45 μm-filtered Instant Ocean (Aquarium Systems, Mentor, Ohio) containing 1 mM 3-amino-1,2,4-triazole (ATA) (Showman and Foerder, 1979) and fertilized with a dilute sperm suspension. After settling to the bottom of a large beaker, fertilized eggs were resuspended in Ca^{2+}-free sea water containing 1% (w/v) ATA and allowed to develop at 16°C with gentle stirring. Mitotic apparatuses were isolated in the presence of Triton-X-100 as described by Silver et al. (1980).

Immunoprecipitation assay

EMAP was immunoprecipitated as follows: 50 μl MTP (~3 mg/ml) in PME (100 mM Pipes, pH 6.8, 1 mM EGTA, 1 M $MgSO_4$, 0.2 mM PMSF, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A) or 50 μl embryo extract in homogenization buffer (100 mM Pipes, pH 7.3, 4 mM EGTA, 1 mM $MgSO_4$, 0.2 mM PMSF, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A) were mixed on a rotary mixer with 10 μl of anti-EMAP antiserum (A58) and 200 μl of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.8, 1% (v/v) NP-40, 250 mM NaCl, 100 mM Na_4PO_7-$10H_2O$, 5 mM NaF, 5 mM EDTA, 0.1 mM PMSF, and 10 μg/ml leupeptin) for 2 hours at 5°C. For the immunoprecipitation of ^{32}P-labeled EMAP, 1 mM ATP with 20 μM $ZnCl_2$ and 0.1 μM okadaic acid as phosphatase inhibitors, were added to the immunoprecipitation buffer. Immune complexes were collected on Protein A-Sepharose beads, washed 4 times with 200 μl of immunoprecipitation buffer and once with 200 μl of 20 mM Tris, pH 7.8, with 20 mM NaF. The immune complexes were solubilized with 50 μl 5× Laemmli sample buffer, boiled for 2 minutes, and analyzed by SDS-PAGE and autoradiography.

Histone H1 kinase assay

The assay was carried out in a total volume of 50 μl containing 40 mM Hepes, pH 7.3, 10 mM EGTA, 20 mM $MgCl_2$, 5 μCi [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont NEN, Boston, MA), 0.26 μM ATP, 10 μM protein kinase inhibitor (PKI), 1 mg/ml histone H1, and 30 μg third-cycle microtubule protein for a total of 10 minutes at 20°C (Turner et al., 1995). The reaction was terminated by adding 2 volumes of 2× Laemmli sample buffer and boiling for 2 minutes. Phosphorylation was assayed by SDS-PAGE followed by autoradiography.

Metabolic labeling and in vivo phosphorylation assay

Sea urchin eggs were collected, washed three times in filtered Instant Ocean, dejellied in Mg^{2+}-free sea water (500 mM NaCl, 27 mM KCl, 2 mM EDTA, pH 7.8), and resuspended as a 5% (v/v) solution in filtered Instant Ocean. Unfertilized eggs were preloaded with [^{32}P] prior to fertilization (Ballinger and Hunt, 1981) by a 4 hour incubation in 2.5 mCi [^{32}P]orthophosphate/ml egg suspension at 15°C with constant stirring. For a typical cell cycle time course, 0.5 ml of eggs were resuspended in 10 ml of filtered-Instant Ocean containing 25 mCi [^{32}P]orthophosphate

(9,120 Ci/mmol; Dupont NEN, Boston, MA). The labeled eggs were washed extensively (6-7) times to remove unincorporated [^{32}P]orthophosphate. The washed eggs (0.5 ml) were resuspended in 10 ml filtered Instant Ocean containing 5 mM para-aminobenzoic acid (PABA), a peroxidase inhibitor used to soften the fertilization envelopes. Sperm were diluted 1:100 in filtered Instant Ocean-5 mM PABA, and 20 μl of this dilute sperm suspension was added to 0.5 ml of labeled eggs in 10 ml of filtered-Instant Ocean-5 mM PABA. Fertilization was monitored by the elevation of the fertilization envelope. One ml of embryo culture was briefly centrifuged (12,000 ***g***) and frozen in liquid nitrogen to collect embryos at each time point.

Egg or embryo extracts were prepared by homogenizing 50 μl packed eggs in 50 μl of homogenization buffer containing 1 mM ATP, on ice with a 1 cm^3 syringe and a 26 G needle. Cold ATP was added at the time of cell lysis to compete for [^{32}P]ATP during sample preparation and immunoprecipitation (Hollenbeck, 1993). In addition, the post-lysis activity of phosphatases was curtailed by the inclusion of NaF, $ZnCl_2$ and okadaic acid (see above), as phosphatase inhibitors. The homogenate was centrifuged in a microcentrifuge for 10 minutes at 12,000 ***g*** at 5°C. The supernatant was removed and centrifuged again, and the resulting extract was used for immunoprecipitation. The cell cycle time course of in vivo phosphorylation was carried out on four different batches of embryos.

For some experiments, sea urchin embryos were labeled with [^{35}S]methionine/cysteine as described by Evans et al. (1983). EMAP was immunoprecipitated from embryo extracts and assayed by SDS-PAGE and autoradiography. No labeled ^{35}S-labeled EMAP was detected under these assay conditions.

Measurement of the ATP pools

ATP pools were measured pre- and post-fertilization in order to determine the specific activity of [^{32}P]ATP in unfertilized eggs and post-fertilization embryos. Nucleotides were extracted with 0.5 M perchloric acid and separated by HPLC on a Phenomenex Partisil 10 SAX 250 mm × 4.6 mm column as previously described (Mejillano et al., 1990). On average (n=4), the specific activity of ATP was 24,098±8,537 cpm ^{32}P/μM ATP and 23,547±10,516 cpm ^{32}P/μM ATP, in unfertilized egg extracts and metaphase embryo extracts, respectively. The large standard errors are due to differences in the uptake of [^{32}P]orthophosphate among batches of eggs.

Two-dimensional phosphoamino acid analysis and phosphopeptide mapping

^{32}P-labeled EMAP was extracted from Immobilon-P nylon membranes for phosphoaminoacid analysis as described by Kamps and Sefton (1989). For phosphopeptide mapping, ^{32}P-labeled EMAP was separated by SDS-PAGE and transferred to nitrocellulose. The ^{32}P-EMAP-containing band was processed for tryptic digestion and phosphopeptide separation exactly as described (van der Geer et al., 1993). The phosphoamino acid analysis and phosphopeptide mapping were done on three different batches of embryos.

Immunofluorescence microscopy

Cleavage-stage sea urchin embryos were prepared for immunofluorescence as previously described (Suprenant et al., 1993). For immunofluorescence, the anti-cdc2 kinase antiserum was used at a final concentration of 1:50 and was visualized with a Texas red labeled goat anti-rabbit IgG secondary antibody, also diluted 1:50 in 15 mM Tris-HCl, pH 7.4, 150 mM NaCl containing 0.05% (v/v) Tween-20. Images were collected with a Bio-Rad MRC 1000 laser scanning microscope based on a Nikon inverted microscope.

Electrophoresis, immunoblotting, autoradiography, and protein determination

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) with 8 or 13% acrylamide gels stained with Coomassie blue.

For immunoblotting, proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979), probed with anti-EMAP, anti-cdc2 and anti-PSTAIRE antibodies, and developed with alkaline-phosphatase-conjugated secondary antibodies as previously described (Suprenant et al., 1993) or visualized with the enhanced chemiluminescence (ECL) system (Amersham Life Sciences, Arlington Heights, IL) for HRP-conjugates.

For autoradiography, dried gels, TLC plates, and western blots were exposed to Kodak X-OMAT film with intensifying screens at –80°C.

Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit as described by the manufacturer (Pierce, Rockford, IL) with bovine serum albumin as a standard.

RESULTS

The 77 kDa EMAP is phosphorylated in unfertilized eggs in vivo

To determine whether EMAP is phosphorylated in vivo, eggs were metabolically labeled with [^{32}P]orthophosphate. Because of the high levels of phosphate in the egg, and the slow uptake of phosphate from sea water (Whitely and Chambers, 1966), it was necessary to pre-incubate unfertilized eggs in 2.5 mCi $^{32}PO_4$/ml artificial sea water for 4 hours. Subsequently, the eggs were washed free of unincorporated label and fertilized. This labeling protocol was designed to yield a constant specific activity of phosphorylated precursors in the unfertilized and fertilized egg. Nucleotides were extracted and separated by HPLC for quantitation. During the first cleavage cycle the specific activity of the ATP pool remained constant at 24,000 cpm/μM ATP (see Materials and Methods).

Under these labeling conditions the ^{32}P-label was taken up slowly into unfertilized *Strongylocentrotus purpuratus* eggs and incorporated into several proteins. After washing out of the ^{32}P-labeled sea water, egg extracts were prepared and the 77 kDa EMAP was immunoprecipitated with the anti-EMAP antiserum (A58). Buffer conditions were chosen to block the post-lysis activity of phosphatases as well as promiscuous phosphorylation during the immunoprecipitation (see Materials and Methods). Fig. 1 illustrates that EMAP is constitutively phosphorylated in the unfertilized egg during the 4 hour incubation in $^{32}PO_4$. With the exception of the high molecular mass phosphoprotein at the top of the gel, no other phosphoproteins co-immunoprecipitate with EMAP in unfertilized egg extracts. The identity of the high molecular mass phosphoprotein has not been pursued because a similar molecular mass phosphoprotein appears in control immunoprecipitations as well (Fig. 1, lane 3). The molar stoichiometry of phosphate incorporation was determined to be 5.2±3.2 mol phosphate/mol EMAP (n=3) in the unfertilized egg.

Cell cycle-dependent phosphorylation of EMAP

To examine EMAP phosphorylation during the first embryonic cell cycle, unfertilized eggs were preloaded with [^{32}P]orthophosphate, washed free of unincorporated label, and fertilized. EMAP immunoprecipitates were prepared at 30 minute intervals during the first cell cycle and examined by autoradiography and immunoblotting (Fig. 2). Under these labeling conditions, EMAP is phosphorylated in the unfertilized egg and remains phosphorylated throughout the cell cycle. The level of phosphorylation increases during the cell cycle,

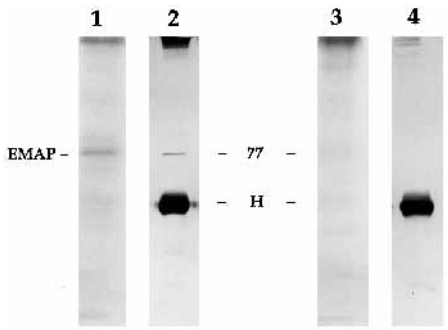

Fig. 1. EMAP is phosphorylated in unfertilized eggs in vivo. EMAP was immunoprecipitated from unfertilized eggs preloaded with $^{32}PO_4$ and processed for autoradiography (lane 1) and immunoblotting with the A58 anti-EMAP antiserum (lane 2). The 77 kDa EMAP is the only phosphoprotein specifically immunoprecipitated from ^{32}P-labeled egg extracts. The high molecular mass phosphoprotein at the top of gel is found in both immune and preimmune control samples. The autoradiograph of the preimmune control immunoprecipitation is shown in lane 3 and the corresponding control immunoblot with the anti-EMAP antiserum in lane 4. The immune and preimmune samples were prepared identically for autoradiography and immunoblotting. The positions of the immunoglobulin heavy chain (H) and the 77 kDa EMAP are noted.

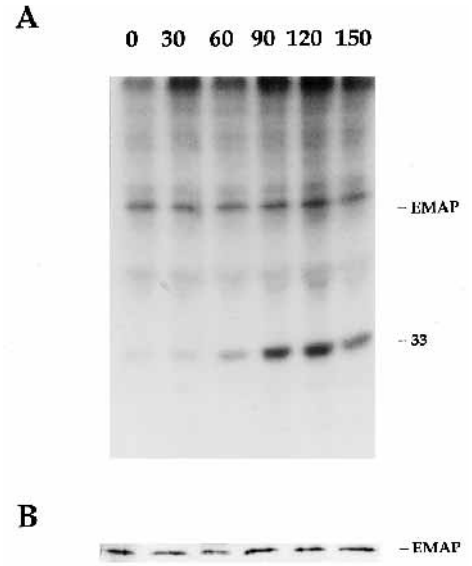

Fig. 2. Time course of EMAP phosphorylation in eggs preloaded with $^{32}PO_4$. (A) EMAP was immunoprecipitated from eggs and embryos at 30 minute intervals during the first cell cycle. The autoradiograph indicates that EMAP remains phosphorylated throughout the cell cycle. The absolute level of EMAP phosphorylation increases and decreases in a cell cycle-dependent manner such that EMAP is maximally phosphorylated at first mitotic metaphase (120 minutes post-fertilization, under these labeling conditions). In addition to phosphorylated EMAP, a high molecular mass phosphoprotein and a 33 kDa phosphoprotein copurify with EMAP during the first cell cycle. The identity of the high molecular mass protein has not been followed up because a high molecular mass phosphoprotein was detected in the preimmune controls (see Fig. 1). In contrast, the 33 kDa phosphoprotein is not immunoprecipitated with the preimmune serum and coprecipitates with EMAP in a cell cycle-dependent manner. The 33 kDa phosphoprotein is detected at 60 minutes and is maximally phosphorylated at mitotic metaphase (120 minutes). (B) The corresponding immunoblot with the anti-EMAP antiserum indicates that approximately equal amounts of EMAP are immunoprecipitated during this time course and that the increase in EMAP phosphorylation was not due to an increase in EMAP in the immunoprecipitates.

reaching a maximum at mitotic metaphase (120 minutes post-fertilization) and decreases during first cleavage (150 minutes post-fertilization). Progression through the cell cycle is delayed in eggs preloaded with 2.5 mCi $^{32}PO_4$/ml sea water, when compared to control batches of eggs fertilized in the absence of ^{32}P. In this particular batch of eggs, cleavage occurred at 150 minutes post-fertilization in the presence of 2.5 mCi $^{32}PO_4$/ml sea water and at 120 minutes in the unlabeled control batch of embryos.

The molar stoichiometry of phosphate incorporation in EMAP was compared in the unfertilized egg and at the first mitotic metaphase. On average, there is a twofold increase in the molar stoichiometry of phosphate incorporation, from $5.2^{\pm}3.2$ mol phosphate/mol EMAP (n=3) in the unfertilized egg to $9.5^{\pm}3.2$ mol phosphate/mol EMAP (n=3) in mitotic embryos. Although the level of phosphorylation increased at mitosis, the amount of EMAP detected in each immunoprecipitate remains approximately the same.

The cyclic nature of EMAP phosphorylation can be accounted for by the activity of a cell cycle kinase and phosphatase or the synthesis and degradation of EMAP during the first cell cycle. The absolute levels of EMAP remain relatively constant during the time course of this experiment, indicating that EMAP is not destroyed in bulk following each mitosis. Furthermore, there is little detectable EMAP synthesis during the first cleavage cycle (see Materials and Methods) Together these results indicate that EMAP is cyclically phosphorylated during the cell cycle and the increase and decrease in phosphorylation levels probably results from the coupled activity of a cell cycle-regulated protein kinase and phosphatase.

EMAP is phosphorylated on multiple serine residues during the cell cycle

The characteristics of EMAP phosphorylation in vivo were analyzed by two-dimensional phosphoamino acid analysis (Fig. 3). For this experiment, eggs were preloaded to a constant specific activity with 2.5 mCi [^{32}P]orthophosphate/ml sea water. EMAP immunoprecipitates were prepared from unfertilized eggs and from embryos at the first mitotic metaphase and acid hydrolysates were prepared and analyzed as described in Materials and Methods. Two-dimensional phosphoamino acid analysis reveals that EMAP is phosphorylated predominantly at serine residues in unfertilized eggs as well as in mitotic embryos (Fig. 3). There is no detectable phosphothreonine or phosphotyrosine at either of these developmental stages.

To determine the number of EMAP peptides containing phosphorylated serine residues and to observe changes in EMAP phosphorylation patterns during the cell cycle, we subjected the ^{32}P-labeled EMAP proteins to phosphopeptide mapping analysis. For this analysis, tryptic-digests of ^{32}P-labeled EMAP were examined by two-dimensional-high voltage thin layer electrophoresis/thin layer chromatography (Fig. 4). Only two prominent phosphopeptides are obtained from EMAP phosphorylated in unfertilized eggs in vivo. These

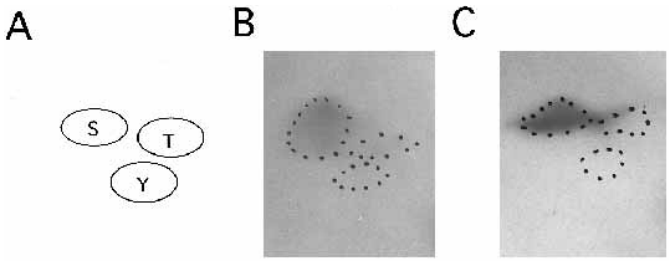

Fig. 3. EMAP is phosphorylated on serine residues in vivo. Two-dimensional phosphoamino acid analysis of EMAP immunoprecipitated from unfertilized eggs (B), and EMAP immunoprecipitated from mitotic extracts (C). The positions of phosphoserine, phosphothreonine and phosphotyrosine are noted in A, and by corresponding dotted lines in B and C.

two phosphopeptides, peptides 1 and 2, are the predominant phosphopeptides observed at each 30 minute interval throughout the first mitotic cell cycle. However, at the onset of nuclear envelope breakdown (90 minutes post-fertilization in this batch of labeled eggs), a third prominent phosphopeptide is acquired (peptide 3). Phosphopeptide 3 is detected during the formation of the mitotic apparatus (120 minutes post-fertilization), as well as the cleavage furrow (150 minutes). Thus, the phosphorylation of EMAP changes both quantitatively and qualitatively during the first mitotic cell cycle. These results indicate that EMAP is phosphorylated at different sites during the cell cycle.

Association of the cdc2 kinase with the 77 kDa EMAP

In the immunoprecipitates shown in Fig. 2, a 33 kDa phosphoprotein copurifies with the 77 kDa EMAP. Unlike the 77 kDa phosphoprotein, the 33 kDa protein is not dramatically phosphorylated until 60 minutes post-fertilization. The phosphorylation of the 33 kDa protein reaches a maximum at 90-120 minutes and then decreases dramatically following mitosis. The relative molecular mass of this polypeptide and its cyclic phosphorylation indicated that the 33 kDa polypeptide might be the $p34^{cdc2}$ kinase and that the $p34^{cdc2}$ kinase might associate with EMAP during the cell cycle.

To determine whether the $p34^{cdc2}$ kinase was capable of associating with sea urchin microtubules, histone H1 kinase activity was assayed in microtubules purified from unfertilized sea urchin eggs (Fig. 5). A high level of H1 kinase activity is found in third-cycle microtubule preparations indicating that the $p34^{cdc2}$ kinase may associate with microtubules. In microtubule preparations, the $p34^{cdc2}$ kinase migrates as a 33 kDa polypeptide that reacts with both anti-cdc2 and anti-PSTAIRE antibodies (Fig. 6). Immunoprecipitation was used to demonstrate that the $p34^{cdc2}$ kinase is associated with the 77 kDa EMAP. EMAP immunoprecipitates from unfertilized egg extracts and mitotic extracts are highly enriched with a 33 kDa polypeptide that reacts strongly and specifically with both anti-cdc2 and anti-PSTAIRE antibodies (Fig. 6). Control immunoprecipitates with pre-immune serum contain neither the 77 kDa EMAP nor the 33 kDa/$p34^{cdc2}$ kinase. Approximately the same amount of the 33 kDa/$p34^{cdc2}$ kinase appears to coprecipitate with EMAP at either stage of the cell cycle regardless of the phosphorylation state of the $p34^{cdc2}$ kinase. We were unable to detect EMAP in the reciprocal anti-cdc2 and anti-PSTAIRE immunoprecipitates.

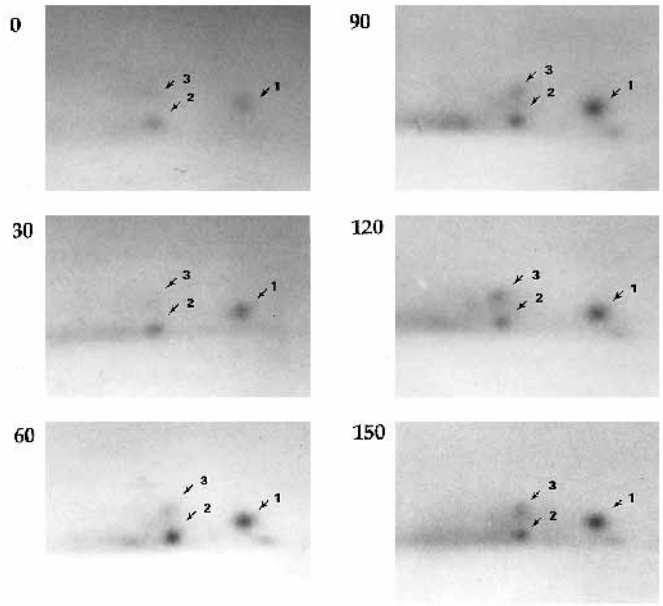

Fig. 4. EMAP is phosphorylated on different sites during the cell cycle. Autoradiograms of two-dimensional tryptic phosphopeptide maps of EMAP phosphorylated in vivo at 30 minute intervals during the cell cycle. Initially, only two phosphopeptides are detected in the unfertilized egg and throughout the early cell cycle. By 90 minutes post-fertilization, a third peptide, phosphopeptide 3 is detected and remains during mitotic metaphase (120 minutes post-fertilization) and cleavage (150 minutes post-fertilization).

This may be because the epitope recognized by the antibody is masked in the EMAP-$p34^{cdc2}$ kinase complexes.

The $p34^{cdc2}$ kinase is enriched in isolated mitotic apparatuses. Proteins in mitotic apparatus preparations were resolved by SDS-PAGE, transferred to nitrocellulose and probed with the anti-cdc2 kinase antiserum (Fig. 7). A prominant 33 kDa polypeptide was recognized by the anti-cdc2 antiserum in both the microtubule and mitotic apparatus preparations.

To ascertain the location of the cdc2 kinase within the mitotic apparatus, cleavage-stage embryos were immunostained with the anti-cdc2 antiserum. Surprisingly, the cdc2 kinase was localized to the spindle poles and not throughout the mitotic apparatus (Fig. 8). In contrast, previous immunolocalization studies have demonstrated that the 77 kDa EMAP is localized throughout the mitotic apparatus, in both the asters and central spindle (Vallee and Bloom, 1983; Suprenant et al., 1993). These results indicate that the cdc2 kinase does not colocalize strictly with the 77 kDa EMAP.

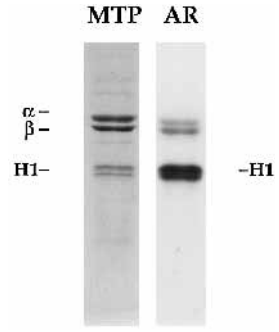

Fig. 5. H1 kinase activity copurifies with sea urchin egg microtubule protein. MTP: SDS-PAGE of third-cycle microtubule protein with histone H1 (H1) added as a substrate. The migration of α and β tubulin are noted to the left. AR: corresponding autoradiograph following an H1 kinase phosphorylation assay. The major phosphoprotein is the exogenously added histone H1.

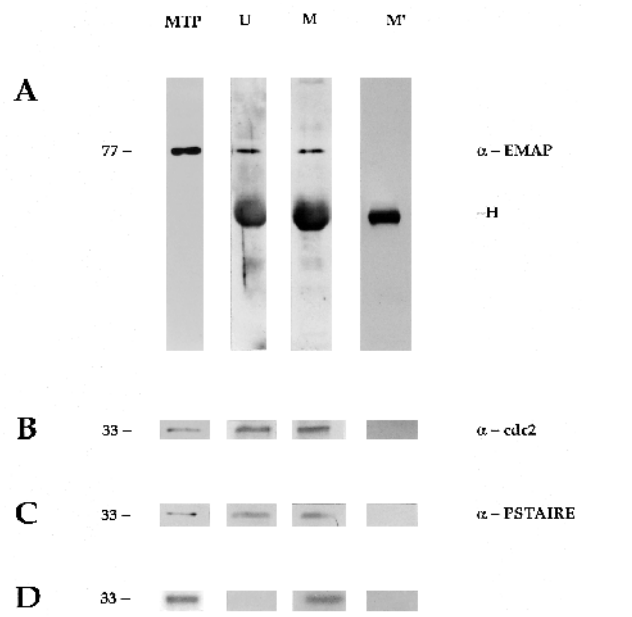

Fig. 6. Identification of a 33 kDa homolog of the p34^{cdc2} kinase in sea urchin MTP and EMAP immunoprecipitates. (A) Third cycle sea urchin microtubule protein (MTP), EMAP-immunoprecipitate from an unfertilized egg extract (U), EMAP-immunoprecipitate from a mitotic embryo extract (105 minutes post-fertilization) (M), and a pre-immune control immunoprecipitate from the same mitotic embryo extract (M′), were separated by SDS-PAGE and immunoblotted with the A58 anti-EMAP antiserum. EMAP is detected in MTP and anti-EMAP immunoprecipitates, but not in the preimmune control precipitates. The position of the immunoglobulin heavy chain is noted to the right. Corresponding immunoblots with the anti-cdc2 antibodies (B) and anti-PSTAIRE antibodies (C) are shown below. The conserved PSTAIRE sequence is found in all cdc 2/'cdc 2-like' proteins. The p34^{cdc2} kinase is highly enriched in the EMAP immunoprecipitates when compared to third-cycle microtubule protein. Note that the p34^{cdc2} kinase is not detected in the control immunoprecipitations. In D, an autoradiograph of the 33 kDa/p34^{cdc2} kinase region of the gel is shown. The 33 kDa/p34^{cdc2} kinase is phosphorylated only in mitotic extracts and microtubule preparations.

Two models are proposed for the interaction of EMAP and the cdc2 kinase within the mitotic apparatus (Fig. 9). In the first model, the p34^{cdc2} kinase does not bind directly to the 77 kDa EMAP, but rather to an adaptor protein that is localized to the mitotic poles. In the second model, the p34^{cdc2} kinase binds directly to a subset of EMAP, post-translationally modified and localized to the spindle poles. In this model, we have indicated that the modification could be a cell cycle-dependent phosphorylation event.

DISCUSSION

Numerous protein kinases and phosphatases play important regulatory roles during mitosis; however, the targets for these enzymes are only beginning to be identified and characterized (reviewed by Nigg, 1993). Identification of the physiological substrates for these kinases and how phosphorylation influences their behavior is essential to understanding how microtubules carry out their fundamental role during mitosis.

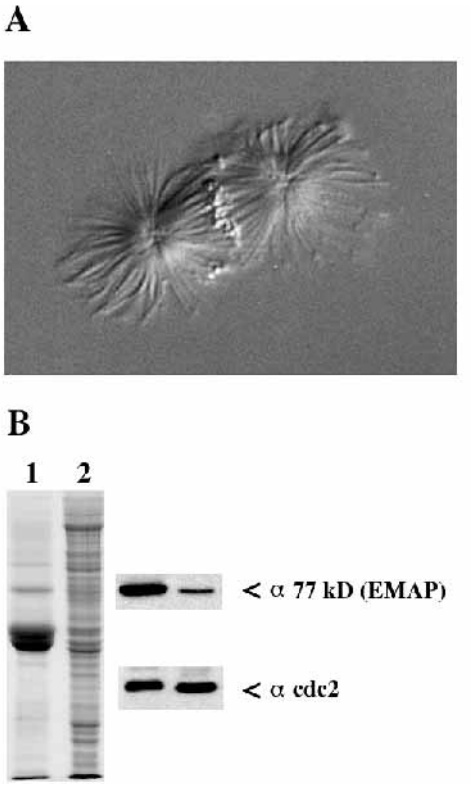

Fig. 7. The cdc2 kinase copurifies with the mitotic apparatus. A protein cross-reacting to antibodies against the p34^{cdc2} kinase is present in mitotic spindles isolated from sea urchin embryos. (A) Mitotic apparatuses were isolated in a microtubule-stabilizing buffer and extensively washed with a non-ionic detergent, Triton X-100. (B) SDS-PAGE and corresponding immunoblot of purified sea urchin microtubule protein (lane 1) and mitotic apparatuses (lane 2). Immunoblots with the affinity-purified anti-EMAP antibodies or the anti-cdc2 kinase antiserum are shown to the right.

Two phosphorylation events for EMAP

To understand how phosphorylation may regulate microtubule function during mitosis, we investigated whether the 77 kDa EMAP was phosphorylated in sea urchin embryos in vivo. We determined that EMAP was phosphorylated both in the unfertilized egg and during the first mitotic cell cycle. Both the extent and sites of EMAP phosphorylation change during the transition from interphase to metaphase.

In the unfertilized egg, EMAP is constitutively phosphorylated on 5 serine residues (on average). Although the unfertilized egg is considered to be metabolically inactive, protein kinases are active at this time. Considering that there are few detectable microtubules in the unfertilized egg (Bestor and Schatten, 1981), these phosphorylation events may favor microtubule disassembly. In addition, a constitutive level of phosphorylation may be necessary for the proper folding of the EMAP polypeptide. The serine/threonine kinase(s) responsible for EMAP phosphorylation in the unfertilized egg are unknown at this time.

The second mitotic phosphorylation event is detected quantitatively by a twofold increase in ^{32}P-incorporation, from 5 mol phosphate/mol EMAP in the unfertilized egg to 10 mol phosphate/mol EMAP at mitotic metaphase. In addition, a new tryptic phosphopeptide is detected as the embryo enters

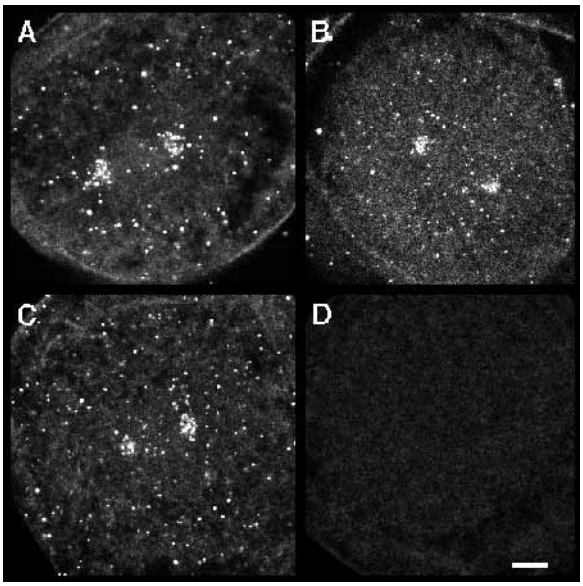

Fig. 8. Immunolocalization of the cdc2 kinase in mitotic sea urchin embryos. The location of the cdc2 kinase is revealed in three different mitotic embryos shown in A, B and C. In all cases, the cdc2 kinase is concentrated at the mitotic spindle poles. The staining pattern is punctate at the spindle poles and throughout the cytoplasm. (D) The low level of background staining with the Texas red-conjugated secondary antibody alone. Bar, 10 μm.

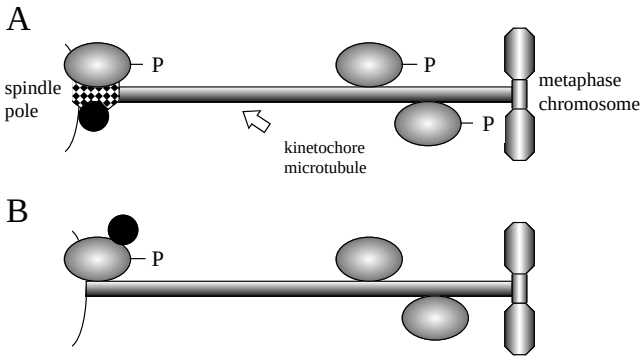

Fig. 9. Two models for the association of the cdc2 kinase with the mitotic apparatus. In model A, phosphorylated EMAP (shaded oval) binds to microtubules throughout the mitotic apparatus. The cdc2 kinase (black circle) binds to the spindle pole through an adapter protein (checked polygon) that binds to EMAP. In model B, the cdc2 kinase binds directly to a subset of phosphorylated EMAP localized at the spindle pole.

mitosis. The change in EMAP phosphorylation corresponds to the time of nuclear envelope breakdown, the formation of the mitotic apparatus, and an increase in microtubule dynamics.

The phosphorylation of EMAP during mitosis is consistent with EMAP being a target for the $p34^{cdc2}$ kinase or a kinase activated downstream of the $p34^{cdc2}$ kinase. Sequence analysis of a cDNA clone for EMAP reveals a number of phosphorylation consensus sites corresponding to several different serine/threonine kinases including two consensus sites for the $p34^{cdc2}$ kinase and one for the mitogen-activated protein (MAP) kinase, all within the first 50 amino acids of EMAP (Li and Suprenant, 1994). This proline-rich (20%) and arginine-rich (16%) domain is very basic with a calculated pI of 13 and has been postulated to be the microtubule-binding domain (Li and Suprenant, 1994). In future work, we intend to identify the kinases responsible for these phosphorylation events and determine the effects of phosphorylation on microtubule assembly dynamics.

EMAP-associated $p34^{cdc2}$ kinase

In this study, we demonstrate directly that the $p34^{cdc2}$ kinase is phosphorylated in vivo. When eggs were preloaded with $^{32}PO_4$, phosphorylated $p34^{cdc2}$ kinase is not detected in the unfertilized egg, but as the fertilized eggs passed through S-phase and entered M-phase, phosphates accumulated on the EMAP-associated $p34^{cdc2}$ kinase. The pattern of ^{32}P-incorporation is similar to the pattern of tyrosine phosphorylation detected on the $p34^{cdc2}$ kinase with anti-phosphotyrosine antibodies with the notable exception that tyrosine phosphorylation peaks during G_2 (Meijer et al., 1991; Edgecombe et al., 1991) and the ^{32}P-incorporation is maximal during M-phase (this report). It is likely that the ^{32}P-incorporation reflects the sum of the inhibitory tyrosine phosphorylation cycle superimposed upon the activating threonine phosphorylation cycle (reviewed by Coleman and Dunphy, 1994).

The $p34^{cdc2}$ kinase was identified as a phosphoprotein that co-immunoprecipitated with the 77 kDa EMAP during the embryonic cleavage cycles. In addition, both the kinase and the EMAP are present in purified microtubule preparations as well as the mitotic apparatus. Because the 77 kDa polypeptide is a microtubule-associated protein, it is tempting to speculate that the 77 kDa EMAP targets the kinase to the mitotic apparatus.

In cultured primate cells the $p34^{cdc2}$ kinase is targeted to microtubules through an interaction between cyclin B and the proline-rich region of MAP-4 (Ookata et al., 1995). In sea urchin embryos, it seems unlikely that cyclin B mediates the interaction between the $p34^{cdc2}$ kinase and the EMAP. There is little detectable cyclin B in the unfertilized sea urchin egg (Evans et al., 1983; Meijer et al., 1989), however, EMAP immunoprecipitates from unfertilized eggs and mitotic embryos contain the same amount of the $p34^{cdc2}$ kinase. If the associations were dependent upon cyclin B, the $p34^{cdc2}$ kinase association with EMAP should follow the cell cycle-dependent synthesis and degradation of cyclin (Evans et al., 1983). In addition, the $p34^{cdc2}$ kinase is fairly abundant in sea urchin eggs and embryos and is estimated to be present at a concentration of 2-5 μM (Meijer et al., 1989). This is an order of magnitude greater than needed to complex all the cyclin B (0.25 μM) at mitosis, implying that the $p34^{cdc2}$ kinase may interact with other as yet unidentified proteins (Meijer et al., 1989).

The molecular nature of the EMAP-cdc2 kinase interaction is unknown at this time. In the model presented in Fig. 9A, we hypothesize that EMAP and the $p34^{cdc2}$ kinase are attached to a hypothetical adapter protein that is localized at the spindle pole. EMAP binds directly to microtubules and is localized throughout the mitotic apparatus (Vallee and Bloom, 1983; Suprenant et al., 1993). In this model, the $p34^{cdc2}$ kinase localization to the spindle pole is dependent upon the association of a spindle pole-specific adapter protein with the 77 kDa EMAP. In contrast, the $p34^{cdc2}$ kinase may associate with EMAP directly (Fig. 9B). This model requires that the EMAP at the

pole is structurally or biochemically different from the EMAP localized throughout the spindle. Since it is not known what fraction of the EMAP population is phosphorylated at mitosis, we suggest that a post-translationally modified fraction of EMAP is localized to the spindle pole where it binds directly to the $p34^{cdc2}$ kinase.

Whether the kinase binds to EMAP directly or indirectly is not known, however, we speculate that EMAP targets the $p34^{cdc2}$ kinase to the mitotic apparatus where several events may happen. EMAP may target an active $p34^{cdc2}$ kinase to a potential substrate localized at the mitotic spindle poles such as γtubulin (Stearns et al., 1991), NuMA (Tousson et al., 1991) or pericentrin (Doxsey et al., 1994). In this regard, it is intriguing that the microtubule-severing protein katanin also localizes to the spindle poles (McNally et al., 1996). As previously suggested, activation of katanin by the $p34^{cdc2}$ kinase could be responsible for an increase in microtubule turnover at the G_2 to M transition (Vale, 1991; Karsenti, 1993). Once targeted to the spindle poles, the $p34^{cdc2}$ kinase may be inactivated. In mammalian cells, it has been demonstrated that microtubules are required for the inactivation of the cdc2 kinase during the metaphase to anaphase transition (Andreassen and Margolis, 1994). If a similar mechanism operates in sea urchins, EMAP may direct the $p34^{cdc2}$ kinase to the mitotic apparatus where it becomes inactivated by a mitotic apparatus-associated tyrosine phosphatase. Other phosphatases such as protein phosphatase 1 (Johnston et al., 1994) and protein phosphatase 2A (Songtag et al., 1995) have been localized to the mitotic apparatus, so it is not unreasonable to expect a mitotic apparatus-associated tyrosine kinase.

In summary, we have demonstrated that the 77 kDa EMAP is phosphorylated in vivo in a manner consistent with it being a substrate for the $p34^{cdc2}$ kinase or a kinase activated downstream such as the MAP kinase. In addition to being a potential substrate for these kinases, EMAP may act as an anchoring protein to spatially segregate the $p34^{cdc2}$ kinase to a region of the mitotic apparatus (Faux and Scott, 1996).

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