

# Cyclin B Interaction with Microtubule-associated Protein 4 (MAP4) Targets p34<sup>cdc2</sup> Kinase to Microtubules and Is a Potential Regulator of M-phase Microtubule Dynamics

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**Abstract.** We previously demonstrated (Ookata et al., 1992, 1993) that the p34<sup>cdc2</sup>/cyclin B complex associates with microtubules in the mitotic spindle and premeiotic aster in starfish oocytes, and that microtubule-associated proteins (MAPs) might be responsible for this interaction. In this study, we have investigated the mechanism by which p34<sup>cdc2</sup> kinase associates with the microtubule cytoskeleton in primate tissue culture cells whose major MAP is known to be MAP4. Double staining of primate cells with anti-cyclin B and anti-MAP4 antibodies demonstrated these two antigens were colocalized on microtubules and copartitioned following two treatments that altered MAP4 distribution. Detergent extraction before fixation removed cyclin B as well as MAP4 from the microtubules. Depolymerization of some of the cellular microtubules with nocodazole preferentially retained the microtubule localization of both cyclin B and MAP4. The association of p34<sup>cdc2</sup>/cyclin B kinase with microtubules was also shown biochemically to be mediated by MAP4. Cosedimentation of purified

p34<sup>cdc2</sup>/cyclin B with purified microtubule proteins containing MAP4, but not with MAP-free microtubules, as well as binding of MAP4 to GST-cyclin B fusion proteins, demonstrated an interaction between cyclin B and MAP4. Using recombinant MAP4 fragments, we demonstrated that the Pro-rich C-terminal region of MAP4 is sufficient to mediate the cyclin B-MAP4 interaction. Since p34<sup>cdc2</sup>/cyclin B physically associated with MAP4, we examined the ability of the kinase complex to phosphorylate MAP4. Incubation of a ternary complex of p34<sup>cdc2</sup>, cyclin B, and the COOH-terminal domain of MAP4, PA<sub>4</sub>, with ATP resulted in intracomplex phosphorylation of PA<sub>4</sub>. Finally, we tested the effects of MAP4 phosphorylation on microtubule dynamics. Phosphorylation of MAP4 by p34<sup>cdc2</sup> kinase did not prevent its binding to microtubules, but abolished its microtubule stabilizing activity. Thus, the cyclin B/MAP4 interaction we have described may be important in targeting the mitotic kinase to appropriate cytoskeletal substrates, for the regulation of spindle assembly and dynamics.

**P**ROGRESSION through M-phase of the cell cycle is controlled by M-phase promoting factor (MPF)<sup>1</sup>, which consists of a complex of p34<sup>cdc2</sup> and cyclin B (for

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1. *Abbreviations used in this paper:* CBB, Coomassie brilliant blue; MPF, M-phase-promoting factor; MAP, microtubule-associated protein.

reviews see Kishimoto, 1988; Hunt, 1989; Murray and Kirschner, 1989; Nurse, 1990; Maller, 1991). The complex displays histone H1 kinase activity specifically at the onset of mitosis. Active p34<sup>cdc2</sup> kinase is required to generate many M-phase-specific events, including chromosome condensation, nuclear envelope breakdown, and reorganization of the microtubule network. While the mechanism by which p34<sup>cdc2</sup> kinase is activated at mitosis has been extensively studied in recent years (see Maller, 1991), relatively little is known about the spatial targeting of p34<sup>cdc2</sup> kinase that gives rise to these M-phase-specific events.

The localization of p34<sup>cdc2</sup> kinase is known to be regulated before mitosis, and may be important for its action at the G2/M transition. For example, a portion of the p34<sup>cdc2</sup>/cyclin B complex has been reported to translocate

from the cytoplasm into the nucleus just before nuclear envelope breakdown (Pines and Hunter, 1991; Bailly et al., 1992; Gallant and Nigg, 1992; Ookata et al., 1992), while another portion accumulates at asters in the cytoplasm (Maldonado-Codina and Glover, 1992; Ookata et al., 1992). In metaphase cells, p34<sup>cdc2</sup>/cyclin B is shown to concentrate in mitotic spindles (Rattner et al., 1990; Pines and Hunter, 1991; Tombes et al., 1991; Maldonado-Codina and Glover, 1992; Ookata et al., 1992). In the nucleus, p34<sup>cdc2</sup> kinase is involved in chromosome condensation, by phosphorylating histone H1 and other proteins, as well as in nuclear envelope breakdown, by phosphorylating nuclear lamins (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990; Luscher et al., 1991; Ohsumi et al., 1993). In addition, p34<sup>cdc2</sup> kinase associated with asters and mitotic spindles may be involved in the rearrangement of the microtubule network at mitosis, by phosphorylating centrosomal proteins (Bailly et al., 1989; Riabowol et al., 1989; Buendia et al., 1992; Ohta et al., 1993). It may also be involved in the change of microtubule dynamics at the onset of mitosis, by phosphorylating microtubule-associated proteins (Belmont et al., 1990; Verde et al., 1990; Ookata et al., 1993). However, except for nuclear lamins, whose filaments are disassembled by phosphorylation with cdc2 kinase, no clear evidence indicating a functional relationship between M-phase events and phosphorylation of p34<sup>cdc2</sup> kinase substrates has been obtained.

In this study, we have focused our attention on the regulation of microtubule dynamics at mitosis, concentrating on the following questions: (a) How does p34<sup>cdc2</sup> kinase associate with asters and spindles? (b) Is any microtubule protein substrate(s) phosphorylated by p34<sup>cdc2</sup> kinase in vivo and/or in vitro? (c) If so, how does this phosphorylation contribute to alteration of microtubule dynamics during mitosis? To begin to address these questions, we previously used starfish oocytes, and demonstrated that the p34<sup>cdc2</sup>/cyclin B complex associated with microtubules via binding to MAPs (Ookata et al., 1993). However, in starfish oocytes we could not readily identify microtubule-associated proteins (MAPs) associated with p34<sup>cdc2</sup>/cyclin B. To analyze the mechanism by which p34<sup>cdc2</sup>/cyclin B associates with microtubules, then, we chose HeLa cells, because they are typical proliferating cells showing cyclic activation of p34<sup>cdc2</sup> kinase at mitosis (Draetta and Beach, 1988), and their MAP composition has been analyzed previously. HeLa MAPs consist mainly of a heat stable 210-kD MAP (MAP4), although a 125-kD MAP has also been detected (Bulinski and Borisy, 1980a; Weatherbee et al., 1982; Murofushi et al., 1987; Bulinski and Bossler, 1994). MAP4 is the most abundant, well-characterized MAP in proliferating cultured cells (reviewed by Olmsted, 1986; Vallee, 1990). Moreover, blotting with a monoclonal antibody (MPM2) that recognizes M-phase-specific phosphoproteins has demonstrated that HeLa MAP4 is phosphorylated at mitosis (Vandre et al., 1991).

Double immunostaining of primate cultured cells with anti-cyclin B and anti-MAP4 antibodies showed correlation of their distributions under several conditions, suggesting that binding of the p34<sup>cdc2</sup> kinase complex to microtubules occurs in a MAP4-dependent manner. We report here that p34<sup>cdc2</sup> kinase associates with microtubules through the binding of cyclin B to the proline-rich region of MAP4. HeLa MAP4 associated with p34<sup>cdc2</sup> kinase in vitro was

phosphorylated by the kinase complex. Darkfield microscopy of single microtubules demonstrated that this level of phosphorylation altered the dynamics of MAP4-stabilized microtubules.

## Materials and Methods

### Cell Culture and Synchronization

HeLa cells (strain S3) were cultured as a monolayer at 37°C in DME supplemented with 5% fetal calf serum. Cells were synchronized at the G1/S boundary by treatment with 2.5 mM thymidine for 24 h. 8 h after wash out of the thymidine to release the block, cells were treated with 0.4 µg/ml nocodazole for 5 h and mitotic cells were collected. African green monkey kidney cells (TC-7 line) were cultured as described previously (Bulinski and Borisy, 1980b).

### Preparation of MAP4, Tubulin and p34<sup>cdc2</sup>/cyclin B Kinase

Microtubules were polymerized with the aid of taxol (20 µM) in the mitotic HeLa cell extract and sedimented through a 10% sucrose cushion, essentially as described by Vallee (1982).

Crude HeLa MAP4 was prepared by boiling a MAP fraction eluted from microtubules with 0.5 M NaCl (Vallee, 1982). Purified MAP4 was prepared by one more cycle of microtubule-binding and heat treatment of the crude MAP4. Bovine MAP4 was purified from adrenal cortex according to Murofushi et al. (1986).

Microtubule protein was prepared from porcine brains by two cycles of temperature-dependent polymerization/depolymerization (Shelanski et al., 1973). Tubulin was purified from microtubules by either phosphocellulose or DEAE-cellulose column chromatography (Murphy and Borisy, 1975; Weingarten et al., 1975).

The expression of MAP4 fragments in *Escherichia coli* strain MM294 and their purification were performed according to Aizawa et al. (1991a).

Cyclin B-dependent p34<sup>cdc2</sup> kinase was purified from starfish oocytes at the first meiotic M-phase by p13<sup>suc1</sup> affinity chromatography; p34<sup>cdc2</sup>/cyclin B complex was eluted from the column with 0.5 M NaCl and 50% ethyleneglycol (Kusubata et al., 1992; Ookata et al., 1993; Okumura and Kishimoto, manuscript in preparation).

### Preparation of GST-fusion Proteins and Conjugation to GSH-Sepharose 4B-beads

GST-cyclin B- and GST-cyclin D-immobilized Sepharose beads were prepared as follows: A 1.4-kb BamHI fragment of starfish cyclin B cDNA, encoding amino acids 37–396 (Tachibana et al., 1990) was ligated in frame to the unique BamHI site of pGEX-2T (Pharmacia LKB Nuclear, Gaithersburg, MD). A 1.1-kb EcoRI fragment of cyclin D2 cDNA, encoding amino acids 27–289 (Tachibana et al., unpublished data) was ligated in frame to the unique EcoRI site of pGEX-2T.

Cultures of *E. coli* JM109 transformed with pGEX-2T plasmids containing either cyclin B or cyclin D2 cDNA were diluted 1:10 in 400 ml of fresh LB medium (10 g/l NaCl, 10 g/l Bactotryptone, 5 g/l yeast extract, pH 7.2) containing ampicillin (150 µg/ml) and incubated for 4 h. After 1 h of growth, isopropyl-1-thio-β-D-galactopyranoside (TAKARA shuzo, Japan) was added to a final concentration of 1 mM. Bacterial cultures were centrifuged and the resulting bacterial pellet was resuspended in 40 ml of 100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 0.5% Nonidet P-40 (NETN). The cells were lysed on ice by mild sonication and centrifuged at 27,000 g for 1 h at 4°C. The supernatant was mixed at 4°C in a 50 ml polypropylene tube on rotating platform with 1 ml glutathione-Sepharose-beads, which had been previously washed three times and resuspended (final concentration 1:1 [vol/vol]) in NETN. After adsorption for 1 h, beads were collected by centrifugation at 500 g and washed three times with 50 ml NETN. There are more amounts of cyclin D compared with cyclin B on the beads.

### GST-cyclin-Beads Binding Assay

HeLa MAP4 (10 µg/ml) or expressed bovine MAP4 fragments (5 µg/ml) were incubated with GST-cyclin B or D-beads in 20 mM Pipes, pH 6.8, 1 mM MgSO<sub>4</sub>, 1 mM EGTA, 0.1 M NaCl, and 0.1% Nonidet P-40 for 20

min at 4°C. Beads were pelleted by a brief centrifugation (12,000 g, 5 s) and suspended in SDS-sample buffer after washing three times with the above binding buffer. The association of MAP4 with beads was analyzed by immunoblot with anti-MAP4 antibody (Murofushi et al., 1987).

### Microtubule Binding Assay

Microtubules were polymerized from purified brain tubulin (0.9 mg/ml) with 20  $\mu$ M taxol and 0.5 mM GTP in the presence or absence of MAP4 (28  $\mu$ g/ml). The purified starfish p34<sup>cdc2</sup>/cyclin B complex (~10  $\mu$ g/ml) was added to the microtubule suspension and incubated at 30°C for 10 min. The bound p34<sup>cdc2</sup>/cyclin B complex was separated from unbound material by sedimentation of microtubules, and cyclin B was detected in supernatant and pellet by immunoblotting with anti-starfish cyclin B antibody (Ookata et al., 1992). The amount of cyclin B associated with microtubules was estimated by densitometric scanning (ACI-Japan, Tokyo, Japan) of the cyclin B band, detected by immunoblotting.

### Kinase Assay

Microtubule-associated kinase activity was measured with or without histone H1 (0.2 mg/ml) as an external substrate, in a reaction mixture consisting of 0.1 M Pipes, pH 6.8, 1 mM EGTA, and 1 mM MgCl<sub>2</sub> (PEM), and 0.5 mM [ $\gamma$ -<sup>32</sup>P]ATP in the presence or absence of cAMP-dependent protein kinase inhibitor (5  $\mu$ g/ml) for 15 min at 30°C. The reaction was stopped by adding SDS-sample buffer. For some experiments, the reaction was stopped by boiling for 5 min in 0.5 M NaCl, and the heat-stable MAP4 was recovered in the supernatant fraction following centrifugation at 100,000 g for 30 min. Proteins were separated on 5–15 or 8% polyacrylamide gradient SDS-PAGE followed by autoradiography on Kodak X-MAT x ray film.

To immunodeplete p34<sup>cdc2</sup>/cyclin B complex, the 0.5 M NaCl-extracted MAP4 fraction was dialyzed against 20 mM Pipes, pH 6.9, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, and 0.1% Triton X-100, mixed with 15  $\mu$ l of anti-human cyclin B antibody prebound to protein A-Sepharose beads, and incubated for 30 min at 4°C. The supernatant fraction following centrifugation at 3,000 g for 2 min was used to assess phosphorylation of MAP4.

The crude MAP4 (50–110  $\mu$ g/ml) was phosphorylated by starfish oocyte p34<sup>cdc2</sup> kinase (5  $\mu$ g/ml) in PEM at 30°C for 60 min. The reaction was stopped by addition of SDS-sample buffer and the samples were analyzed on 5–15% polyacrylamide SDS-PAGE. The level of incorporated phosphate was estimated by Cerenkov radiation of excised MAP4 bands. The amount of MAP4 was estimated by densitometric scanning of the gel stained with Coomassie brilliant blue (CBB), using BSA as a standard. Molecular mass of HeLa MAP4 was estimated to be 110 kD, according to the sequence data of bovine MAP4 (Aizawa et al., 1990).

The purified p34<sup>cdc2</sup>/cyclin B complex was bound to pI<sup>3suc1</sup>-conjugated Sepharose-beads by incubation in Buffer A (80 mM Na- $\beta$ -glycerophosphate, 20 mM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, pH 7.3) for 30 min at 4°C (Ookata et al., 1992). After washing with Buffer A, the PA<sub>4</sub> fragment of bovine MAP4 (0.17 mg/ml; see diagram in Fig. 5 for terminology) was added and incubated in PEM at 4°C for 30 min. Unbound PA<sub>4</sub> was removed by washing twice with 20 mM Pipes, pH 6.8, 100 mM NaCl, 0.1% Nonidet P-40 containing 1 mg/ml bovine serum albumin, and once with PEM. PA<sub>4</sub> was phosphorylated by incubating with 0.2 mM [ $\gamma$ -<sup>32</sup>P]-ATP at 30°C for 60 min and the reaction was stopped by the addition of SDS-sample buffer. The samples were boiled for 2 min and centrifuged briefly to remove Sepharose beads. The supernatants were electrophoresed on 10% SDS-PAGE and autoradiographed.

### Observation of Microtubule Dynamics

The crude MAP4 (100  $\mu$ g/ml) or purified MAP4 (31  $\mu$ g/ml) was phosphorylated at 30°C for 60 min with starfish p34<sup>cdc2</sup> kinase. To inactivate and remove p34<sup>cdc2</sup> kinase following this incubation, the reaction mixture was boiled for 3 min after addition of 0.5 M NaCl. After centrifugation at 100,000 g for 30 min, the supernatant was dialyzed against PEM and used as phosphorylated MAP4.

Microtubule dynamics were analyzed by monitoring the change in microtubule length from the images recorded on videotapes, by the procedure described previously (Horio and Hotani, 1986) with some modifications. Brain MAPs were shown to stabilize microtubules under the same conditions used here for MAP4 (Itoh and Hotani, 1994). Briefly, 50  $\mu$ l of tubulin (3.7 mg/ml) was incubated at 37°C for 5 min to allow polymerization of microtubules. Prewarmed MAP4 was added to the microtubule solution, and 5  $\mu$ l was immediately transferred to a glass slide. And then, microtubules were observed at room temperature (24–26°C). The final tubulin and

MAP concentrations were about 1.8 mg/ml and 0 to 50  $\mu$ g/ml, respectively. The change in length at each end of a given microtubule was followed by determining the distance between each end and an arbitrary fixed point on the microtubule as a function of time.

### Immunofluorescence Microscopy

Mitotic HeLa cells on coverslips were treated with a microtubule stabilizing buffer (25 mM imidazole, pH 6.9, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 10 mM EGTA, 20% glycerol) containing 0.5% Triton X-100 and fixed with cold methanol for 10 min, followed by washing with TBS. HeLa cells were stained with monoclonal anti-cyclin B<sub>1</sub> (clone 2H1-H6; Oncogene Science, Inc., Manhasset, NY) or anti-human cyclin B anti-serum (2,000-fold dilution, a generous gift from Dr. J. Pines, Pines and Hunter, 1991), or anti-bovine adrenal cortex MAP4 (500-fold dilution, Murofushi et al., 1987), followed with secondary FITC-conjugated anti-mouse or rabbit IgG (Capel Labs., Cochranville, PA). DNA was stained with 0.1  $\mu$ g/ml DAPI. Specimens were observed under an epifluorescence microscope (Olympus, Tokyo, Japan).

For some experiments, a 1/100 dilution of an anti-HeLa MAP4 antibody previously described (Chapin and Bulinski, 1991) and undiluted hybridoma supernatants of anti-cyclin B monoclonal antibodies (GNS1, GNS7, and GNS11, generous gifts of Dr. Steven Schiff, Rockefeller University, NY) were used to stain African green monkey kidney cells (TC-7 line) fixed directly in methanol. Staining protocols were described previously (Chapin and Bulinski, 1991).

### Western Blotting and Protein Determination

SDS-PAGE was performed according to Laemmli (1970) with 12.5% polyacrylamide gel for p34<sup>cdc2</sup> kinase and cyclin B, and with 5–15% polyacrylamide gradient gel for MAP4. Following electrophoresis, the proteins were transferred to nitrocellulose membrane or Immobilon (Millipore Corp., Bedford, MA) by the method of Towbin et al. (1979). The blot was probed with anti-human p34<sup>cdc2</sup> carboxy-terminal peptide (a generous gift from Dr. F. Matsumura, Rutgers University, New Brunswick, NJ), anti-cyclin B<sub>1</sub> (Oncogene Science, Inc.) and anti-bovine adrenal cortex MAP4 antibodies, and developed using an alkaline phosphatase-conjugated secondary antibody and a BCIP/NBT phosphate substrate system (KPL).

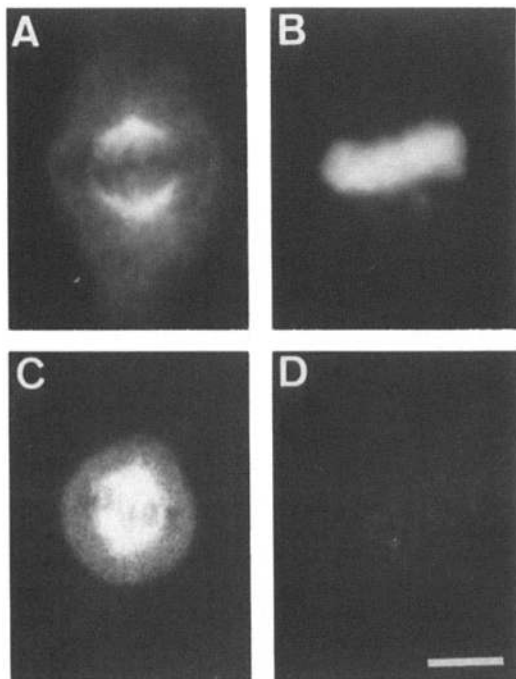
Protein concentration was determined by bicinchoninic acid protein assay reagent (Pierce Chem. Co., Rockford, IL), using bovine serum albumin as a standard.

## Results

### Colocalization and Copartitioning of Cyclin B with MAP4 in Primate Cultured Cells

p34<sup>cdc2</sup>/cyclin B associated with microtubules in a MAP-dependent manner in starfish oocytes (Ookata et al., 1993). To identify the MAP responsible for the binding, we chose primate tissue culture cells whose major MAP is MAP4. First, mitotic HeLa cells were stained with anti-cyclin B and compared with those stained with anti-MAP4 (Fig. 1). Both cyclin B (Fig. 1 A) and MAP4 (Fig. 1 C) were concentrated in mitotic spindles as reported previously (Bulinski and Borisy, 1980b; Murofushi et al., 1987; Pines and Hunter, 1991).

To examine further the colocalization of cyclin B and MAP4 at various stages of the cell cycle, we used standard double-labeling of African green monkey cells (TC-7) with anti-cyclin B and anti-MAP4 antibodies. MAP4 immunofluorescence was colocalized with microtubules irrespective of the cell cycle, while cyclin B, whose cellular concentration increased during the cell cycle, appeared with varying intensity along the microtubules of interphase cells (Fig. 2 A, a, b, e, and f). As reported previously (Pines and Hunter, 1991), at the G2/M border, most of the cyclin B was translocated to the nucleus (Fig. 2 A, b). In contrast, MAP4 was colocalized with microtubules at this stage, and remained in



**Figure 1.** Immunolocalization of cyclin B and MAP4 in mitotic HeLa cells. HeLa cells were extracted with a microtubule-stabilizing buffer containing 0.5% Triton X-100 and 20% glycerol, fixed with cold methanol, and stained with anti-human cyclin B (Oncogene Science, Inc.) (A), anti-bovine MAP4 (C), and non-immune rabbit antiserum (D). Cyclin B was concentrated in the mitotic spindle as was MAP4. (B) DAPI staining of HeLa cell shown in (A). Bar, 10  $\mu\text{m}$ .

the cytoplasm (Fig. 2 A, f). After cyclin B's transient foray into the nucleus, cyclin B was found associated with mitotic spindle fibers following nuclear envelope breakdown (Fig. 2 A, c); MAP4 was also present on spindle fibers (Fig. 2 A, g). At the onset of anaphase, as previously reported, cyclin B was degraded, while MAP4 remained codistributed with microtubules, as before (Fig. 2 A, d and h).

To ascertain the colocalization of MAP4 and cyclin B, we tried two treatments of cells in which MAP4 shows unusual behavior relative to tubulin or other MAPs, and asked whether cyclin B copartitioned with MAP4. The first type of experiment consisted of treating cells with a low concentration of nocodazole (0.13  $\mu\text{M}$  for 30 min) in order to depolymerize a portion of the cellular microtubules. Under these conditions, because of the tubulin monomer released from depolymerizing microtubules, the tubulin immunofluorescence pattern was bright and diffuse, and the remaining microtubules were obscured (Fig. 2 B, a and c). In contrast, both MAP4 (Fig. 2 B, b) and cyclin B (Fig. 2 B, d) were mostly localized to the microtubules remaining after drug treatment. Second, we extracted cells with a nonionic detergent, saponin, in the absence of glycerol, before fixation. Although the microtubules withstood the detergent extraction (Fig. 2 C, a and c), both MAP4 and cyclin B were solubilized by the extraction (Fig. 2 C, b and d) and only the faint staining of cyclin B was observed after extraction (Fig. 2 C, d). In contrast to MAP4, 125-kD MAP (*ensconsin*) is resistant to the detergent extraction and remains associated with microtubules in detergent-treated cells (Bulinski and Bossler,

1994). These correlative results are consistent with the participation of MAP4 in the binding of cyclin B, presumably as a complex of p34<sup>cdc2</sup>/cyclin B, to microtubules in vivo.

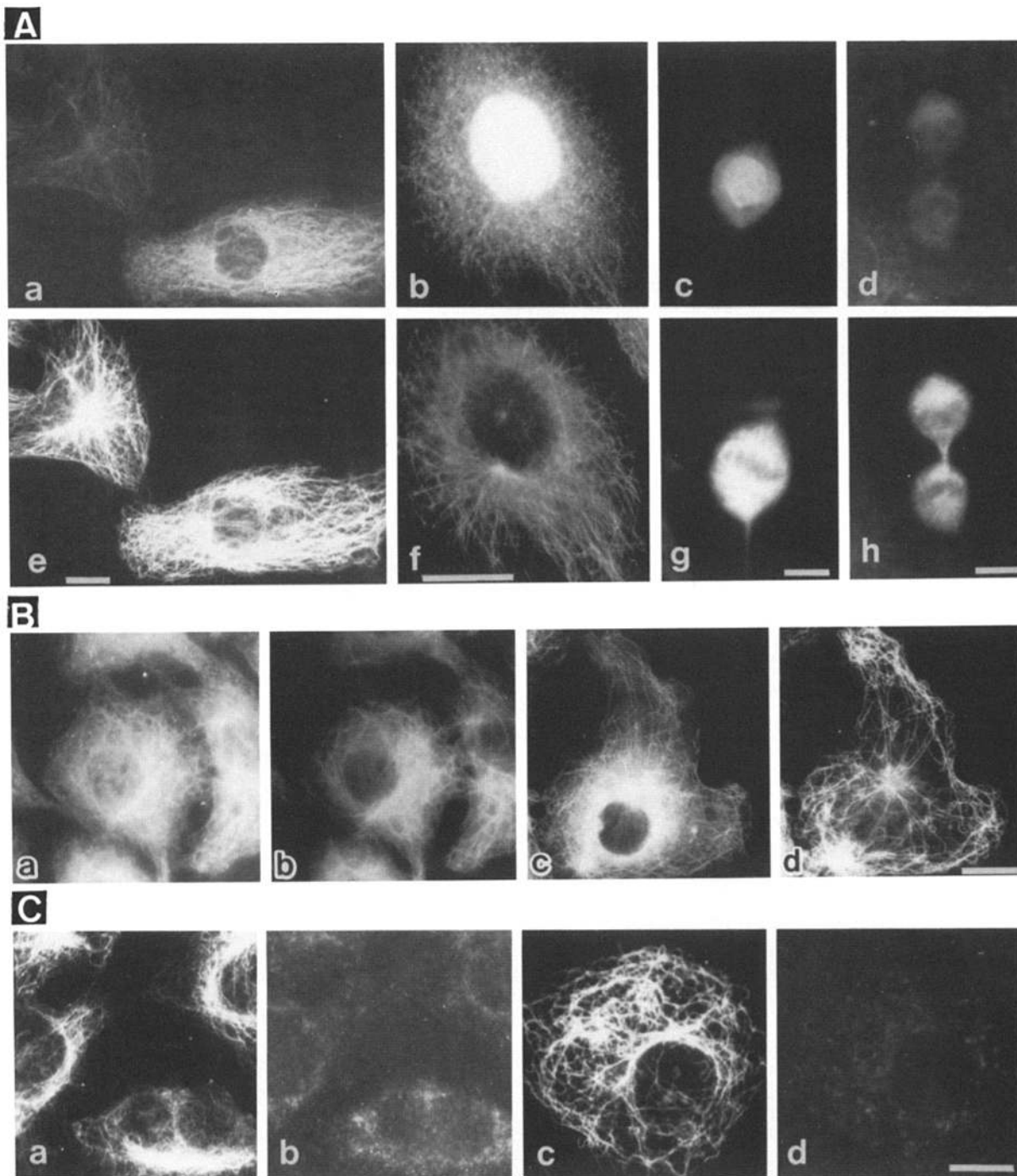
#### **Cosedimentation of p34<sup>cdc2</sup>/cyclin B Complex with Microtubules in HeLa Cell Extracts**

To examine the association of p34<sup>cdc2</sup>/cyclin B complex with microtubules in HeLa cells biochemically, we prepared taxol-polymerized microtubules from mitotic HeLa cells and immunoblotted these with anti-human p34<sup>cdc2</sup> and anti-cyclin B antibodies as we had done previously with starfish oocytes (Ookata et al., 1993). Protein staining of HeLa microtubule proteins is shown in Fig. 3 A, lane 1. Tubulin (indicated by Tu) was a major protein in the fraction. A band that was visible at  $\sim 200$  kD by CBB staining probably corresponds to MAP4 as suggested by blotting with anti-MAP4 (labeled MAP4 in Fig. 3 A, lane 2). p34<sup>cdc2</sup> and cyclin B proteins present in the microtubule preparations are shown by blotting with anti-human p34<sup>cdc2</sup> (Fig. 3 A, lane 3) and anti-cyclin B<sub>1</sub> antibodies (Fig. 3 A, lane 4). Almost all of the p34<sup>cdc2</sup>/cyclin B complex in the HeLa cell extracts sedimented with microtubules (although a portion of cyclin B in HeLa cells sedimented with membrane particles by centrifugation of the homogenate), and this p34<sup>cdc2</sup>/cyclin B complex was released from microtubules by a high salt solution together with MAP4 (data not shown).

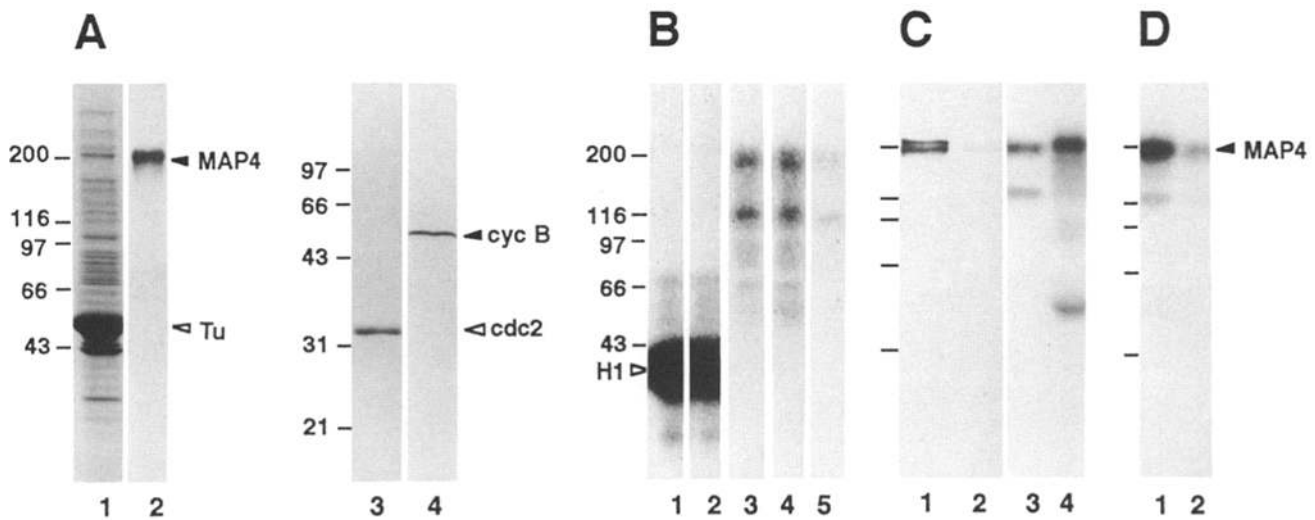
Since the active form of p34<sup>cdc2</sup>/cyclin B complex possesses high histone H1 kinase activity, we examined the histone H1 kinase activity of the microtubule-associated p34<sup>cdc2</sup>/cyclin B complex. When histone H1 was added to the microtubule fraction as an exogenous substrate, heavy phosphorylation of histone H1 was detected (Fig. 3 B, lane 1). This histone H1 phosphorylation was not inhibited by cAMP-dependent protein kinase inhibitor (Fig. 3 B, lane 2), eliminating the contribution of cAMP-dependent protein kinase. Since Ca<sup>2+</sup> was depleted by EGTA in the reaction mixture, contribution of protein kinase C and Ca<sup>2+</sup>/calmodulin-dependent protein kinase was not expected. These results suggest this histone H1 kinase activity is mostly due to p34<sup>cdc2</sup> kinase associated with microtubules.

Possible endogenous substrates for this kinase were examined by phosphorylation of the crude microtubule pellet in the absence of any exogenous substrates. Two major distinct phosphorylated bands were detected (Fig. 3 B, lane 3). The broad higher molecular mass band was shown to be composed of at least two proteins: a heat stable 200-kD protein and a little larger heat labile protein (Fig. 3 C, lanes 3 and 4). We identified the heat stable 200-kD protein as MAP4 because of its electrophoretic mobility, anti-MAP4 immunoreactivity, as well as, of course, its heat-stability (Fig. 3 C, lanes 1 and 3). The identities of the other phosphorylated species are less certain. The heat labile phosphoprotein could be a myosin heavy chain or a 210-kD heat-labile microtubule-interacting protein reported recently by Draberova and Draber (1993). The lower phosphorylated protein, migrating at around 120 kD, may correspond to 125-kD MAP, which was recently purified and named *ensconsin* (Bulinski and Bossler, 1994).

Phosphorylation of the 200-kD species was not inhibited by cAMP-dependent protein kinase inhibitor (Fig. 3 B, lane 4) but was abolished by the addition of Histone H1 (see Fig. 3 B, lanes 1 and 2), suggesting that contribution of MAP



**Figure 2.** Colocalization and copartitioning of cyclin B with MAP4 in African green monkey kidney cell (TC-7). (A) Colocalization of cyclin B (a–d) and MAP4 (e–h) at several stages of cell cycle. TC-7 cells were methanol-fixed and double stained with monoclonal anti-cyclin B (a generous gift from Steven Schiff, Rockefeller University, NY) and polyclonal anti-human MAP4. The cell cycle stages depicted in the micrographs are interphase (a and e), G<sub>2</sub>/M transition (b and f), metaphase (c and g), and telophase (d and h). (B) Association of cyclin B and MAP4 with subsets of microtubules remaining after partial depolymerization with 0.1  $\mu$ M nocodazole for 30 min. Double staining with anti-tubulin (a) and anti-MAP4 (b). Double-staining of methanol fixed cells with anti-tubulin (c) and anti-cyclin B (d). Note that the tubulin staining appeared bright and diffuse because of depolymerized, monomeric tubulin, while both MAP4 and cyclin B were associated with the remaining microtubule polymers. (C) Coextraction of MAP4 and cyclin B from the microtubule cytoskeleton by detergent treatment. TC-7 cells were extracted with the detergent, saponin (0.2 mg/ml) in PEM buffer (without glycerol) for 2 min before methanol fixation. Double staining with anti-tubulin (a) and anti-MAP4 (b). Double staining with anti-tubulin (c) and anti-cyclin B (d). Note that, as previously reported (Schliwa et al., 1981; Bulinski and Bossler, 1994), MAP4 was extracted from microtubules during detergent extraction. Cyclin B showed the same behavior as MAP4 upon extraction. Bar, 10  $\mu$ m.



**Figure 3.** Detection of p34<sup>cdc2</sup>/cyclin B and MAP4 in the microtubule fraction prepared from mitotic HeLa cells. HeLa cells were synchronized at S-phase by thymidine block and then at M-phase by nocodazole treatment. Microtubules were polymerized with the aid of taxol in the mitotic HeLa cell extract and precipitated through 10% sucrose cushion by centrifugation. (A) Immunoblotting of microtubule-proteins with anti-MAP4, anti-human p34<sup>cdc2</sup> and anti-cyclin B<sub>1</sub> antibodies. Lane 1, CBB staining of microtubule-proteins; lane 2, blotting with anti-MAP4; lane 3, blotting with anti-human p34<sup>cdc2</sup> antibody; lane 4, anti-cyclin B<sub>1</sub>. (B) Autoradiograph showing phosphorylation of excess histone H1 and endogenous proteins by microtubule-associated kinase. Phosphorylation of histone H1 by microtubule-associated kinase in the absence (lane 1) and presence (lane 2) of protein kinase A inhibitor, and phosphorylation of endogenous proteins by microtubule-associated kinase in the absence (lane 3) and presence (lane 4) of protein kinase A inhibitor. Note that phosphorylation of 200-kD bands was inhibited in the presence of histone H1 (lanes 1 and 2). Inhibition of phosphorylation was also observed when 0.5 mg/ml p34<sup>cdc2</sup> kinase peptide (CKASPEKAKSPVKE) was added to the reaction mixture (lane 5). (C) Identification of a heat-stable phosphorylated 200-kD species as MAP4. HeLa microtubule proteins were incubated in the presence of [ $\gamma$ -<sup>32</sup>P]ATP at 30°C for 20 min. The phosphorylation reaction was stopped by boiling for 5 min in the presence of 0.5 M NaCl. The heat-stable supernatant (lanes 1 and 3) and -labile precipitate fractions (lanes 2 and 4) were autoradiographed (lanes 3 and 4) and immunoblotted with anti-MAP4 antibody (lanes 1 and 2) after 7.5% SDS-PAGE. (D) The effect of immunodepletion of p34<sup>cdc2</sup>/cyclin B kinase on MAP4 phosphorylation. p34<sup>cdc2</sup>/cyclin B kinase was removed from the 0.5 M NaCl-extracted MAP4 fraction by immunoprecipitation with anti-human cyclin B. The supernatant fractions were incubated to allow for phosphorylation of exogenously added MAP4 (lane 2). The control experiment, in which the precipitation was carried out without first antibody is shown in lane 1. MAP4, tubulin, cyclin B, p34<sup>cdc2</sup> and histone H1 are indicated by MAP4, Tu, cyc B, cdc2, and H1, respectively.

kinase in MAP4 phosphorylation, if present, was minor. Synthetic peptide containing the Ser-Pro-Glu(Val)-Lys [SPE(V)K] amino acid sequence, which represents a consensus sequence for phosphorylation by p34<sup>cdc2</sup> kinase (Hisanaga et al., 1991) abolished phosphorylation of MAP4 (Fig. 3 B, lane 5) as did a p34<sup>cdc2</sup> kinase inhibitor, butyrolactone I (Kitagawa et al., 1993) (data not shown). Whether MAP4 kinase in the microtubule fractions is due to p34<sup>cdc2</sup>/cyclin B kinase was further confirmed by its immunodepletion from the MAP fraction with an anti-cyclin B antibody. To detect the kinase activity distinctly, HeLa MAP4 prepared from interphase cells was added as a substrate to the reaction mixture. Immunodepletion decreased MAP4 phosphorylation to about 10% of the control (Fig. 3 D). These results indicate that p34<sup>cdc2</sup> kinase associated with microtubules is capable of phosphorylating MAP4, which is apparently a detectable p34<sup>cdc2</sup> kinase substrate, among HeLa microtubule proteins.

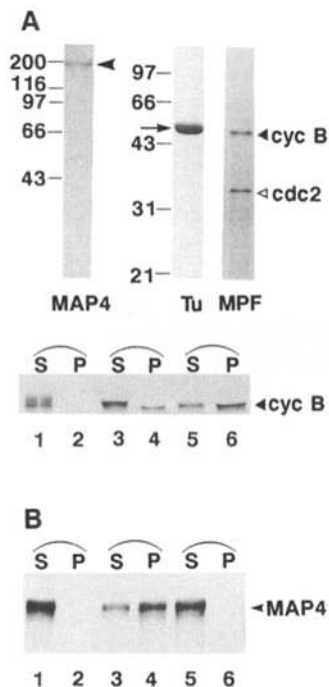
#### **p34<sup>cdc2</sup>/cyclin B Binds to Microtubules through the Interaction of MAP4 and Cyclin B**

To determine whether the binding of the p34<sup>cdc2</sup>/cyclin B complex to microtubules results from binding directly to tubulin or to MAP4, the cosedimentation experiment was repeated using purified proteins. MAP4, tubulin, and

p34<sup>cdc2</sup>/cyclin B were purified from HeLa cells, porcine brain and starfish oocytes, respectively, as described in Materials and Methods (Fig. 4 A, top). The binding to microtubules was detected by Western blotting with anti-cyclin B or anti-p34<sup>cdc2</sup> antibody. p34<sup>cdc2</sup>/cyclin B alone did not sediment when centrifuged in the presence of 0.1% Nonidet P-40 (Fig. 4 A, lanes 1 and 2). A small portion (18%) of p34<sup>cdc2</sup>/cyclin B was detected in the microtubules sedimented in the absence of MAPs (Fig. 4 A, lanes 3 and 4), while 70% of the complex added sedimented with microtubules in the presence of purified MAP4 (Fig. 4 A, lanes 5 and 6) when estimated with the amount of cyclin B. This result indicates that the p34<sup>cdc2</sup>/cyclin B complex associates with microtubules in vitro by virtue of its binding to MAP4.

Association of p34<sup>cdc2</sup>/cyclin B complex with MAP4 could be brought about by binding of either p34<sup>cdc2</sup> or cyclin B to the MAP4. Accordingly, we tested the ability of cyclin B to bind to MAP4. We prepared GST-starfish cyclin B beads and, as controls, glutathione-Sepharose beads and GST-Xenopus cyclin D beads (K. Tachibana and T. Kishimoto, unpublished observation), and tested which of these was capable of binding to MAP4. MAP4 was bound to GST-cyclin B beads (Fig. 4 B, lane 4) but was not bound to either of the control beads (Fig. 4 B, lanes 2 and 6). These results





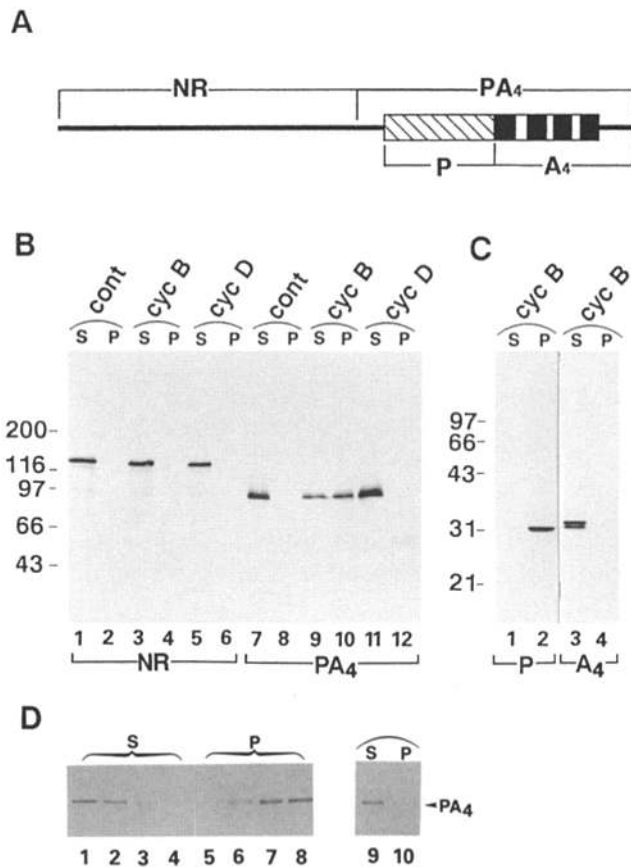
**Figure 4.** Association of the p34<sup>cdc2</sup>/cyclin B complex with microtubules is mediated by the interaction between MAP4 and cyclin B proteins. (A) In vitro cosedimentation of p34<sup>cdc2</sup>/cyclin B with microtubules in the presence and absence of MAP4. Upper gels represent purified proteins used for the cosedimentation experiment. MAP4, tubulin (*Tub*) and p34<sup>cdc2</sup>/cyclin B (*MPF*) were purified from mitotic HeLa cells, porcine brains and starfish oocytes, respectively, as described in Materials and Methods. p34<sup>cdc2</sup>/cyclin B was incubated with microtubules polymerized from porcine brain tubulin in the absence (lanes 3 and 4) and presence of HeLa MAP4 (lanes 5 and 6). After centrifugation, the supernatants (S; lanes 3 and 5) and pellets (P; lanes 4 and 6) were analyzed by immunoblotting with anti-cyclin B. p34<sup>cdc2</sup>/cyclin B alone treated in a similar way is shown in lanes 1 (S) and 2 (P). (B) Immunoblot demonstrating the association of MAP4 with cyclin B protein. GST-starfish cyclin B and GST-*Xenopus* cyclin D fusion protein were expressed in *E. coli* and coupled with glutathione-beads as described in Materials and Methods. MAP4 was incubated with glutathione-beads (lanes 1 and 2), GST-cyclin B-beads (lanes 3 and 4) and GST-cyclin D-beads (lanes 5 and 6). After centrifugation, the supernatants (lanes 1, 3, and 5) and the pellets (lanes 2, 4, and 6) were analyzed by immunoblot with anti-MAP4 antibody. MAP4 bound only to GST-cyclin B-beads (lane 4) but not to glutathione- or cyclin D-beads (lanes 2 and 6).

demonstrate that cyclin B is capable of binding to MAP4, thus bringing about association of the p34<sup>cdc2</sup>/cyclin B complex with microtubules in vitro.

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### Cyclin B Associates with the Proline-rich Region of the MAP4 Molecule

MAP4 is a large protein possessing multiple functional domains (Fig. 5 A). We tested which of these MAP4 domains is capable of p34<sup>cdc2</sup>/cyclin B complex binding. Various fragments of MAP4 were produced in *E. coli* using a bovine MAP4 cDNA (Aizawa et al., 1991a). When GST-cyclin B beads were incubated with the amino-terminal half (NR, amino acid residues 11–533, corresponding to the projection domain) or the carboxy-terminal half (PA<sub>4</sub>, amino acid residues 534–1072, corresponding to the microtubule-binding domain) (Fig. 5 A) and then precipitated by brief centrifugation, only PA<sub>4</sub> was detected in the precipitated GST-cyclin B beads (Fig. 5 B, lanes 3, 4, 9, and 10). When NR or PA<sub>4</sub> was incubated with glutathione-Sepharose 4B beads or GST-cyclin D beads, no binding of MAP4 domains was detected with either of these control beads (Fig. 5B, lanes 1, 2, 5–8, 11, and 12), demonstrating the specificity of binding of PA<sub>4</sub> to cyclin B beads.



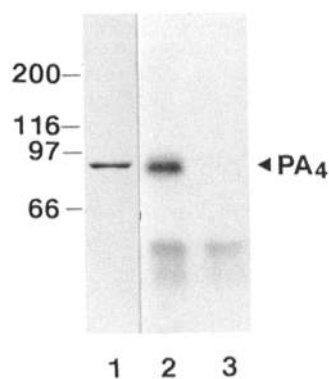
**Figure 5.** The domain structure of bovine MAP4 and association of cyclin B with the Pro-rich region of MAP4. (A) The domain structure of bovine MAP4 and fragments used in the binding experiments. The NH<sub>2</sub>-terminal half and the COOH-terminal half are represented by NR and PA<sub>4</sub>, respectively. The Pro-rich region (P) located in the NH<sub>2</sub>-terminal half of PA<sub>4</sub> is indicated as a shaded area. The A<sub>4</sub> fragment (A<sub>4</sub>) consists of four imperfectly repeated sequences within the assembly promoting (AP) region, which is indicated by black and white stripes, as well as a tail region. These MAP4 fragments were expressed in *E. coli*, using bovine MAP4 cDNA (Aizawa et al., 1991a). (B) NR (lanes 1 to 6) and PA<sub>4</sub> (lanes 7–12) of MAP4 were incubated with glutathione-beads (lanes 1, 2, 7, and 8), GST-cyclin B-beads (lanes 3, 4, 9, and 10) and GST-cyclin D-beads (lanes 5, 6, 11, and 12), respectively. After brief centrifugation, the supernatants (S) and the pellets (P) were analyzed by immunoblot with anti-MAP4. (C) P (lanes 1 and 2) and A<sub>4</sub> (lanes 3 and 4) fragments were incubated with GST-cyclin B-beads, and after centrifugation the supernatants (lanes 1 and 3) and the pellets (lanes 2 and 4) were analyzed by immunoblot with anti-MAP4. (D) The binding of PA<sub>4</sub> to GST-cyclin B beads as a function of GST-cyclin B bead concentration. 5 μg/ml PA<sub>4</sub> was incubated for 20 min at 4°C with GST-cyclin B beads diluted 8- (lanes 2 and 6), 4- (lanes 3 and 7), or undiluted bead preparation (lanes 4 and 8), respectively, with control Sepharose CL-4B beads. Control Sepharose CL-4B beads alone are shown in lanes 1 and 5. After washing with 20 mM Pipes, pH 6.8, 1 mM MgSO<sub>4</sub>, 1 mM EGTA, 0.1 M NaCl, and 0.1% Nonidet P-40, PA<sub>4</sub> in the unbound fractions (supernatants; lanes 1–4) and GST-cyclin B beads in the bound fractions (pellets; lanes 5–8) was analyzed by immunoblotting with anti-bovine MAP4. Salt (0.35 M NaCl) extraction of GST-cyclin B beads–PA<sub>4</sub> complex, followed by centrifugation of the beads, dissociated PA<sub>4</sub> from cyclin B beads, as was shown in lane 9 (extracted supernatant fraction) and lane 10 (precipitated bead fraction).

To define further the subdomain of MAP4 that interacts with cyclin B, two subfragments of PA<sub>4</sub>, the amino-terminal half of PA<sub>4</sub> (P, amino acid residues 647–865) and the carboxy-terminal half of PA<sub>4</sub> (A<sub>4</sub>, amino acid residues 870–1072) (Fig. 5 A), were prepared and their binding to GST-cyclin B beads was examined. The P fragment corresponds to the positively charged MAP4 region enriched in Pro residues (Pro-rich region), and the A<sub>4</sub> fragment consists of four imperfect repeat sequences (AP-sequence region) that contribute to the assembly-promoting ability of MAP4 (Aizawa et al., 1991a) and a hydrophobic tail region. When GST-cyclin B beads were incubated with each of these fragments, only the P fragment was detected in the fraction bound to the GST-cyclin B beads (Fig. 5 C, lane 2), indicating that the Pro-rich region is the fragment of the MAP4 molecule sufficient to mediate cyclin B association.

The binding of PA<sub>4</sub> to GST-cyclin B beads was studied by varying the concentration of GST-cyclin B beads (Fig. 5 D, lanes 1–8). The concentration of GST-cyclin B beads was adjusted by dilution with control Sepharose beads. The amount of PA<sub>4</sub> bound to beads increased according to the amount of GST-cyclin B beads. When undiluted GST-cyclin B beads were used, most of PA<sub>4</sub> (5 μg/ml) bound to the beads and PA<sub>4</sub> was not detected in the supernatant fraction (lanes 4 and 8). To determine whether PA<sub>4</sub>-cyclin B binding is ionic, we treated a PA<sub>4</sub>-cyclin B bead pellet with 0.35 M NaCl. Following a centrifugation step, PA<sub>4</sub> was found only in the supernatant fraction, indicating that it had been dissociated from cyclin B by salt treatment (Fig. 5 D, lanes 9 and 10).

### p34<sup>cdc2</sup> Kinase Phosphorylates MAP4 In Vitro

Observation of a physical association of p34<sup>cdc2</sup> kinase with MAP4 suggested to us that intracomplex phosphorylation of MAP4 might occur within the ternary complex consisting of p34<sup>cdc2</sup>/cyclin B/MAP4. To test this possibility, we performed phosphorylation of the PA<sub>4</sub> fragment of MAP4, which had been bound to the p34<sup>cdc2</sup>/cyclin B complex immobilized on p13<sup>suc1</sup> beads. The p34<sup>cdc2</sup>/cyclin B/PA<sub>4</sub> complex adsorbed to p13<sup>suc1</sup> beads was incubated with [ $\gamma$ -<sup>32</sup>P]ATP, subjected to SDS-PAGE, and autoradiographed. Fig. 6 shows that a labeled band comigrated with PA<sub>4</sub> (lanes



1, CBB staining of PA<sub>4</sub> protein to indicate the position of PA<sub>4</sub> on SDS-PAGE; lane 2, phosphorylation of PA<sub>4</sub> bound to p34<sup>cdc2</sup>/cyclin B-p13<sup>suc1</sup> beads; lane 3, an autoradiograph from a control experiment in which PA<sub>4</sub> was incubated with p13<sup>suc1</sup> beads before the binding of p34<sup>cdc2</sup>/cyclin B to the beads.

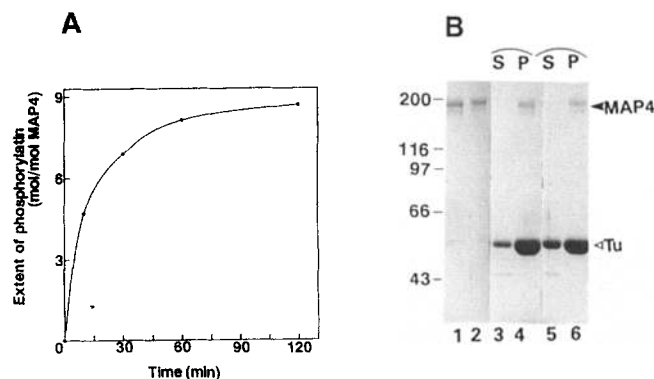
**Figure 6.** Intracomplex phosphorylation of PA<sub>4</sub> by p34<sup>cdc2</sup> kinase in the PA<sub>4</sub>/p34<sup>cdc2</sup>/cyclin B complex. Purified starfish p34<sup>cdc2</sup> kinase was bound to p13<sup>suc1</sup> Sepharose 4B by incubating for 30 min at 0°C. After washing, p34<sup>cdc2</sup> kinase-p13<sup>suc1</sup> Sepharose was further incubated with the PA<sub>4</sub> fragment of MAP4 for 30 min at 0°C. After washing, PA<sub>4</sub> retained on beads was phosphorylated by incubating with [ $\gamma$ -<sup>32</sup>P]ATP. Lane 1,

1 and 2), while no phosphorylation was detected in a control experiment in which PA<sub>4</sub> was incubated with p13<sup>suc1</sup> beads before application of p34<sup>cdc2</sup>/cyclin B (lane 3).

The stoichiometry of phosphorylation was measured by incubating heat stable HeLa MAP4 with purified starfish p34<sup>cdc2</sup> kinase. Approximately eight moles of phosphate was incorporated per mole of MAP4 (Fig. 7 A). This value is in approximate agreement with the number of (S/T)PX(R/K) p34<sup>cdc2</sup> kinase consensus sequences (six sites) found in human MAP4 (Chapin and Bulinski, 1991; West et al., 1991). Furthermore, we observed 3 mol of phosphate incorporated into bovine adrenal cortex MAP4 (data not shown) which contains four consensus sequences (Aizawa et al., 1990). These results suggest that under our reaction conditions most sequences that resemble the (S/T)PX(R/K) consensus within MAP4 are capable of phosphorylation by p34<sup>cdc2</sup> kinase. Fig. 7 B, lanes 1 and 2 represent the protein staining of unphosphorylated (lane 1) and phosphorylated MAP4 (lane 2). Comparison of lanes 1 and 2 shows that phosphorylation by p34<sup>cdc2</sup> kinase decreased the electrophoretic mobility of MAP4 as previously observed by Vandre et al. (1991).

### Phosphorylation of MAP4 by p34<sup>cdc2</sup> Kinase: Effect on Microtubule Dynamics

Since MAP4 is capable of binding to and undergoing phosphorylation by the p34<sup>cdc2</sup>/cyclin B kinase, we determined the effects that this phosphorylation might have on MAP4's ability to bind to and stabilize microtubules. Unphosphorylated MAP4, and MAP4 phosphorylated by p34<sup>cdc2</sup> kinase, were incubated with taxol-stabilized microtubules and the mixtures were centrifuged. Both forms of MAP4 were recovered exclusively in the sedimented microtubules (Fig. 7 B,



**Figure 7.** Phosphorylation of MAP4 by p34<sup>cdc2</sup> kinase. (A) Time course of phosphorylation of HeLa MAP4 by purified p34<sup>cdc2</sup> kinase. HeLa MAP4 was incubated with starfish oocyte p34<sup>cdc2</sup> kinase at 30°C. The amount of label incorporated into MAP4 was expressed in mol Pi/mol protein, assuming the molecular mass of MAP4 to be 110 kD (Aizawa et al., 1990). Lanes 1 and 2 in (B) represent SDS-PAGE of unphosphorylated (lane 1) and phosphorylated MAP4 (lane 2), demonstrating that phosphorylation decreased the electrophoretic mobility of MAP4. (B) Sedimentation of phosphorylated MAP4 with microtubules. Unphosphorylated (lanes 3 and 4) and phosphorylated HeLa MAP4 (lanes 5 and 6) were incubated with microtubules polymerized from porcine brain tubulin with taxol. The mixtures were centrifuged to sediment microtubules, and their supernatants (lanes 3 and 5) and pellets (lanes 4 and 6) were subjected to SDS-PAGE. HeLa MAP4 and tubulin are indicated by MAP4 and Tu, respectively.



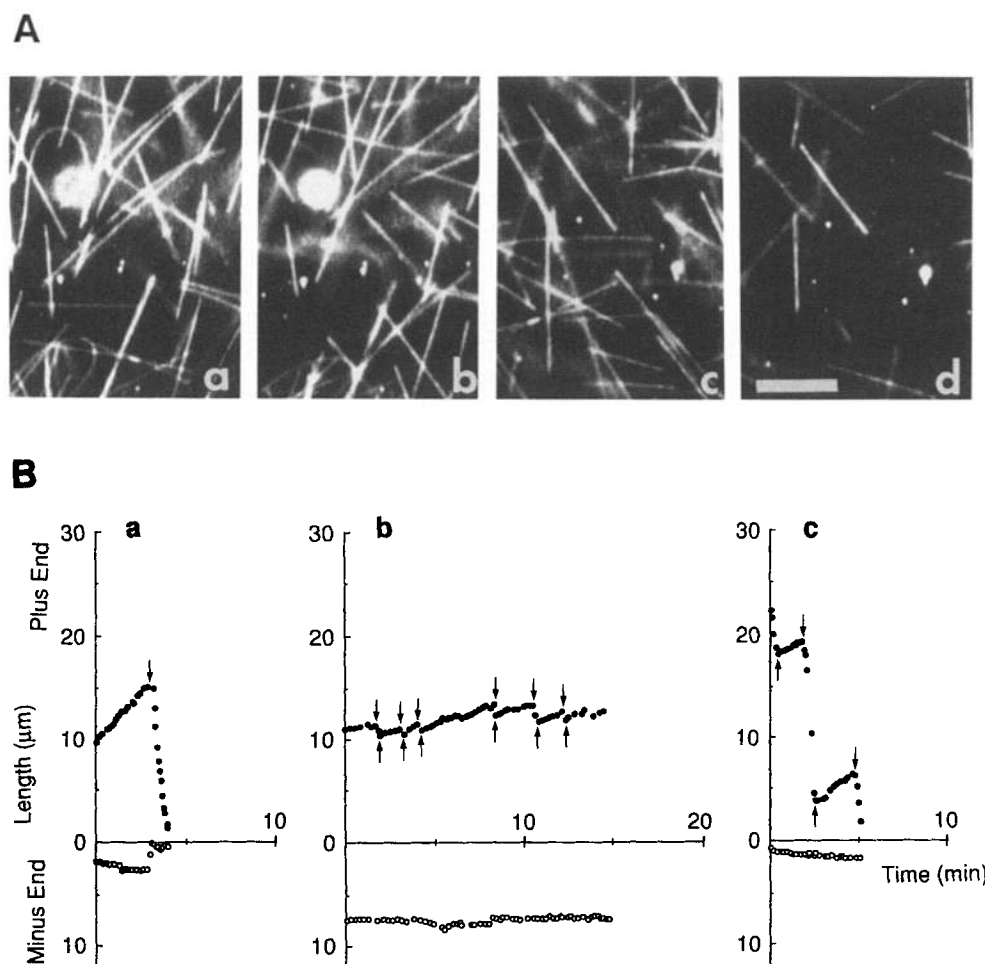
lanes 4 and 6), indicating that, after phosphorylation, MAP4 was still competent to bind to microtubules.

To determine if phosphorylation of MAP4 by p34<sup>cdc2</sup> kinase might play a role in modulating microtubule dynamics, we analyzed the changes in length of individual microtubules by dark-field microscopy. Under conditions described in Materials and Methods, microtubules in the absence of MAP4 were quite labile, and most microtubules disappeared without rescue (the phase transition from depolymerization to polymerization) after catastrophe (the phase transition from polymerization to depolymerization) (Fig. 8 B, a) as has been reported by Walker et al. (1989).

The effects of MAP4 and its phosphorylation on microtubule stabilization are represented as whole CRT (cathode-ray tube) views of the typical image in Fig. 8 A. There were many microtubules at 3 min after addition of MAP4 in either specimens of unphosphorylated (25  $\mu\text{g/ml}$ ) (Fig. 8 A, a) or phosphorylated MAP4 (25  $\mu\text{g/ml}$ ) (Fig. 8 A, c). Micrographs of the same field, 16 min after addition of MAP4, are shown in Fig. 8 A, b and d. In contrast to MAP-free microtu-

bules, all of which disassembled in a few minutes (data not shown), a considerable number of microtubules remained in the presence of unphosphorylated MAP4 (Fig. 8 A, b), indicating that MAP4 possesses the ability to stabilize microtubules. On the other hand, when phosphorylated MAP4 was added, the number of microtubules remaining after 16 min was reduced greatly (Fig. 8 A, d), suggesting that phosphorylation by p34<sup>cdc2</sup> kinase had reduced the ability of MAP4 to stabilize microtubules.

Dynamics of individual microtubules was analyzed by monitoring the change in microtubule length from the images recorded on videotapes as described in Materials and Methods. Typical examples of the dynamics of single microtubules are shown in Fig. 8 B. A plus end was distinguished from a minus end by its more dynamic behavior. In the absence of MAP4, once depolymerization began at the plus end, the microtubule disappeared immediately without rescue (Fig. 8 B, a). In the presence of unphosphorylated MAP4 (50  $\mu\text{g/ml}$ ), microtubules became very stable and almost no changes were observed in the microtubule length (data not



**Figure 8.** Effect of MAP4 phosphorylation by p34<sup>cdc2</sup> kinase on microtubule dynamics. (A) Dark field micrographs showing the effect of phosphorylation with p34<sup>cdc2</sup> kinase on microtubule-stabilizing ability of MAP4. Tubulin solution (3.7 mg/ml) was incubated for five minutes at 37°C to allow the spontaneous formation of microtubules. Unphosphorylated (a and b) or phosphorylated MAP4 (c and d) was then added to the tubulin solution. Final tubulin and MAP4 concentrations were  $\sim 1.8$  mg/ml and 25  $\mu\text{g/ml}$ , respectively. 5  $\mu\text{l}$  of each solution was immediately transferred to a glass slide, and observed with a microscope under dark field illumination. a and c are micrographs taken 3 min, while b and d were taken 16 min, after addition of MAP4. Bar represents 10  $\mu\text{m}$ . (B) Length histories of representative, individual microtubules in the presence of unphosphorylated and phosphorylated MAP4. (See Table I for information on the population of microtubules.) Changes in microtubule length were tracked at both the plus and minus ends of a

single microtubule in the absence (a), and the presence of unphosphorylated (b, 13  $\mu\text{g/ml}$ ) and phosphorylated (c, 30  $\mu\text{g/ml}$ ) MAP4. Filled and open circles indicate the plus and minus end of the microtubules, respectively. Phosphorylation by p34<sup>cdc2</sup> kinase decreased the microtubule-stabilizing ability of MAP4; the microtubule's behavior in the presence of phosphorylated MAP4 is apparently similar to the behavior of a microtubule in the absence of MAP4. To show more dynamic behavior at the plus end of microtubules, results from a low concentration (13  $\mu\text{g/ml}$ ) of unphosphorylated MAP4 were selected for presentation in (b); even in the presence of a high concentration (30  $\mu\text{g/ml}$ ) of phosphorylated MAP4 (c) MAP4 showed almost no microtubule stabilizing capacity. Catastrophe and rescue events are indicated by arrows pointed downward and upward, respectively.

shown). By decreasing the concentration of MAP4, the duration of shortening appeared to increase, resulting in quite frequent conversions between elongation phases and shortening phases at the plus end as shown in Fig. 8 B, b (13  $\mu\text{g/ml}$  unphosphorylated MAP4). From the profile of microtubule length, it could be speculated that microtubule-stabilization by MAP4 is due to enhanced rescue events at the plus end. When phosphorylated MAP4 was added, the rescue frequency decreased remarkably. Even in the presence of a higher concentration of phosphorylated MAP4 (30  $\mu\text{g/ml}$  in Fig. 8 B, c), microtubules were very unstable and only a few rescue events were observed before the microtubules depolymerized completely.

To determine the detailed effect of phosphorylated MAP4 on microtubule dynamics, experiments in which varied MAP4 concentrations were used would be optimal. However, because 30  $\mu\text{g/ml}$  was the highest concentration of phosphorylated MAP4 we could prepare, we limited our comparison of various parameters of microtubule dynamics to lower MAP4 concentrations. Accordingly, we compared dynamics in the presence of 26  $\mu\text{g/ml}$  unphosphorylated MAP4 and 30  $\mu\text{g/ml}$  phosphorylated MAP4 (Table I). The most notable change produced by addition of MAP4, or by its phosphorylation, was the frequency of rescue. Because the frequency of rescue at the plus end was quite low (0.004/s) in the absence of MAP4, most microtubules disappeared without any rescue. Addition of unphosphorylated MAP4 increased the rescue frequency more than 30-fold (0.128/s). When phosphorylated MAP4 was added instead of unphosphorylated MAP4, the frequency of rescue decreased almost five-fold, to 0.027/s. In contrast, the frequency of catastrophe was similar in the presence of phosphorylated MAP4 (0.008/s) and in the presence of unphosphorylated MAP4 (0.01/s). These results support our previous assertion that MAP4 increases the frequency of rescue while its phosphorylation by p34<sup>cdc2</sup> kinase reduces its rescue frequency significantly.

In order to perform many experiments with limited amounts of material, we used two types of MAP4 preparation. We performed this experiment eight times with six different preparations of MAP4; two times with purified MAP4 and six times with crude MAP4 preparations. With all MAP4 preparations, the effect of phosphorylation that we observed was the same, that is, phosphorylation decreased the microtubule-stabilizing ability of MAP4. Data presented in Fig. 8 and in Table I were obtained from experiments with

these crude MAP4 preparations. Although there were several contaminating proteins in the crude MAP4 fractions, MAP4 was the only microtubule binding protein present, i.e., the only protein that bound to taxol-stabilized microtubules (data not shown; also see Fig. 4, Chapin and Bulinski, 1994). Although the potency of microtubule-stabilizing ability varied somewhat among MAP4 preparations, our data suggested that this difference may represent differences in the fraction of active MAP4 within each preparation.

## Discussion

### p34<sup>cdc2</sup> Kinase Is Targeted to Microtubules at Mitosis

We have shown here in primate tissue culture cells that the p34<sup>cdc2</sup>/cyclin B complex associates with microtubules through the binding between cyclin B and MAP4. Considering that the microtubule cytoskeleton is one of the major targets of p34<sup>cdc2</sup> kinase at mitosis, this binding could be a possible mechanism for p34<sup>cdc2</sup> kinase to perform its action on the microtubule cytoskeleton efficiently.

In our work, we have demonstrated that cyclin B binds to the Pro-rich region of the MAP4 molecule. Although the MAP4 fragments used in this experiment were synthesized in *E. coli* from bovine MAP4 cDNA instead of from human (HeLa) MAP4 cDNA, the same result would be expected with HeLa MAP4, because bovine MAP4 has been shown to have an amino acid sequence very similar to that of human MAP4. For example, the identity of amino acid residues in the Pro-rich region is 76% overall (Fig. 9, Aizawa et al., 1990; Chapin and Bulinski, 1991), and the amino-terminal half of the Pro-rich region is >85% conserved between bovine and human. This region is of special interest because of the high proportion of basic amino acid residues (18%) and the complete conservation of their positions between human and bovine MAP4. Although the exact site within the Pro-rich region that interacts with cyclin B has not yet been determined, ionic interactions might cause their binding, as suggested from the fact that a moderate salt concentration dissociated cyclin B-MAP4 binding (Fig. 5 D and Bulinski, J. C., S. Hisanaga, K. Tachibana, and T. Kishimoto, unpublished results).

Association with microtubules seems to be a unique characteristic of p34<sup>cdc2</sup> kinase complexed with cyclin B. No other cdc2- (or cdk-) complexes involved in cell cycle progression have been found to associate with microtubules

Table I. Effect of MAP4 and Its Phosphorylation on Microtubule Dynamics

	Growth rate	Shortening rate	Catastrophe frequency	Rescue frequency	Growth* length	Shortening* length
	$\mu\text{m}/\text{min}$	$\mu\text{m}/\text{min}$	$\text{sec}^{-1}$	$\text{sec}^{-1}$	$\mu\text{m}$	$\mu\text{m}$
Plus end						
MAP-free	1.31 $\pm$ 0.43 (n = 22)	10.61 $\pm$ 2.88 (n = 22)	0.006	0.004	3.5	40.40
Native MPA4 <sup>‡</sup>	0.85 $\pm$ 0.39 (n = 30)	8.94 $\pm$ 3.55 (n = 29)	0.01	0.128	1.25	0.93
Phosphorylated MAP4 <sup>§</sup>	0.93 $\pm$ 0.32 (n = 26)	9.42 $\pm$ 3.94 (n = 26)	0.008	0.027	2.05	3.94

\* Growth or shortening length was calculated by dividing the total number of transitions by the summed length of growth or shortening.

<sup>‡</sup> 26  $\mu\text{g/ml}$  unphosphorylated MAP4.

<sup>§</sup> 30  $\mu\text{g/ml}$  phosphorylated MAP4.

human	694	PP	<span style="border: 1px solid black; padding: 2px;">SPEKKT</span> <span style="border: 1px solid black; padding: 2px;">TKPLATTQPAKTSTSKAKTQPTSLPKQAPPTTIGGLNKKPMSL</span>
mouse	665	---	<span style="border: 1px solid black; padding: 2px;">A</span> -----S-----
bovine	641	---	-----L-S-----
human	744	ASGLVPAAPPKRP	AVAS**ARPSILPSKDVKPKPIADAKAPEKRA <span style="border: 1px solid black; padding: 2px;">SPSKP</span>
mouse	715	---S---	H---A-TAT---T-AR---TE-VA---T---
bovine	691	---S---	A-T**S---T---T---V-E-I---V---
human	792	ASAPASRSGSKSTQTVAKTTTAAAVASTGPSSRSPSTLLPKKPTAIKTEG	
mouse	765	S---	LKP-P-T-P-S-A-SPSTLV-----A-T---R-S-----
bovine	739	---	VKP-----A-P-APAT-TL- <u>SP</u> -ST--NL- <u>TP</u> ---R-----
human	842	KPAEVKMTAKSVPADLSRPKSTSTSSMKKTTTSLGTAPAAGVVP	SRVKA
mouse	815	---D-R---	AS-----S-T-A-V-RN-PT-A-P-MTST---P
bovine	789	---	I---AT-A-----T---V-S-V-P---P-*A---ARP
human	892	TPMPSRPSTT	PFIDKKPTSAKPSSTTPRLSRLATNTSAPDLKN 934
mouse	865	MSA---	S-GALSV-----T---SA-V-----TV-----S 907
bovine	838	AT-P---	G-PV-----A---T-SA---G-V-A-A----- 880

**Figure 9.** Comparison of amino acid sequences of Pro-rich region among human, mouse and bovine MAP4. The Pro-rich region of human MAP4, amino acid position 694-934, is aligned with a homologous sequence in mouse MAP4, amino acid position 665-907, and the corresponding region of bovine MAP4, amino acid positions 641-880. Identical amino acids within human, mouse and bovine MAP4 are represented by a bar and deletions are indicated by asterisk. Possible phosphorylation sites, which are composed of consensus sequences (Ser-Pro-X-Lys) for p34<sup>cdc2</sup> kinase, are enclosed in boxes. Ser-Pro and Thr-Pro sequences, which were suggested by Aizawa et al. (1991b) to be sites for phosphorylation of bovine MAP4 by p34<sup>cdc2</sup> kinase, are indicated by a double underline.

or to phosphorylate them, and none colocalize with microtubules in vivo. Cyclin A shows predominantly nuclear localization during S to G2 phase, although a small fraction associates with centrosomes (Pines and Hunter, 1991). After nuclear envelope breakdown, cyclin A becomes dispersed throughout the cell (Pines and Hunter, 1991) and is not detected in the isolated mitotic spindles (Tombs et al., 1991). Although the cellular distribution of other cyclins has not been studied in detail, cyclin D, which is one of the G1 cyclins (for review see Hunter and Pines, 1991) is found in the nucleus (Xiong et al., 1992), and in our assays, cyclin D did not show binding to either HeLa MAP4 or any fragments of the bovine MAP4 molecule. Specific association of only cyclin B/p34<sup>cdc2</sup> with MAP4 and microtubules might have been predicted, since the microtubule network changes dramatically only at the beginning of M-phase when cyclin B-associated p34<sup>cdc2</sup> kinase is activated, and shows no apparent response to changes in activity of interphase cdk/cyclin complexes.

Most of the p34<sup>cdc2</sup>/cyclin B complex translocates into the nucleus from the cytoplasm before nuclear envelope breakdown and accumulates on the chromosomes, nucleolus, and nuclear lamina (Pines and Hunter, 1991; Bailly et al., 1992; Gallant and Nigg, 1992; Ookata et al., 1992). This translocation raises two interesting mechanistic questions: How does p34<sup>cdc2</sup>/cyclin B release from microtubules at the G2/M border? In addition, does cyclin B's interaction with MAP4 and microtubules serve to direct a subset of p34<sup>cdc2</sup> kinase toward specific subcellular substrates at appropriate stages of mitosis? Association of p34<sup>cdc2</sup> kinase with chro-

mosomes and nuclear lamina has also been demonstrated by the detection of histone H1 kinase activity and by immunoblotting of isolated chromatin and nuclei (Chambers and Langan, 1990; Dessev et al., 1991). Each of these nuclear components shows dramatic structural or functional changes at the onset of mitosis, and each component is thought to possess proteins that are substrates for phosphorylation by p34<sup>cdc2</sup>/cyclin B kinase. Our results raise the possibility that cyclin B generally serves to anchor the p34<sup>cdc2</sup>/cyclin B complex to the specific subcellular structures on which it must act in order to drive the cell from an interphase to a mitotic state.

#### Possible Phosphorylation Sites of MAP4 by p34<sup>cdc2</sup> Kinase

Microtubule dynamics is generally thought to be regulated by MAPs (Olmsted, 1986; Pryer et al., 1992). Heat stable brain MAPs, MAP2 and tau, as well as MAP4, contain three, four, or five imperfect repeated sequences in their microtubule assembly promoting region near their carboxy-termini (see Chapin and Bulinski, 1992 for a review, Aizawa et al., 1990; West et al., 1991; Chapin et al., 1994). Phosphorylation of MAP2 and tau proteins has been shown to reduce their microtubule stabilizing ability (Lindwall and Cole, 1984; Faruki et al., 1992; Drechsel et al., 1992). Since MAP4 is the predominant MAP in HeLa cells (Bulinski and Borisy, 1980a), and it is known to be phosphorylated at mitosis (Tombs et al., 1991; Vandre et al., 1991), we suspected that MAP4 phosphorylation might be responsible for modulating microtubule dynamics at M-phase. In support of this notion, Olmsted et al. (1989) previously demonstrated from FRAP analysis of fluorescent-labeled MAP4 microinjected into cultured cells that the rate of MAP4 exchange on microtubules is a function of time in the cell cycle.

In this work, we have tested the hypothesis that p34<sup>cdc2</sup> kinase directly affects the microtubule-stabilizing ability of MAP4 by phosphorylating the MAP at mitosis. Association of p34<sup>cdc2</sup> kinase with MAP4 in vitro and probably in vivo suggests that this hypothesis is likely. Further support for this hypothesis derives from our demonstration that (a) MAP4 did become phosphorylated in microtubule fractions by a microtubule-associated histone H1 kinase that is likely to correspond to p34<sup>cdc2</sup> kinase; (b) purified p34<sup>cdc2</sup>/cyclin B kinase efficiently phosphorylated purified MAP4 in vitro and PA<sub>4</sub> in a ternary complex of p34<sup>cdc2</sup>/cyclin B/PA<sub>4</sub>; and (c) phosphorylation by p34<sup>cdc2</sup> kinase reduced the ability of MAP4 to stabilize microtubules, consistent with the increase in microtubule dynamics that has been noted in mitotic cells (Salmon et al., 1984).

The sequence (Ser/Thr)-Pro-X-(Lys/Arg) is generally accepted to be the phosphorylation consensus sequence for p34<sup>cdc2</sup> kinase (Moreno and Nurse, 1990). There are two consensus sequences for phosphorylation by p34<sup>cdc2</sup> kinase within the Pro-rich region of MAP4 (enclosed in boxes in Fig. 9); in contrast, no such sequence is present in the AP sequence region. These two Ser-Pro-X-Lys sequences are completely conserved among human, bovine and mouse MAP4, while the Ser-Pro and Thr-Pro sequences (indicated by double underlines in Fig. 9), which were initially suggested as putative phosphorylation sites in bovine MAP4 by Aizawa et al. (1991b), are not found in human MAP4 (Aizawa et al., 1990; Chapin and Bulinski, 1991; West et al.,

1991). Preliminary results using antibodies that recognize the phosphorylated form of these putative phosphorylation sites suggest that one or both of these is indeed phosphorylated during mitosis (our unpublished observation). Further studies will be needed in order to establish whether phosphorylation of SPXK sites is crucial for the M-phase regulation of MAP4 function.

MAP4 has been reported to be phosphorylated by protein kinase C (Mori et al., 1991) and MAP kinase (Hoshi et al., 1992) and phosphorylation by either kinase decreases the ability of MAP4 to stimulate polymerization of microtubules in vitro. The protein kinase C-phosphorylation site has been shown to be within the Pro-rich region (Mori et al., 1991). It is reasonable to expect that introduction of negative charges into the Pro-rich region by phosphorylation might result in a suppression of the microtubule-stabilizing ability of MAP4.

### **MAP4 Stabilizes Microtubules by Enhancing the Rescue Frequency and Phosphorylation by p34<sup>cdc2</sup> Kinase Diminishes Its Stabilizing Effect**

We have documented the effects of MAP4 on microtubule dynamics, for the first time. MAP4 concentrations used were insufficient to stimulate microtubule polymerization initially; the 24–26°C temperature of observation was also relatively unfavorable for microtubule-polymerization. Therefore, in our experiments both the rate of microtubule-polymerization and the frequency of rescue events were lower than those obtained by Walker et al. (1988). However, even under these conditions, HeLa MAP4 strongly stabilized microtubules, and this stabilization was shown to be mainly due to the increase in rescue frequency at the plus end of microtubules.

In contrast to results with unphosphorylated MAP4, microtubules depolymerized completely in the presence of phosphorylated MAP4. Although this result is overtly similar to results obtained with microtubules devoid of bound MAP4, we found that MAP4 phosphorylation did not lead to a complete loss of binding of MAP4 to microtubules. The occurrence of a reduced frequency of rescue in the presence of bound, phosphorylated MAP4 is in contrast to the complete lack of rescue in the absence of MAP4. It is possible that MAP4 phosphorylation decreases the microtubule-stabilizing ability of MAP4 by reducing its affinity to bind microtubules, as was suggested to occur with tau protein (Drechsel et al., 1992). If MAP4's affinity for microtubules were reduced, the MAP might be ineffective at stopping microtubule depolymerization, regardless of the fact that it was still bound to microtubules. Moreover, a lower affinity of binding could decrease the number (density) of MAP4 molecules on microtubules and, as a result, the shortening distance (perhaps the distance between MAP4 molecules on the microtubule) would increase.

The decrease in MAP4's microtubule-stabilizing ability brought about by phosphorylation was shown to correspond to a decrease in the frequency of rescue at the plus end of microtubules. Other dynamic parameters were not similarly affected by phosphorylation. In particular, those of the minus end appeared relatively insensitive to the presence or absence of MAPs (Itoh and Hotani, 1994) and to the phosphorylation of MAP4. A similar decrease in rescue fre-

quency was reported by Verde et al. (1992) with *Xenopus* egg extracts that were brought to mitotic phase from interphase by addition of cyclin B or cyclin A, as well as by Glicksman et al. (1992) with sea urchin egg extract that was induced to a mitotic-like state by okadaic acid. In other words, phosphorylation of MAP4 by p34<sup>cdc2</sup> kinase apparently renders MAP4-stabilized microtubules more dynamic, analogous to what has been observed for mitotic microtubules by microinjection of fluorescently labeled tubulin (Salmon et al., 1984; Hamaguchi et al., 1985; Belmont et al., 1990; Verde et al., 1992) and MAPs (MAP4 and MAP2) (Olmsted et al., 1989).

We thank Dr. N. Lomax (Drug Synthesis and Chemistry Branch, National Cancer Institute, MD) for providing taxol, A. Okuyama (Bnayu Tsukuba Research Institute, Tsukuba, Japan) for providing butyrolactone I, and Drs. J. Pines (Cambridge, UK), Steven Schiff and F. Matsumura for supplying antiserum against human cyclin B, monoclonal antibodies to cyclin B, anti-human p34<sup>cdc2</sup> carboxy-terminal peptide, respectively. The authors would like to express their thanks to Dr. K. Ohsumi for his valuable suggestions and discussions and to K. Yamada for his help in a large scale culture of HeLa cells.

This work was supported by grants from the Yamada Science Foundation (to S. Hisanaga), the American Cancer Society (to J. C. Bulinski), the Ministry of Education, Science, and Culture, Japan, the Toray Science Foundation, and the Mitsubishi Science Foundation (to T. Kishimoto).

Received for publication 18 August 1994 and in revised form 14 November 1994.

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