Association of p34^cdc2/cyclin B complex with microtubules in starfish oocytes

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SUMMARY

The microtubular cytoskeleton exhibits a dramatic reorganization, progressing from interphase radial arrays to a mitotic spindle at the G2/M transition. Although this reorganization has been suspected to be caused by maturation promoting factor (MPF: p34^cdc2/cyclin B complex), little is known about how p34^cdc2 kinase controls microtubule networks. We provide evidence of the direct association of the p34^cdc2/cyclin B complex with microtubules in starfish oocytes. Anti-cyclin B staining of detergent-treated oocytes, isolated asters and meiotic spindles revealed fluorescence associated with microtubule fibers, chromosomes and centrosomes. Microtubules prepared from starfish oocytes were associated with cyclin B and p34^cdc2 proteins. Microtubule-bound p34^cdc2 and cyclin B were released from microtubules by a high-salt solution and possessed a complex form as shown by the adsorption to sucl-beads and by immunoprecipitation with the anti-cyclin B antibody. The p34^cdc2/cyclin B complex associated to microtubules had high histone H1 kinase activity at meiotic metaphase. However, it was not necessary for the p34^cdc2/cyclin B complex to be active for microtubule binding, as an inactive form in immature oocytes was also observed to bind to microtubules. The coprecipitation of sucl-column purified p34^cdc2/cyclin B with purified porcine brain microtubules in the presence of starfish oocyte microtubule-associated proteins (MAPs) indicates that the association of p34^cdc2/cyclin B with microtubules in vitro is mediated by MAPs.

Key words: cdc2, cyclin B, microtubules, MAPs, starfish oocytes

INTRODUCTION

In eukaryotic cells, the onset of M-phase is controlled by maturation promoting factor (MPF). MPF consists of at least two subunits; p34^cdc2, the 34 kDa product of the cdc2/CDC28 gene, and a 45-62 kDa protein known as cyclin B (for reviews, see Kishimoto, 1988; Murray and Kirschner, 1989; Nurse, 1990). p34^cdc2 has been identified as the catalytic subunit of histone H1 kinase, which is specifically activated at G2/M transition and inactivated at the onset of anaphase. Extensive studies have been conducted to elucidate the mechanisms leading to activation and inactivation of p34^cdc2 kinase during the cell cycle. The association with cyclin B and the phosphorylation/dephosphorylation of p34^cdc2 have been shown to represent key regulatory steps (Gould and Nurse, 1989; Solomon et al., 1990; Krek and Nigg, 1991; also see Maller, 1991; Millar and Russell, 1992, for reviews).

Profound structural changes, including chromosome condensation, nuclear envelope breakdown and cytoskeletal reorganization are observed in cells during the G2 to M-phase transition (Gerhart, 1980). M-phase-specific events are thought to be downstream of the MPF action. Although a great deal is known about the activation mechanism of p34^cdc2 kinase, relatively little is known about the mechanisms by which MPF regulates M-phase events. Further, how one enzyme can modulate so many events is still unknown. Although several candidate substrates for p34^cdc2 kinase have been reported (see Moreno and Nurse, 1990, for a review), the identification of in vivo substrate proteins remains to be performed.

Reorganization of the microtubule cytoskeleton is an M-phase-specific event. Mitotic spindle microtubules assembled after dissolution of the interphase network of microtubules acquire a highly dynamic property (Belmont et al., 1990; Verde et al., 1990). It is believed that microtubules are destabilized during mitosis as a result of the phosphorylation of microtubule-associated proteins (MAPs). Although p34^cdc2 kinase is known to phosphorylate MAPs in vitro (Erikson and Maller, 1989; Aizawa et al., 1991) and p34^cdc2 kinase has been shown to affect microtubule dynamics in cell-free extracts of Xenopus eggs (Verde et al., 1990), definitive proof that the kinase directly phosphorylates MAPs in vivo is still lacking.

If p34^cdc2 kinase is responsible for the reorganization of microtubule networks, how it regulates such processes is
unknown. One possible mechanism is through the approximation of the kinase for specific MAP proteins, which are phosphorylated at mitosis. In fact, numerous reports have described the localization of the p34\(^{cdk2}\)/cyclin B complex on the mitotic spindle, especially around centrosomes (Riabowol et al., 1989; Bailly et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Pines and Hunter, 1991; Maldonado-Codina and Glover, 1992). However, all of these reports were based on immunofluorescence observations, and there is little evidence to reveal the direct interaction between p34\(^{cdk2}\) kinase and microtubule proteins.

During the course of our study on the relocation of p34\(^{cdk2}\)/cyclin B at meiosis reinitiation in starfish oocytes, we observed that the p34\(^{cdk2}\)/cyclin B complex accumulated in premeiotic asters and spindles (Ookata et al., 1992b). However, it was unclear whether the staining was associated with microtubules themselves or with other materials within the asters and spindles. To determine whether the association of the p34\(^{cdk2}\)/cyclin B complex with microtubules is direct or not, we isolated asters and meiotic spindles from maturing oocytes and stained them with anti-cyclin B. We further investigated the association of p34\(^{cdk2}\)/cyclin B with microtubules in vitro by using oocyte extracts and purified proteins. As a result, we are able to present evidence to indicate that the p34\(^{cdk2}\)/cyclin B complex associates with microtubules via microtubule-associated proteins (MAPs).

**MATERIALS AND METHODS**

**Animals and oocyte preparation**

Starfish, Asterina pectinifera, collected during the breeding season, were kept in laboratory aquaria supplied with circulating seawater (14°C). Immature oocytes without follicle cells were released from isolated ovaries after treatment with Ca\(^{2+}\)-free seawater. Meiosis reinitiation of starfish oocytes was induced by the addition of 1 mM 1-methyladenine to seawater at 22°C (Tachibana et al., 1990). Rattner et al., 1990; Pines and Hunter, 1991; Maldonado-Codina and Glover, 1992).

**Protein preparation**

Starfish oocyte microtubules were prepared using modifications of the method of Vallee and Bloom (1983). Starfish oocytes were homogenized in 3 vol of 0.1 M Pipes, pH 6.8, 2 mM EGTA, 1 mM MgCl\(_2\) (PEM) containing 0.9 M glycerol, 1 mM PMSF, 10 μg/ml leupeptin and 0.1 mM DTT at 4°C, and were centrifuged at 100,000 g for 60 min at 4°C. Taxol and GTP were added to the supernatant to final concentrations of 20 μM and 0.5 mM, respectively. The solution was warmed to 30°C for 10 min and then chilled on ice for 10 min. Polymerized microtubules were collected by centrifugation at 40,000 g for 30 min at 4°C through a cushion of PEM containing 10% sucrose. The resulting pellets were suspended in PEM containing 0.75 M NaCl, 10 μM taxol and 0.5 mM GTP at 30°C. After centrifugation at 100,000 g for 30 min at 30°C, the supernatant was dialyzed against PEM containing protease inhibitors, and was used as the crude microtubule-associated proteins (MAPs). The p34\(^{cdk2}\)/cyclin B complex isolated in the MAPs fraction was depleted by treatment with p13c~-Sepharose beads.

The p34\(^{cdk2}\)/cyclin B complex was purified from starfish oocytes by p13c~-affinity column chromatography (Kusubata et al., 1992; E. O. and T. K., unpublished). Starfish oocytes at the first meiotic metaphase were crushed in 240 mM sodium β-glycerophosphate, 60 mM EGTA, 45 mM MgCl\(_2\), 1 mM DTT, 10 μg/ml cytochalasin B, pH 7.3, by centrifugation at 130,000 g for 2 h at 4°C (Kishimoto and Kondo, 1986). The supernatant was mixed with an equal vol. of extraction Buffer A (160 mM sodium β-glycerophosphate, 40 mM EGTA, 30 mM MgCl\(_2\), 2 mM DTT, 1 mM ATP, S, 40 μg/ml leupeptin, 0.6 mM phenylmethylsulfonyl fluoride, pH 7.3) containing 200 mM sucrose and 200 mM KCl, and centrifuged at 100,000 g for 1 h. The supernatant was removed and applied to a p13c~-Sepharose 4B column equilibrated with a half concentration of Buffer A containing 0.1% Brij 35 (Buffer B). After washing the column with Buffer B containing 0.5 M NaCl, the p34\(^{cdk2}\)/cyclin B complex was eluted with Buffer B containing both 0.5 M NaCl and 50% ethylene glycol. p13c~-Sepharose beads were prepared as described previously (Ookata et al., 1992b; Kusubata et al., 1992). Porcine brain tubulin was purified from two-cycled microtubule proteins using a phosphocellulose column according to the method of Weingarten et al. (1975).

**Immunofluorescent staining of frozen sections, permeabilized oocytes and isolated asters and meiotic spindles**

Immunofluorescent staining of frozen sections and permeabilized oocytes was performed as described previously (Ookata et al., 1992b).

1-Methyladenine-activated oocytes were treated with 20 μM taxol for 20 min to promote premeiotic aster development and stabilization. At 30 min and 45 min after 1-methyladenine addition, premeiotic asters and first meiotic spindles, respectively, were isolated by vigorous shaking of the oocytes (Hisanaga et al., 1987) in a microtubule-stabilizing buffer (25 mM imidazole, pH 6.9, 10 mM KCl, 10 mM EGTA, 20% glycerol, 1% Triton X-100, Shirai et al., 1990).

Isolated premeiotic asters and meiotic spindles were air-dried on a glass slide and then treated with methanol for 10 min at −20°C. After blocking with 3% skim milk in Tris-buffered saline (TBS), each preparation was incubated for 1 h at room temperature with affinity-purified anti-cyclin B antibody (Ookata et al., 1992b) or monoclonal anti-tubulin antibody, followed by washing with TBS containing 0.01% Triton X-100. The specimens were reacted with FITC-conjugated anti-mouse IgG (Cappel) diluted to 1:100 in TBS for 1 h at room temperature, then washed with TBS and mounted with TBS containing 20% glycerol and 100 μg/ml 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Aldrich). Specimens were observed with an epifluorescence microscope (Olympus, Tokyo).

**Cosemination assay**

The association of the purified p34\(^{cdk2}\)/cyclin B complex with microtubules was examined with a cosemination assay. The purified p34\(^{cdk2}\)/cyclin B complex, which was dialyzed against PEM containing 0.1% Nonidet P-40 (inclusion of non-ionic detergent to prevent non-specific aggregation of the purified p34\(^{cdk2}\)/cyclin B was essential), was incubated with microtubules polymerized from porcine brain tubulin with the aid of taxol in the presence or absence of starfish oocyte MAPs, at 30°C for 10 min. The mixture was then centrifuged at 40,000 g for 30 min at 2°C, and the resulting supernatant and pellet were analyzed by western blotting with antibodies against starfish cyclin B and PSTAIR peptide.

**Histone H1 kinase assay**

Histone H1 kinase activity was assayed as described previously (Hisanaga et al., 1991). The microtubule precipitate and the supernatant from oocyte extracts were incubated with 1.2 mg/ml histone H1 in PEM containing 0.1 mM [γ-\(^{32}\)P]ATP for 6 min at 25°C. Reactions were stopped by the addition of SDS-sample buffer and boiled for 2 min. Samples were run on a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The gel was autora-
RESULTS

Electrophoresis, western blotting, immunoprecipitation, and protein determination
SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) with 8%, 10% or 12.5% acrylamide gels stained with Coomassie blue.

For immunoblotting, proteins were transferred onto nitrocellulose according to Towbin et al. (1979). The membrane was blocked with TBS containing 3% skim milk and subsequently incubated with the affinity-purified anti-cyclin B antibody or monoclonal anti-PSTAIR antibody for 1 h at room temperature. After washing with TBS, the membrane was incubated with alkaline phosphatase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (DAKO) at a dilution of 1:400 for 30 min. The antibodies were located with a BCIP/NBT phosphatase substrate assay system (KPL) after washing with TBS.

Immunoprecipitation of the p34cdc2/cyclin B complex with anticyclin B antibodies was performed as described previously (Ookata et al., 1992b). A 40 µl sample of the high-salt extract from the microtubule precipitate, diluted 2-fold with PEM, was incubated at 4°C for 60 min with 10 µl anti-cyclin B serum, which had been diluted 10-fold with TBS. At the end of incubation, 30 µl of a 50% slurry of Protein A-Sepharose CL4B was added and the mixture was further incubated for 90 min at 4°C. The antibody-containing pellet was recovered by centrifugation at 12,000 g for 3 min at 4°C, washed three times in 1 ml of the immunoprecipitation buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM Na pyrophosphate, 0.1 mM Na-orthovanadate, 10 mM p-nitrophenylphosphate and 0.1 mM ZnCl2) and added to 40 µl of 2× concentrated SDS-PAGE sample buffer. After boiling for 5 min, 10 µl of the supernatant was processed for SDS-PAGE and western blotting with anti-PSTAIR antibody.

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

RESULTS

Colocalization of cyclin B protein with microtubules in premeiotic asters and meiotic spindles
As has also been reported with HeLa cells (Pines and Hunter, 1991; Bailly et al., 1992) and Drosophila embryos (Maldonado-Codina and Glover, 1992), we showed in a previous paper with starfish oocytes (Ookata et al., 1992b) that the p34cdc2/cyclin B complex accumulated in meiotic spindles at M-phase. To reveal the relationship of p34cdc2/cyclin B localization with microtubules more clearly, we performed immunofluorescent staining of starfish oocytes with anti-cyclin B antibody using three different preparations of specimens. Since all cyclin B formed a complex with p34cdc2 in starfish oocytes at the first meiotic cycle (Ookata et al., 1992b), anti-cyclin B is a probe for the detection of the p34cdc2/cyclin B complex. The anti-cyclin B antibody used in this study was affinity-purified with Immobilon membranes blotted with Escherichia coli-expressed starfish cyclin B protein (Tachibana et al., 1990; Ookata et al., 1992b).

Fig. 1A, B and C show a frozen section of starfish oocytes at the first meiotic metaphase stained with anti-cyclin B, anti-tubulin and DAPI, respectively. Cyclin B staining was evident in the spindle region where intense tubulin staining was observed. Because of the poor resolution, however, it was difficult to correlate the cyclin B staining with spindle microtubules.

To attain a higher staining resolution in the meiotic spindles, soluble proteins including the soluble form of the p34cdc2/cyclin B complex were removed by treatment with detergent. Whole-mount immunofluorescent staining with anti-cyclin B has already been presented in our previous paper (Ookata et al., 1992b), but here the tubulin staining was carried out simultaneously to correlate the cyclin B localization with microtubules more clearly. The first meiotic metaphase oocyte shown in Fig. 1D, F (and F inset) was triple-stained with anti-cyclin B, anti-tubulin and DAPI, respectively. The accumulation of cyclin B in the meiotic spindle was observed more distinctly than in the frozen sections and the staining of cyclin B almost overlapped with that of tubulin except for chromosome-staining (Fig. 1D and F). A control oocyte reacting only with FITC-conjugated second antibody is shown in Fig. 1E; no specific signal was obtained. Specific staining was also not observed with a premekine serum of anti-cyclin B, as reported previously (Ookata et al., 1992b).

We continued further to confirm this association with isolated meiotic spindles. Premeiotic asters were also isolated to verify our previous observation that part of cyclin B accumulated in premeiotic asters when p34cdc2/cyclin B is translocated into the nucleus from the cytoplasm (Fig. 7A of Ookata et al., 1992b). Since premeiotic asters are too small for isolation, taxol was used for their enlargement and stabilization. The affinities of MAPs for microtubules are known not to be changed by taxol (Vallee, 1986).

Isolated asters and spindles were stained with the affinity-purified anti-cyclin B (Fig. 2B and D) as for the staining of permeabilized oocytes (Fig. 1D). Although double staining with anti-cyclin B and anti-tubulin was not performed in Fig. 2, the staining of microtubule fibers with anti-cyclin B was clearly observed along the periphery of asters and spindles (arrowheads in Fig. 2B and D), suggesting the association of p34cdc2/cyclin B with microtubule fibers. In contrast to tubulin staining (Fig. 2A and C), with which chromosomes and the centrosomal region failed to stain, the antibody against cyclin B also stained the chromosomes aligned at the equatorial line in isolated spindles (Fig. 2B, arrow) and the centrosomal region of isolated asters (Fig. 2D). These results are consistent with reported observations that p34cdc2 and/or cyclin B also associates with centrosomes and chromosomes (Riabowol et al., 1989; Bailly et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Pines and Hunter, 1991; Ookata et al., 1992b; Maldonado-Codina and Glover, 1992).

Microtubules prepared from the starfish oocyte extract is associated with p34cdc2/cyclin B complex
The association of p34cdc2 and cyclin B with microtubules was examined in isolated microtubules by immunoblotting with anti-cyclin B and anti-PSTAIR antibodies. Microtubule proteins were prepared from maturing oocytes with the aid of taxol. Starfish oocyte microtubule proteins were
composed of tubulin (indicated by Tu in Fig. 3A, lane 2) and many minor proteins with molecular masses higher than tubulin. Some of these minor proteins may very well represent MAPs of starfish oocytes. Thus far only one MAP has been identified in starfish oocytes, a 70 kDa protein described by Hosoya et al. (1990), which likely corresponds to the 70 kDa protein seen in our preparations (arrow in Fig. 3A, lane 2).

While the affinity-purified anti-cyclin B recognized only a 48 kDa cyclin B protein, the monoclonal anti-PSTAIR antibody reacted with three polypeptides, which were designated as the upper (U), middle (M) and lower (L) bands of p34cdc2 (Ookata et al., 1992b), depending on the stage of oocyte maturation. The U band in the immature oocytes changed to the L band after reinitiation of maturation, probably by dephosphorylation. Among the three polypeptides, the U and L bands bound to p13suc1-conjugated Sepharose 4B together with cyclin B, indicating that U and L, but not M, represent a component of MPF (Ookata et al., 1992b).

The microtubule precipitate from the first meiotic metaphase oocytes contained proteins stained with anti-cyclin B and anti-PSTAIR antibodies. Certain amounts, but not all, of cyclin B and p34cdc2 in the crude extract were recovered in the microtubule pellet (Fig. 3B, lanes 1 and 2). In a control experiment without taxol, in which oocyte microtubules were not polymerized, only trace amounts of cyclin B and p34cdc2 were detected in the precipitates (data not shown), indicating that the precipitation of p34cdc2 and cyclin B was a microtubule-dependent phenomenon. More importantly, the L band of p34cdc2, which is a component of the active MPF, was selectively precipitated with microtubules, while the majority of the M band remained in the supernatant.

A characteristic of most MAPs is their extractability from microtubules by increased salt concentrations. If the association of p34cdc2 and cyclin B with microtubules is ionic as that of most MAPs, or alternatively, if p34cdc2 and/or cyclin B associate with microtubules via MAPs, they would be sensitive to salt extraction. To investigate this possibility, isolated microtubules from maturing oocytes were treated with PEMG buffer containing various concentrations of NaCl, and the resulting supernatant and precipitate were analyzed by immunoblotting. Both p34cdc2 and cyclin B began to appear in the supernatant at 0.3 M NaCl together with 70 kDa MAP, and almost the same amounts were recovered in the supernatants at 0.5 and 0.75 M NaCl extraction. The result obtained with 0.75 M NaCl treatment is shown in Fig. 3C. In contrast to the M band, which was only partly removed from the precipitate with a high-salt solution, the L band of p34cdc2 as well as cyclin B were shown to be released into the high-salt supernatant (Fig. 1.)
This also indicates that the precipitation of the L band, but not the M band, of p34^{cdc2} with microtubules was not a result of nonspecific aggregation.

Whether microtubule-bound p34^{cdc2} and cyclin B form a complex was examined by adsorption to p13^{suc1}-conjugated Sepharose 4B and by immunoprecipitation with the anti-cyclin B antibody. It was clearly seen that cyclin B bound to suc1-beads together with p34^{cdc2} (Fig. 3C, lanes 3 and 4). Further, p34^{cdc2} was observed to precipitate together with cyclin B protein by anti-cyclin B antibody (Fig. 3C, lanes 5 and 6). These results are consistent with our previous observation that all cyclin B forms a complex with p34^{cdc2} in the starfish oocyte (Ookata et al., 1992b), and clearly also indicate that a complex is formed between microtubule-bound p34^{cdc2} and cyclin B.

To assess whether the association of the p34^{cdc2}/cyclin B complex with microtubules depends on its kinase activity, microtubules were prepared from immature oocytes and were immunoblotted as described above. Starfish immature oocytes reportedly contain a stockpile of the p34^{cdc2}/cyclin B complex in an inactive form, pre-MPF (Pondaven et al., 1990; Strausfeld et al., 1991; Ookata et al., 1992b; E.O. and T.K., unpublished observation). Both p34^{cdc2} and cyclin B in the immature oocytes also precipitated in a microtubule-dependent manner (Fig. 3B, lanes 3 and 4). More p34^{cdc2}/cyclin B tended to coprecipitate with microtubules in immature oocytes than in maturing oocytes. In this case, the U band of p34^{cdc2}, which forms a complex with cyclin B as pre-MPF, was specifically coprecipitated with microtubules. This result indicates that the association of the p34^{cdc2}/cyclin B complex with microtubules is independent of histone H1 kinase activity.

To determine the extent of p34^{cdc2} kinase association

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Fig. 2. Cyclin B and tubulin immunofluorescent staining of asters and meiotic spindles isolated from maturing starfish oocytes. Meiotic spindles (A and B) and asters (C and D) were isolated from starfish oocytes at the first meiotic metaphase or from oocytes treated for 20 min with 20 µM taxol 10 min after the addition of 1-methyladenine as described in Materials and Methods. Tubulin was detected with a mouse monoclonal antibody and fluorescein-conjugated second antibody (A and C). Cyclin B was detected with anti-cyclin B antibody and FITC-conjugated goat antibody to rabbit IgG (B and D). Microtubules stained with anti-cyclin B are indicated by arrowheads in (B) and (D). Chromosomes aligned at the equatorial region are indicated by the arrow in (B). Bar, 20 µm.

Fig. 3. Immunoblots representing the co-precipitation of p34^{cdc2} and cyclin B with microtubules in starfish oocyte extracts. Microtubules were isolated from high-speed supernatants with the aid of taxol. (A) Protein staining of the microtubule precipitate (lane 2) and supernatant (lane 1) from maturing oocytes (30 min after addition of 1-methyladenine). Tubulin and 70 kDa MAP are indicated by Tu and the arrow in lane 2, respectively. (B) Immunoblots of the microtubule precipitates (lanes 2 and 4) and the supernatants (lanes 1 and 3) from maturing oocytes (30 min after addition of 1-methyladenine). Tubulin and 70 kDa MAP are indicated by Tu and the arrow in lane 2, respectively. (B) Immunoblots of the microtubule precipitates (lanes 2 and 4) and the supernatants (lanes 1 and 3) from maturing oocytes (30 min after addition of 1-methyladenine). Tubulin and 70 kDa MAP are indicated by Tu and the arrow in lane 2, respectively. (B) Immunoblots of the microtubule precipitates (lanes 2 and 4) and the supernatants (lanes 1 and 3) from maturing oocytes (30 min after addition of 1-methyladenine). Tubulin and 70 kDa MAP are indicated by Tu and the arrow in lane 2, respectively. (B) Immunoblots of the microtubule precipitates (lanes 2 and 4) and the supernatants (lanes 1 and 3) from maturing oocytes (30 min after addition of 1-methyladenine). Tubulin and 70 kDa MAP are indicated by Tu and the arrow in lane 2, respectively. (B) Immunoblots of the microtubule precipitates (lanes 2 and 4) and the supernatants (lanes 1 and 3) from maturing oocytes (30 min after addition of 1-methyladenine). Tubulin and 70 kDa MAP are indicated by Tu and the arrow in lane 2, respectively.
with microtubules, histone H1 kinase activities in the microtubule precipitate and supernatant were measured (Fig. 4). More than 90% of Ca\(^{2+}\) and cyclic nucleotide-independent histone H1 kinase activity in starfish oocytes at the first meiotic M- phase has been shown to be due to p34\(^{cdc2}\) kinase (Labbe et al., 1988). As expected, since MPF (or histone H1 kinase) is inactive in immature oocytes, histone H1 phosphorylation was not detected in the microtubule fraction obtained from immature oocytes (Fig. 4, lane 3). On the other hand, microtubules obtained from maturing oocytes (lanes 2 and 4) at 30 min after the addition of 1-methyladenine. Microtubule precipitate was suspended in one-third vol. of supernatant. Equal vols of the microtubule suspension and supernatant were processed for histone H1 kinase measurement. Histone H1 is indicated by H1. Size standards (in kDa) are shown on the left.

**Association of purified p34\(^{cdc2}\)/cyclin B complex with microtubules reconstructed from porcine brain tubulin and starfish MAPs**

The binding of the p34\(^{cdc2}\)/cyclin B complex to microtubules was ascertained with purified proteins. P34\(^{cdc2}\)/cyclin B was purified from starfish oocytes at the first meiotic metaphase with a p13\(^{suc1}\)-affinity column (Fig. 5A, lane 1); tubulin was purified from porcine brain (Fig. 5A, lane 2). The high-salt extract of starfish oocyte microtubules was used as the starfish MAPs (Fig. 5A, lane 3). The p34\(^{cdc2}\)/cyclin B complex contained in the starfish MAPs fraction was depleted by incubation with p13\(^{suc1}\) beads. When the p34\(^{cdc2}\)/cyclin B complex was incubated with microtubules in the presence or absence of starfish MAPs and centrifuged, a greater amount of the complex coprecipitated with microtubules in the presence of starfish MAPs than in their absence (Fig. 5B, lanes 4 and 6). This was more clearly demonstrated by measuring the histone H1 kinase activity recovered in the microtubule precipitates and the supernatants (Fig. 5C). A similar result was also obtained with porcine brain whole MAPs or purified HeLa MAP4 (in preparation). These results suggest that the association of the complex with microtubules is mediated by MAPs.

A small amount of the purified p34\(^{cdc2}\)/cyclin B precipitated with MAP-free microtubules. Although we cannot rule out the possibility that a small fraction of p34\(^{cdc2}\)/cyclin B could bind to tubulin, we suspect that this may represent a non-specific aggregation of the p34\(^{cdc2}\)/cyclin B complex. The purified p34\(^{cdc2}\)/cyclin B tended to aggregate in a low salt solution in the absence of Nonidet P-40.

**Microtubule proteins contain substrates for p34\(^{cdc2}\) kinase**

To determine if the microtubule precipitates contained a substrate for p34\(^{cdc2}\) kinase, microtubule proteins from the first meiotic metaphase were incubated with [\(\gamma\)\(^{32}\)P]ATP without exogenous substrate. Several proteins of higher
molecular mass than tubulin were phosphorylated in the precipitated microtubules obtained from maturing oocytes (Fig. 6, lane 4). Although a band corresponding to tubulin was also phosphorylated to some extent, this was probably not due to p34cdc2 kinase, because phosphorylation of the band was observed with immature oocytes as well (Fig. 6, lane 2). Further, p13ync1-column-purified p34cdc2 kinase did not phosphorylate tubulin obtained from porcine brain (data not shown). However, phosphorylation of other proteins was mediated by p34cdc2 kinase in the microtubule fraction, as similar bands in microtubule precipitates prepared from immature oocytes were phosphorylated by the addition of purified p34cdc2 kinase (Fig. 6, lane 3).

DISCUSSION

Despite the many reports concerning the colocalization of p34cdc2 and/or cyclin B with the mitotic spindle (Riabowol et al., 1989; Bailly et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Pines and Hunter, 1991; Ookata et al., 1992b; Maldonado-Codina and Glover, 1992), it is still unresolved whether p34cdc2 kinase is associated directly with microtubules or is localized to a spindle matrix such as that described by Steffen and Link (1992). Previous reports have relied on immunofluorescent staining using anti-p34cdc2 or anti-cyclin protein, and therefore, until now, there has been no biochemical evidence to resolve this issue.

We examined the association of p34cdc2/cyclin B complex with microtubules by three different procedures: (1) immunofluorescence localization of cyclin B protein in frozen sections of starfish oocytes, detergent-treated oocytes and isolated microtubular cytoskeletons (premeiotic asters and meiotic spindles); (2) the coprecipitation of p34cdc2/cyclin B complex with microtubules polymerized in oocyte extracts; and (3) the coprecipitation of purified p34cdc2/cyclin B complex with reconstituted microtubules in vitro. The results obtained here indicate that the p34cdc2/cyclin B complex associates with microtubules themselves and that the association can be mediated by MAPs.

Tombes et al. (1991) also reported the presence and partial purification of cdc2-like M-phase kinase from isolated mitotic spindles of CHO cells. Immunolocalization with anti-p34cdc2 and anti-cyclin B revealed the staining of microtubules, poles and kinetochores of isolated mitotic spindles. However, the absence of stainable chromosomes, this staining pattern is similar to that of isolated starfish meiotic spindles. On the other hand, Bailly et al. (1992) reported that the p34cdc2/cyclin B complex binds to detergent-resistant substances independently of the state of microtubules in HeLa cells. Cyclin B staining along microtubules was observed only after treatment with detergent but not in intact cells. Their interpretation was that the detergent treatment induced a collapse of cyclin B-associated structures on microtubule bundles, though they did recognize the partial colocalization of cyclin B1 to the mitotic spindle. In the case of the starfish oocyte, it appears certain that at least a part of p34cdc2/cyclin B associates with microtubular structures, as its accumulation in spindles was also observed in frozen sections of intact cells (Fig. 1A).

More distinct staining was obtained with lysed oocytes (Fig. 6, lane 4), and isolated meiotic spindles (Fig. 2). This may be due partly to the release of the soluble p34cdc2/cyclin B complex for the lysed oocytes and the further removal of p34cdc2/cyclin B associated with detergent-resistant cytoskeletons for the isolated asters and spindles, although we cannot rule out the possibility that the lysis or isolation procedure using detergent increased the amount of p34cdc2/cyclin B associated with asters and spindles.

The association of p34cdc2/cyclin B with microtubules was biochemically shown here for the first time. This association may be mediated by MAPs as indicated by in vitro reconstitution experiments using purified p34cdc2/cyclin B. In these experiments we did not specify the oocyte MAP(s) with which the p34cdc2/cyclin B complex associates, but we have already obtained evidence in HeLa cells that p34cdc2/cyclin B associates with MAP4, the most predominant, heat stable HeLa MAP (Ookata et al., 1992a). In agreement with our results, based on observations that MAP4 and MAP1B were major endogenous spindle substrates for the M-phase kinase, Tombes et al. (1991) concluded that cdc2-like M-phase kinase in isolated CHO mitotic spindles associated with microtubules via MAPs.

In contrast to the spindle localization of p34cdc2/cyclin B complex at mitosis (Bailly et al., 1989, 1992; Riabowol et al., 1989; Pines and Hunter, 1991; Ookata et al., 1992b), distinct colocalization of p34cdc2 with microtubules was not observed in the interphase cells. This might suggest the involvement of cyclin B protein in the association of p34cdc2 kinase with microtubules. In fact, the interaction of cyclin with microtubules has been genetically indicated with the cdc13 mutant of Schizosaccharomyces pombe (Booher and Beach, 1988). The cdc13-117 mutant, which is hypersensitive to the tubulin inhibitor thiabendazole, failed to form the mitotic spindle but has cytologically normal cytoplasmic interphase microtubules. Then, the cdc13 gene product, which is a yeast homologue of cyclin B protein, is
suggested to have an essential function for mitotic microtubules.

Most of the p34\(^{cd2}/cyclin B\) complex coprecipitated with microtubules when the purified p34\(^{cd2}/cyclin B\) was used for the binding assay, suggesting that p34\(^{cd2}/cyclin B\) has an intrinsic ability to bind to microtubules. However, in vivo interactions of p34\(^{cd2}/cyclin B\) with microtubules seem to be more intricate. Only about one-third of the p34\(^{cd2}/cyclin B\) complex in the oocyte extract coprecipitated with microtubules even when all tubulin was polymerized into microtubules by taxol. Although the possibility exists that there are insufficient MAPs in the oocyte for binding all of the p34\(^{cd2}/cyclin B\) complex, it seems more probable that p34\(^{cd2}/cyclin B\) binds to other proteins that do not interact with microtubules. In addition to microtubular cytoskeletons, anti-cyclin B staining showed its binding to centrosomes, the nuclear matrix and detergent-resistant cytoskeletons in the starfish oocyte (Ookata et al., 1992b) and in HeLa cells (Bailly et al., 1992). Associations of p34\(^{cd2}\) kinase with chromosomes, nuclear lamina and detergent-resistant cytoskeletons have also been demonstrated biochemically (Chambers and Langan, 1990; Chou et al., 1990; Dessev et al., 1991; Kusubata et al., 1992; K. Kishimoto, S. H., and T. K., unpublished observation). Considering that these subcellular structures could have their organization affected by p34\(^{cd2}\) kinase at mitosis, the association might represent a possible targeting mechanism for p34\(^{cd2}\) kinase toward specific substrate proteins.

One potential role of microtubule-bound p34\(^{cd2}/cyclin B\) may be the reorganization of the microtubule cytoskeleton, i.e. conversion from interphase cytoplasmic microtubules to spindle microtubules at the G2/M transition. It has been reported that spindle microtubules are more dynamic than interphase microtubules (Salmon et al., 1984a,b; Hamaguchi et al., 1985; Belmont et al., 1990). Microtubule dynamics are affected by MAPs (Itoh and Hotani, 1988), and the microtubule-polymerizing ability of MAPs is affected by their phosphorylation states (Jameson et al., 1980; Murthy and Flavin, 1983). Further, it was recently reported that the phosphorylation states of MAP4 of tissue culture cells change during the mitotic cycle (Vandre et al., 1991). CHO MAP4 was shown to be phosphorylated at the entry into mitosis and dephosphorylated upon completion of mitosis. Moreover, it is generally believed that phosphorylation of MAPs at M-phase is induced by M-phase-specific kinase, p34\(^{cd2}\) kinase. In fact, 190K MAP, a counterpart of HeLa MAP4 in adrenal medulla, was shown to be phosphorylated by cd2 kinase in vitro and to change the microtubule-polymerizing ability by phosphorylation (Aizawa et al., 1991; Ookata et al., 1992a). In the case of starfish oocytes, several proteins associated with microtubules were phosphorylated by p34\(^{cd2}\) kinase. It appears that phosphorylation changes the microtubule organization during meiotic maturation.

The microtubule-nucleating activity was also shown to be regulated by MPF in Xenopus eggs (Verde et al., 1990). The M-phase extract of Xenopus eggs had a much more potent ability to restore and increase the microtubule-nucleating activity of the inactivated centrosomes than the interphase extract. Furthermore, the ability of the interphase extract to increase the centrosome activity was greatly enhanced by the addition of purified MPF (Buendia et al., 1992; Ohta et al., 1993). Taken together, the reorganization of the microtubule cytoskeleton at the G2/M transition may be considered to be under dual regulation of MPF: phosphorylation of MAPs for the microtubule dynamics, and that of a centrosomal protein(s) for the microtubule nucleation.

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REFERENCES


Bailly, E., Doree, M., Nurse, P. and Bornens, M. (1989). p34\(^{cd2}\) is located in both nucleus and cytoplasm: part is centrosomally associated at G2/M and enters vesicles at anaphase. EMBO J. 8, 3985-3995.


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