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Original Article

Association of p34^{cdc2} kinase and MAP kinase with microtubules during the meiotic maturation of *Xenopus* oocytes

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ABSTRACT **p34^{cdc2} protein is found in prophase, metaphase and activated *Xenopus* oocytes at a similar level whereas its kinase activity oscillates within meiosis. Using an anti-PSTAIRE antibody that recognizes *Xenopus* p34^{cdc2}, it was demonstrated that the major part of p34^{cdc2} was associated with microtubules isolated *in vitro* from *Xenopus* oocytes. Conversely, tubulin was recovered in association with p34^{cdc2} in p13-Sepharose pellets. The abundance of the fraction of p34^{cdc2} which was associated with microtubules did not oscillate during the meiotic maturation and the activation process. By contrast, the histone H1 kinase activity of p34^{cdc2} estimated in microtubular oocyte pellets was much higher in metaphase than in prophase oocytes. Cyclin B, which is associated *in vivo* with p34^{cdc2} in prophase and metaphase oocytes, was also present in the microtubular fractions. However, cyclin was not necessary for the binding of p34^{cdc2} to microtubules since p34^{cdc2} from activated eggs, where cyclin was missing, still copurified with microtubules. Purified MAP2, but not tubulin, was able to bind to p34^{cdc2}, demonstrating that the association between p34^{cdc2} and microtubules was mediated by microtubule-associated proteins. During the meiotic maturation of *Xenopus* oocytes, several protein kinases were activated, among them MAP kinase. MAP kinase also associated with microtubules. It was demonstrated that both p34^{cdc2} kinase and MAP kinase purified from *Xenopus* oocytes were able to phosphorylate *in vitro* rat brain MAP2. However both protein kinases phosphorylated different domains of MAP2, suggesting that they might regulate microtubules in different ways.**

KEY WORDS: *Xenopus oocyte, meiotic maturation, p34^{cdc2}, microtubules, MAP2, MAP kinase*

Introduction

The G2/M transition of the cell cycle in eukaryotic cells is regulated by a factor called MPF (M-phase promoting factor). MPF is a complex between a 34 kDa Ser/Thr protein kinase called p34^{cdc2} (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Lohka *et al.*, 1988) and cyclin B (Labbé *et al.*, 1988; Lohka *et al.*, 1988). Although p34^{cdc2} is present in the cells at a similar level during the whole cell cycle, its histone H1 kinase activity is activated specifically during M-phase (Arion *et al.*, 1988; Draetta and Beach, 1988; Labbé *et al.*, 1988). Activation of p34^{cdc2} protein kinase activity is regulated both by its association with cyclin and by phosphorylation (Gautier *et al.*, 1989; Morla *et al.*, 1989; Murray and Kirschner, 1989; Solomon *et al.*, 1992). MPF is known to induce a variety of morphological changes accompanying entry into M-phase, such as chromosome condensation (Earnshaw, 1988), nuclear envelope breakdown (Gerace *et al.*, 1978; Gerace and Blobel, 1980), Golgi apparatus and endoplasmic reticulum vesicularization (Zeligs and Wollman, 1979) and spindle formation (Weber and Osborn, 1979). However, the exact mechanisms by which MPF leads to these morphological changes are still unknown. Several data support the view that microtubular organization could be under the control of p34^{cdc2} kinase (Verde *et al.*, 1990, 1992).

The *Xenopus* oocyte is a particularly suitable cell to study the relationship between MPF activation and microtubular reorganization. *Xenopus* oocytes are physiologically arrested at the G2/M border in the first meiotic prophase. The prophase-blocked oocytes

Abbreviations used in this paper: GCBD, germinal vesicle breakdown; MPF, M-phase or maturation-promoting factor; MAP2, microtubule-associated protein 2; MAPs, microtubule-associated proteins; MAP kinase, mitogen activated protein kinase.

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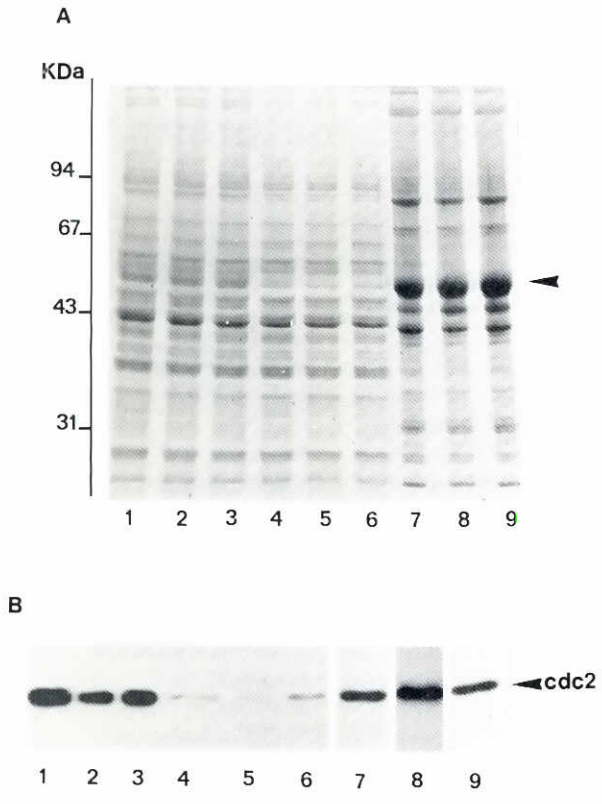

Fig. 1. Association of p34^{cdc2} with microtubules. *Cytosols (lanes 1,2,3), microtubule supernatants (lanes 4,5,6) and microtubular pellets (lanes 7,8,9) were prepared from prophase oocytes (lanes 1,4,7), metaphase oocytes (lanes 2,5,8) and activated eggs (lanes 3,6,9). (1 to 6) Proteins corresponding to 3 oocytes; (7 to 9) proteins corresponding to 10 oocytes.* **(A)** *Coomassie blue staining. Arrow indicates the position of tubulin.* **(B)** *Western blot with the anti-PSTAIR antibody.*

contain a stockpile of inactive pre-MPF, formed by cyclin B and tyrosine 15-phosphorylated p34^{cdc2}. In response to progesterone, inactive pre-MPF is converted into active MPF by dephosphorylation of tyrosine 15 and probably of threonine 14, and prophase-blocked oocytes enter M-phase (reviewed in Jessus and Ozon, 1993). They mature into metaphase II-arrested oocytes. Several protein kinases are activated following MPF activation, as MAP kinase (Haccard *et al.*, 1990; Gotoh *et al.*, 1991a). Metaphase-blocked oocytes can be activated *in vitro* by calcium, a treatment mimicking fertilization. This treatment leads to the destruction of cyclin B, the inactivation of MPF and p34^{cdc2} kinase activity, and the re-entry into an interphase-like state (Murray and Kirschner, 1989; Minshull *et al.*, 1990; Jessus and Beach, 1992). Prophase oocytes contain an extensive array of submembranous cortical microtubules in addition to some scattered microtubules present around the nuclear envelope (Jessus *et al.*, 1986; Huchon *et al.*, 1988). During meiotic maturation, the cortical microtubular array is not altered (Jessus *et al.*, 1986). However, MPF activation induces the apparition of a microtubular cytoplasmic network at the basal part of the disrupting nucleus. This transitory array precedes the formation of the metaphase spindles (Huchon *et al.*, 1981; Jessus *et al.*, 1986). The ability of *Xenopus* oocyte to assemble microtubules is also markedly altered *in vivo* following p34^{cdc2} kinase activation. For example, prophase cytoplasm is unable to organize microtubular cytasters under the action of taxol, an alkaloid agent promoting tubulin polymerization. The situation is dramatically different after p34^{cdc2} kinase activation, when asters can be easily induced by taxol in the metaphase cytoplasm (Heidemann and Gallas, 1980; Heidemann *et al.*, 1985; Jessus *et al.*, 1986, 1987, 1988).

The present paper reports that a direct physical interaction exists between p34^{cdc2} and microtubules of *Xenopus* oocytes and that MAP2 is a good *in vitro* substrate of p34^{cdc2} and MAP kinase, both extracted from *Xenopus* oocytes. However, the MAP2 domains phosphorylated by either p34^{cdc2} kinase or MAP kinase are distinct. Since MAP kinase is also able to associate with microtubules, both kinases might be involved in changes of microtubule organization during the G_2-M transition.

Results

p34^{cdc2} associates with microtubules of Xenopus oocytes

Microtubules were isolated from prophase, metaphase, and activated *Xenopus* oocytes using the taxol procedure which allows a partial purification of tubulin and microtubule associated proteins (Vallee and Bloom, 1983; Jessus *et al.*, 1985). α and ß tubulins are the major proteins recovered in the microtubular pellet. However, a number of proteins remain associated with tubulin in this fraction (Fig. 1A). An affinity-purified rabbit polyclonal antibody directed against the peptide EGVPSTAIRELLKE was used to detect p34^{cdc2} by immunoblotting analysis. This peptide is highly conserved in p34^{cdc2} of all studied species (Lee and Nurse, 1987). As shown in Fig. 1B, the same amounts of p34^{cdc2} were detected in cytosols from prophase and metaphase oocytes and activated eggs. In the microtubular pellets obtained by the taxol procedure, p34^{cdc2} was clearly recognized by the anti-PSTAIRE antibody (Fig. 1B). The amount of p34^{cdc2} associated with microtubules was not significantly different in the three stages. The presence of p34^{cdc2} was also confirmed by using a second antibody directed against the N-terminal part of p34^{cdc2} (gift from Dr. M. Dorée; data not shown) which does not recognize cdk2 protein, a protein kinase homologous to p34^{cdc2}. Only a faint p34^{cdc2} band was detected in the supernatant depleted of microtubules from oocytes of both stages

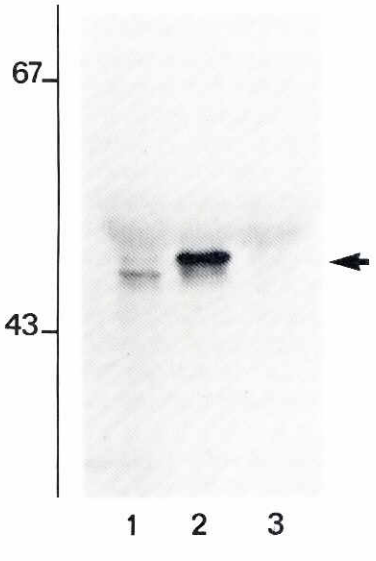

Fig. 2. Detection of cyclin B in microtubules. *Microtubular pellets were prepared from prophase oocytes* **(lane 1)**, *metaphase oocytes* **(lane 2)** *and activated eggs* **(lane 3)** *and western blotted with the anti-cyclin B antibody. Proteins corresponding to 15 oocytes. Arrow indicates the position of cyclin.*

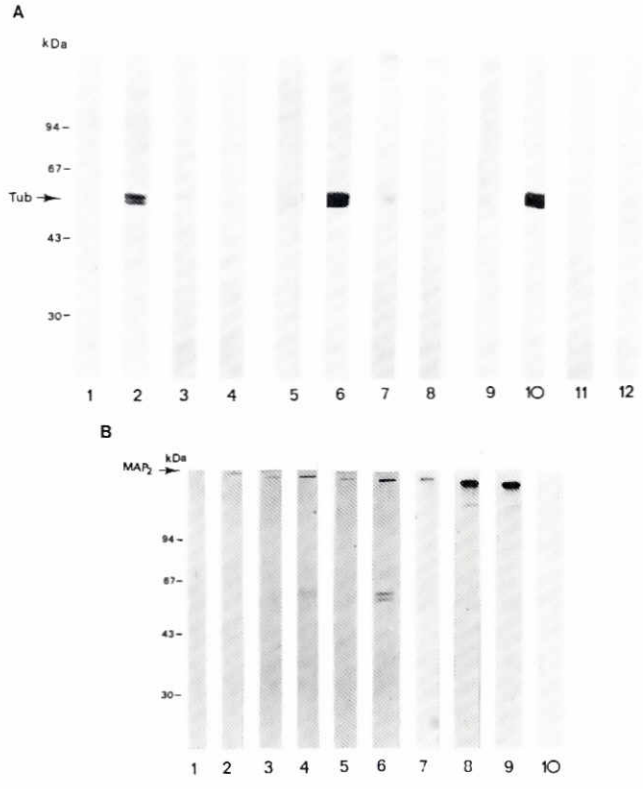

Fig. 3. Association of tubulin and MAP2 with p34^{cdc2} protein kinase. *Aliquots of purified tubulin or MAP2 were incubated with metaphase p34^{cdc2} protein kinase bound to p13-sepharose beads in the presence of γ(32P)ATP. After the phosphorylation reaction, the proteins associated with the p13-sepharose pellet were separated from the proteins present in the supernatant by centrifugation and then submitted to SDS-PAGE.* **(A)** *10 μg/ml (lanes 1,2,3 and 4) or 40 μg/ml of tubulin (lane 5,6,7 and 8) was incubated with p34^{cdc2} kinase bound to p13-beads and γ(32P)ATP. (Lanes 9,10,11 and 12) represent the same experiment as in (5,6,7 and 8) except that p13-sepharose beads do not contain any p34^{cdc2} kinase. Coomassie-blue staining: proteins associated with the beads (lanes 1,5,9) or present in the supernatant (lanes 2,6,10). Radioautography: phosphorylated proteins associated with the beads (lanes 3,7,11) or present in the supernatant (lanes 4,8,12).* **(B)** *10 μg/ml (lanes 1,2,7) or 50 μg/ml (lanes 3,4,8) or 100 μg/ml of MAP2 (lanes 5,6,9) were phosphorylated by p34^{cdc2} bound to p13-beads. Lane 10 represents the same experiment as in 9 except that p13-sepharose beads do not contain any p34^{cdc2} kinase. Coomassie blue staining: proteins associated with beads (lanes 1,3,5) or present in the supernatant (lanes 2,4,6). Radioautography of the phosphorylated proteins associated with beads (lanes 7,8,9 and 10).*

and activated eggs (Fig. 1B), indicating that the major part of the whole amount of p34^{cdc2} was associated with microtubules.

Cyclin B is not required for the interaction between p34^{cdc2} and microtubules

In both prophase and metaphase oocytes, cyclin B is associated with p34^{cdc2} (Minshull *et al.*, 1990; Jessus and Beach, 1992). In contrast, cyclin B is totally degraded in activated eggs (Murray and Kirschner, 1989; Jessus and Beach, 1992). p34^{cdc2} is therefore present in a monomeric form in these interphasic eggs. To determine whether microtubule-associated p34^{cdc2} was monomeric or associated with cyclin B, a western blot was performed by using an anti-cyclin B antibody (Fig. 2). Cyclin B was associated with p34^{cdc2} in microtubules extracted from prophase and metaphase oocytes, but absent in the microtubular pellet prepared from activated eggs (Fig. 2). The difference of molecular weight between prophase and metaphase corresponds to the phosphorylation of cyclin that occurs during meiotic maturation (Gautier *et al.*, 1990; Jessus and Beach, 1992). Moreover, the antibody has a stronger affinity for the metaphase form of cyclin than for the prophase form (Jessus and Beach, 1992), inducing a stronger signal in metaphase pellets than in prophase pellets, despite the presence of a constant amount of cyclin in prophase and metaphase oocytes. The results illustrated in Fig. 2 demonstrate that both pre-MPF and MPF forms were able to associate with prophase and metaphase microtubules. This also demonstrated that cyclin B was not required for the association between p34^{cdc2} and microtubules since monomeric p34^{cdc2} copurified with microtubules of activated eggs (Fig. 1B) in the absence of cyclin B (Fig. 2).

In vitro association of purified rat brain MAP2 and tubulin with Xenopus p34^{cdc2} protein kinase

To further investigate the mechanism of the association between p34^{cdc2} and microtubules, we determined whether either purified tubulin or purified MAP2 were able to associate with p34^{cdc2}. We took advantage of the p13 protein, the product of the *suc1* gene from *Schizosaccharomyces pombe*, which interacts strongly with p34^{cdc2} (Brizuela *et al.*, 1987; Dunphy *et al.*, 1988). Purified tubulin at 2 concentrations (20 or 40 μg/ml) or purified MAP2 at 3 concentrations (10, 50 or 100 μg/ml) was incubated with p34^{cdc2} that had been previously bound to p13-Sepharose beads. The proteins associated with the p13-bound kinase were separated from the non-associated proteins by centrifugation and both fractions were analyzed by SDS-PAGE. Figure 3A (lanes 1 and 5) clearly shows that purified tubulin does not bind to p34^{cdc2} but remains in the supernatant. In contrast, a significant amount of MAP2 associated with the beads (Fig. 3B, lanes 3 and 5). This result suggests that the association of p34^{cdc2} with microtubules is mediated by MAPs rather than by tubulin.

Histone H1 kinase activity is present in microtubules extracted from metaphase oocytes

In its active state, p34^{cdc2} possesses histone H1 kinase activity (Arion *et al.*, 1988; Draetta and Beach, 1988; Labbé *et al.*, 1988). To determine whether microtubule-associated p34^{cdc2} is active or

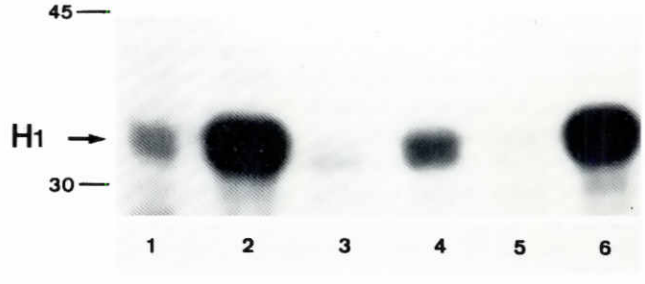

Fig. 4. Histone H1 kinase activity is associated with metaphase microtubules. *Cytosols* **(lanes 1,2)**, *microtubule supernatants* **(lanes 3,4)** *and microtubular pellets* **(lanes 5,6)** *were prepared from prophase oocytes* **(lanes 1,3,5)** *and metaphase oocytes* **(lanes 2,4,6)** *and assayed for histone H1 kinase activity. Phosphorylation of histone H1 is visualized by autoradiography. Proteins corresponding to 10 oocytes.*

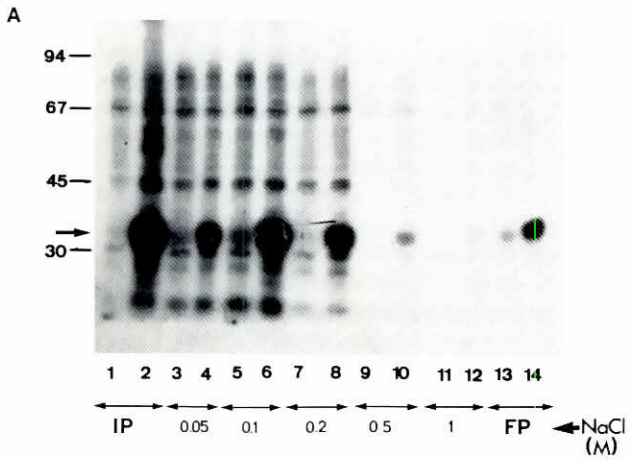

Fig. 5. Elution of histone H1 kinase activity by NaCl from metaphase microtubules. (A) *Microtubular pellets from 20 prophase oocytes (lanes 1,3,5,7,9,11, 13) and from 20 metaphase oocytes (lanes 2,4,6,8,10, 12,14) were resuspended with the indicated increasing NaCl concentrations. The eluted supernatants were assayed for histone H1 kinase activity. Phosphorylation of histone H1 is visualized by autoradiography. IP: histone H1 kinase activity present in initial pellets before NaCl extraction; FP: histone H1 kinase activity present in final pellets after the last 1 M NaCl wash.* **(B)** *Prophase cytosols (lanes 1,2), metaphase cytosols (lanes 3,4) and 0.5 M NaCl extracts from metaphase microtubular pellet (lanes 5,6) were precipitated on p13-sepharose beads (lanes 1,3,5) or control sepharose beads (lanes 2,4,6) and assayed for histone H1 kinase activity. Phosphorylation of histone H1 is visualized by autoradiography.*

inactive, histone H1 kinase activity was assayed in cytosols, supernatants depleted of microtubules and microtubular pellets from both prophase and metaphase oocytes. As shown in Fig. 4, histone H1 kinase activity was high in metaphase cytosol and absent in prophase cytosol. The same pattern of histone H1 kinase activity was found in microtubular pellets (Fig. 4). It can therefore be assumed that most of the histone H1 kinase activity measured under our experimental conditions was due to $p34^{cdc2}$ for three main reasons: first the major protein kinases able to phosphorylate histone H1, i.e. the cAMP-dependent protein kinase, PKA, and the Ca^{2+}-calmodulin dependent protein kinase, were blocked respectively by the presence of the specific inhibitor of PKA (PKI) and EGTA. Moreover these two kinase activities are not cell cycle dependent and therefore could not be responsible for the strong activation of histone H1 kinase activity observed in metaphase cytosol. Second, the other *Xenopus* protein kinases strongly activated in metaphase oocytes, such as MAP kinase or S6 kinase, do not use histone H1 as a substrate (Haccard *et al.*, 1990). Third, the histone H1 kinase activity was eluted from microtubular pellets and shown to be able to associate with p13, a 13k Da protein which presents a strong specific affinity for $p34^{cdc2}$ (see below). The level of histone H1 kinase activity was found dramatically reduced in the supernatants which had been depleted of microtubules. The level of prophase kinase activity, which was very low in the initial cytosol (Fig. 4, lane 1), was absent in prophase supernatant depleted from microtubules and in prophase microtubular pellet (Fig. 4, lanes 3 and 5). The level of the metaphase kinase was reduced 6 times in the metaphase supernatant depleted in microtubules in comparison to the initial cytosol (Fig. 4, lanes 2 and 4). This loss of kinase activity was not due to the inactivation of the enzyme since the histone H1 kinase activity was recovered entirely associated with the microtubular pellet (Fig. 4, lane 6). This result shows that the active form of MPF is able to bind microtubules.

Histone H1 kinase activity eluted from microtubules binds to p13-Sepharose beads

To study the association of $p34^{cdc2}$ with microtubules, the histone H1 kinase activity was eluted from a metaphase microtubular pellet by different NaCl concentrations. As shown in Fig. 5, histone H1 kinase activity was partly released by NaCl concentrations ranging from 50 mM to 200 mM, indicating a weak association between this kinase activity and microtubules. However, a fraction of histone H1 kinase activity was not able to be eluted from microtubules by NaCl concentrations as high as 1 M (Fig. 5A), indicating that another subset of the activity was very strongly associated with microtubules.

It was further investigated whether both forms of histone H1 kinase activities corresponded to $p34^{cdc2}$. A 34 kDa band was detected by western blotting with the anti-PSTAIRE antibody and the antibody recognizing the N-terminal part of $p34^{cdc2}$ in microtubular pellets remaining after 1 M NaCl extraction (not shown), indicating that the histone H1 kinase activity which was strongly bound to microtubules corresponded to $p34^{cdc2}$. The ability of histone H1 kinase activity eluted by NaCl from a metaphase microtubular pellet to bind to p13-Sepharose beads was then investigated. Fig. 5B shows that histone H1 kinase activity released by 0.5 M NaCl from metaphase microtubules was able to bind p13-sepharose beads, demonstrating that the subset of histone H1 kinase activity which binds weakly to microtubules also corresponded to $p34^{cdc2}$.

α- and ß-tubulins co-precipitate with $p34^{cdc2}$ on p13-beads

In the previous experiments, $p34^{cdc2}$ was recovered in association with microtubules that had been polymerized *in vitro* in oocyte extracts. The possibility that $p34^{cdc2}$ is also associated *in vivo* with microtubules was then investigated. $p34^{cdc2}$ was precipitated on p13-Sepharose beads from prophase and metaphase oocytes. The precipitates were then immunoblotted with anti-α and anti-ß tubulin antibodies. As shown on Fig. 6, tubulin was recovered with $p34^{cdc2}$ on p13 beads, indicating that $p34^{cdc2}$ was *in vivo* associated with microtubules. The *in vivo* association between tubulin and $p34^{cdc2}$ was strong, since no tubulin was eluted by NaCl concentrations as high as 3 M (Fig. 6). This might correspond to the strong association between $p34^{cdc2}$ and microtubules, which was resistant to 1 M NaCl wash (Fig. 5, lane 14). The *in vivo* association between $p34^{cdc2}$ and tubulin is probably mediated through MAPs, since it was previously demonstrated that pure tubulin does not bind $p34^{cdc2}$ (Fig. 3A).

In vitro phosphorylation of rat brain MAP2 by $p34^{cdc2}$

In order to elucidate what might be the significance of the association between $p34^{cdc2}$ and microtubules, the ability of p13-

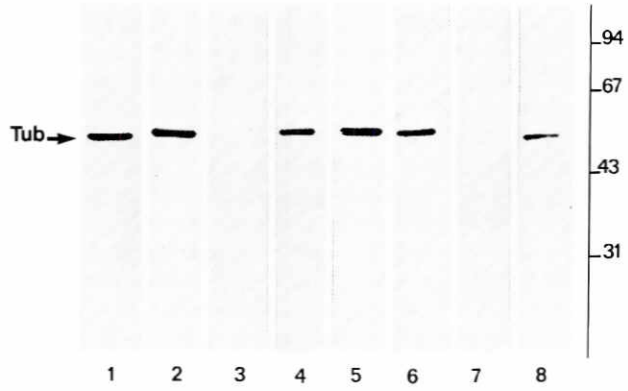

Fig. 6. Association of α- and ß-tubulin with p34^{cdc2} in p13-precipitates. *Prophase* **(lanes 1,2,3,4)** *and metaphase* **(lanes 5,6,7,8)** *cytosols were precipitated on p13-Sepharose beads. The not-bound proteins, the p13-pellets, proteins eluted from the beads by a 3 M NaCl concentration and pellets after the 3M NaCl wash were blotted with a mixture of anti-α- and anti-ß-tubulin antibodies.* **(Lanes 1 and 5)** *Proteins not bound to p13;* **(lanes 2 and 6)** *p13 pellets;* **(lanes 3 and 7)** *proteins eluted by a 3M NaCl wash;* **(lanes 4 and 8)** *p13 pellet after a 3M NaCl wash.*

bound p34^{cdc2} protein kinase from metaphase oocytes to phosphorylate tubulin and the microtubule-associated protein MAP2 was checked. As seen in Fig. 3A (lanes 3, 4, 7 and 8), tubulin was not a substrate of p34^{cdc2}. The complete absence of tubulin phosphorylation could not be explained by the loss of enzymatic activity since MAP2 was phosphorylated under the same conditions (Fig. 3B, lanes 7,8 and 9).

Comparison of MAP2 sites phosphorylated by p34^{cdc2} or by MAP kinase

MAP2 is known to possess two different domains, a projection domain and a tubulin binding domain (Vallee, 1980). Each of them is phosphorylated by a series of different protein kinases, some of them being well characterized (Hernandez *et al.*, 1987). It was therefore interesting to investigate which domain could be phosphorylated by p34^{cdc2} kinase and MAP kinase. As was already demonstrated (Ray and Sturgill, 1988; Silliman and Sturgill, 1989), MAP kinase phosphorylates MAP2 (Fig. 3B, Fig. 7, lane 4). Figure 7 represents the proteolytic fragments of purified rat brain MAP2 phosphorylated by both enzymes.

When MAP2 was first phosphorylated by p34^{cdc2} kinase and then proteolyzed by chymotrypsin for 8 min (Fig. 7, lane 2), a large number of phosphorylated MAP2 fragments of different sizes was obtained. One of these fragments had a molecular weight of 36 kDa and might represent the tubulin binding domain of MAP2 (Vallee, 1980).

To check this possibility, the ^{32}P-labeled MAP2 fragments obtained in Fig. 7 (lane 2) were incubated with purified tubulin and an enhancer dose of non-labeled MAP2 under conditions which allow tubulin assembly into microtubules. Some labeling of the full length 280 kDa MAP2 and of a 55 kDa MAP2 peptide was observed after polymerization in Fig. 7 (lane 3). This labeling might result from a phosphorylation induced by a kinase activity contaminating the tubulin preparations. The 36 kDa MAP2 fragment phosphorylated by p34^{cdc2} kinase was incorporated into microtubules and recovered in the microtubule pellet after centrifugation (Fig. 7, lane 3). The low recovery of the 36 kDa MAP2 fragment in the pellet might be explained by a low tubulin binding capacity of this fragment once phosphorylated by the p34^{cdc2} kinase. Larger phosphorylated MAP2 fragments (67 kDa-250 kDa) were also found in association with the microtubular pellet (Fig. 7, lane 3), suggesting that they contain the tubulin binding domain of MAP2. Due to the dilution, it was difficult to analyze the phosphorylated proteolytic fragments of MAP2 not associated with the microtubules (not shown).

MAP2 was also phosphorylated by MAP kinase purified from *Xenopus* oocytes and then proteolyzed by α-chymotrypsin for 8 min (Fig. 7, lane 5). The proteolysis reaction generated numerous phosphorylated MAP2 fragments markedly different in size from MAP2 fragments phosphorylated by p34^{cdc2} kinase (Fig. 7, compare lanes 2 and 5). Two main phosphorylated fragments of 40 kDa and 32 kDa were found labeled (Fig. 7, lane 5) but no phosphorylated fragment of 36 kDa was obtained. None of the proteolytic phosphorylated fragments were recovered associated with the microtubule pellet (Fig. 7, lane 6), indicating that the phosphorylated fragments generated after action of MAP kinase do not contain the tubulin binding domain of MAP2.

MAP kinase associates with microtubules of Xenopus oocytes

MAP kinase is tyrosine phosphorylated and activated at time of MPF activation in maturing *Xenopus* oocytes (Haccard *et al.*, 1990, 1993a). It could play a role in the microtubular reorganization which takes place at GVBD time. The possibility that MAP kinase asso-

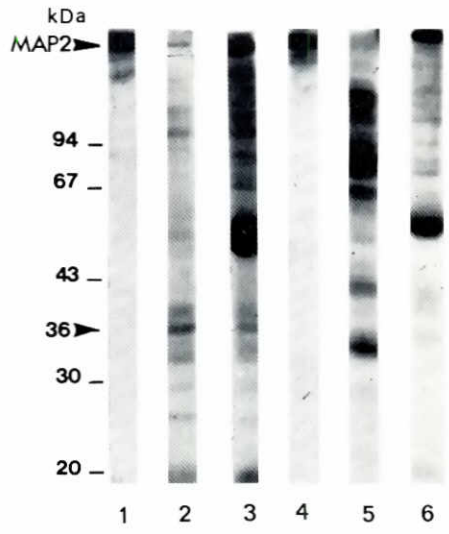

Fig. 7. Phosphorylation of MAP2 by p34^{cdc2} kinase and MAP kinase and ability of α-chymotrypsine phosphorylated fragments of MAP2 to bind to tubulin. *12 µg of purified MAP2 was submitted to phosphorylation by either p34^{cdc2} kinase or MAP kinase and then treated or not treated by α-chymotrypsine: MAP2 phosphorylated by p34^{cdc2} kinase* **(lane 1)** *or MAP kinase* **(lane 4)**; *MAP2 phosphorylated by p34^{cdc2} kinase and digested by α-chymotrypsine for 8 min* **(lane 2)**; *MAP2 phosphorylated by MAP kinase and digested by α-chymotrypsine for 8 min* **(lane 5)**. *The proteolytic fragments obtained in 2 and 5 were incubated with purified tubulin (1 mg/ml) and MAP2 (0.1 mg/ml). The assembled material was separated from the soluble proteins by centrifugation. Radioactive fragments of MAP2 phosphorylated by either p34^{cdc2} kinase or MAP kinase and bound to polymerized tubulin were analyzed by autoradiography in* **lane 3** *and* **lane 6** *respectively.*

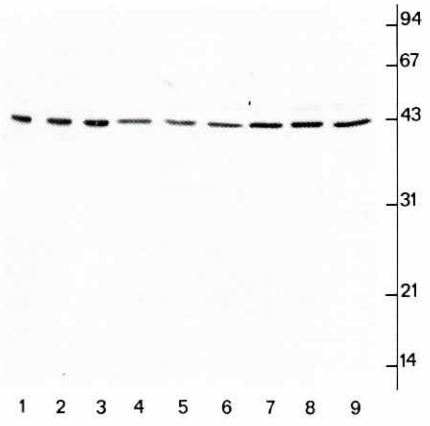

Fig. 8. Association of MAP kinase with microtubules. *Western blot with the anti-MAP kinase antibody. Cytosols* **(lanes 1,2,3)**, *microtubule supernatants* **(lanes 4,5,6)** *and microtubular pellets* **(lanes 7,8,9)** *were prepared from prophase oocytes* **(lanes 1,4,7)**, *metaphase oocytes* **(lanes 2,5,8)** *and activated eggs* **(lanes 3,6,9)**. **(1 to 6)** *Proteins corresponding to 5 oocytes;* **(7 to 9)** *proteins corresponding to 15 oocytes.*

ciates physically with microtubules was tested. Microtubules were isolated from prophase, metaphase and activated *Xenopus* oocytes using the taxol procedure. Initial cytosols, microtubule-depleted supernatants and microtubular pellets were immunoblotted with an anti-MAP kinase antibody (Fig. 8). A significant amount of MAP kinase was recovered in the microtubular pellets (Fig. 8, lanes 7,8 and 9). The quantity of MAP kinase present in supernatants depleted of microtubules (Fig. 8A, lanes 4,5 and 6) was reduced when compared to the quantity present in the initial cytosols (Fig. 8A, lanes 1,2 and 3). However, in contrast to $p34^{cdc2}$ (Fig. 1B), an important part of MAP kinase was not associated with microtubules. It was also possible to demonstrate that MAP kinase was associated *in vivo* with microtubules. By immunocytochemistry, the anti-MAP kinase antibody staining revealed that MAP kinase was concentrated around the metaphase II spindle (data not shown).

Discussion

This article demonstrates that (i) $p34^{cdc2}$ and MAP kinase associate with microtubules; (ii) cyclin B is not required for the $p34^{cdc2}$-microtubule interaction that is probably mediated between MAPs and $p34^{cdc2}$; (iii) MAP2 is a good substrate for both kinases that phosphorylate it on different sites. What could be the biological significance of such an association?

$p34^{cdc2}$ may modulate microtubule organization by phosphorylating MAPs. This attractive hypothesis is supported by different experimental results:

1. It has already been shown in other systems that $p34^{cdc2}$ localizes partly with microtubular structures like centrosomes and spindle pole bodies (Bailly *et al.*, 1989; Riabowol *et al.* 1989; Alfa *et al.*, 1990), kinetochore-to-pole microtubules (Rattner *et al.*, 1990), mitotic apparatus (Pines and Hunter, 1991), and meiotic spindles and asters (Ookata *et al.*, 1992; Kubiak *et al.*, 1993). In this report, it was shown that $p34^{cdc2}$ of *Xenopus* oocytes had a strong affinity for microtubules, since the majority of the protein was recovered with the microtubular pellet after polymerization. Moreover, the presence of tubulin in p13-precipitates demonstrated that $p34^{cdc2}$ was partly associated *in vivo* with microtubules.
2. MAPs, such as MAP2 or tau protein, are good substrates of $p34^{cdc2}$ (this study; Erikson and Maller, 1989; Mawal-Dewan *et al.*, 1992). In a similar manner, the direct phosphorylation of MAPs by such as PKA (Theurkauf and Vallee, 1982) and casein kinase II (Diaz-Nido *et al.*, 1988) and their localization to the microtubule networks (Serrano *et al.*, 1989) previously demonstrated that these kinases were implicated in microtubule regulation. Therefore, the reported phosphorylation of MAPs and the ability of $p34^{cdc2}$ to associate with microtubules may be relevant. Supporting this idea, Shiina *et al.* (1992) showed that both MAP kinase and $p34^{cdc2}$ are able to phosphorylate *in vitro* a *Xenopus* microtubule-associated protein, p220, modifying its capacity to promote *in vitro* tubulin polymerization. These authors proposed that both $p34^{cdc2}$ and MAP kinase could *in vivo* phosphorylate the *Xenopus* microtubule-associated protein p220, contributing via this phosphorylation to the microtubular modifications of M-phase.
3. Lamb *et al.* (1990) showed that microinjection of $p34^{cdc2}$ kinase induces marked changes in cellular structures, closely mimicking the events occurring during early phases of mitosis. However, even if a marked reduction in interphase microtubules was observed, no spindle formation was induced. One can hypothesize that some other kinase activities, such as MAP kinase, contribute normally to the formation of metaphase spindles.
4. Verde *et al.* (1990, 1992) demonstrated that $p34^{cdc2}$ protein kinase has the capacity to regulate *in vitro* microtubule elongation rate and steady-state length in *Xenopus* oocyte extracts, inducing the conversion of an interphase microtubular array into a metaphase network. These authors proposed that the changes in microtubule rearrangements at the interphase-M-phase transition are regulated *in vivo* by $p34^{cdc2}$ kinase. By using MAP kinase, Gotoh *et al.* (1991b) also reproduced the conversion between an interphase and a mitotic microtubular array in *Xenopus* oocyte extracts, demonstrating that, *in vitro*, $p34^{cdc2}$ and MAP kinase are able to generate similar effects on microtubules.

During the meiotic maturation, MPF can therefore regulate the organization of the giant microtubular network that appears at the basal part of the broken nucleus (Jessus *et al.*, 1986; Huchon *et al.*, 1993). It has already been shown that MPF and nuclear components are both necessary for the formation of this structure (Heidemann and Gallas, 1980; Jessus *et al.*, 1988). The present findings, showing that $p34^{cdc2}$ associates with microtubules and phosphorylates MAPs, support this idea. Other protein kinases are also activated during meiosis, and they can contribute to the architectural modifications of M-phase (Cicirelli *et al.*, 1988). Among the activated kinases is the MAP kinase (Haccard *et al.*, 1990; Gotoh *et al.*, 1991a). The roles of this kinase are unclear. Very recently, it has been shown that MAP kinase is a component of the *Xenopus* CSF (Haccard *et al.*, 1993b), responsible for the metaphase arrest and the stabilization of MPF in unfertilized eggs (Masui, 1974). Since MAP kinase affects spindle dynamics in egg extracts (Gotoh *et al.*, 1991b; Shiina *et al.*, 1992), it has been proposed that the targets of MAP kinase could be microtubular components. The present demonstration that MAP kinase associates *in vitro* and *in vivo* with microtubules strongly supports this hypothesis. The phosphorylation of specific sites of MAP2 or MAP2-related proteins by both MAP kinase and $p34^{cdc2}$ might be responsible for microtubule rearrangements during the G_2-M transition and for the stabilization of the metaphase II spindle.

MAP2 protein is a large molecule with two main functional domains (Vallee, 1980). One of them is a small fragment of about 35-36 kDa which contains the tubulin binding region and the second is a large fragment of about 240 kDa which represents the microtubule projection domain. The two domains of MAP2 were shown to be phosphorylated at different sites by several protein kinases (Hernandez *et al.*, 1987). In this report, it was demonstrated that $p34^{cdc2}$ kinase and MAP kinase did not phosphorylate the same domains of MAP2. $p34^{cdc2}$ kinase phosphorylates a fragment of 36 kDa able to bind to tubulin and this strongly suggests that this fragment contains the tubulin binding domain. This 36 kDa fragment was not found in the MAP kinase phosphorylated peptide pattern of MAP2. Moreover the two peptide fragments of about 32 kDa and 40 kDa that are mainly phosphorylated by MAP kinase remain in the soluble fraction after microtubule polymerization, indicating, in agreement with Silliman and Sturgill (1989), that they do not contain the tubulin binding domain. Taken together these results indicate that $p34^{cdc2}$ kinase can phosphorylate at least the tubulin binding domain (one cannot exclude that the projection domain is not phosphorylated), and that MAP kinase is able to phosphorylate mainly the projection domain of MAP2.

The physiological meaning of the differential phosphorylation of the two domains of MAP2 by various specific protein kinases is unknown. However, it is admitted by several authors that the phosphorylation of the tubulin binding domain of MAP2 lowers the affinity of MAP2 for tubulin and thus decreases the level of microtubule assembly (Burns *et al.*, 1984). Some different physiological consequences of the phosphorylation of MAP2 projection domain can be expected. This phosphorylation may alter the interaction of microtubules with organelles such as plasmic membranes. It was demonstrated that MAP2-like proteins were localized in submembranous regions in *Xenopus* oocytes (Fellous *et al.*, 1991) or erythrocytes (Sloboda and Dickersin, 1980). It cannot be excluded that the phosphorylation of some specific sites of MAP2 projection domains may also alter microtubule assembly or at least microtubule stability (Burns *et al.*, 1984). A MAP2-like protein called O-MAP is present in *Xenopus* oocyte and phosphorylated *in vivo* during the meiotic maturation (Fellous *et al.*, 1991). O-MAP is therefore a good candidate as a physiological substrate of $p34^{cdc2}$ and MAP kinase in *Xenopus* oocyte.

It must be noted that $p34^{cdc2}$ and MAP kinase bind to microtubules to the same extent in prophase, metaphase or interphase egg extracts. Apparently, this association is not driven by some associated proteins such as cyclin B. This leads to the hypothesis that $p34^{cdc2}$ and MAP kinase could be associated with microtubules over the entire meiotic maturation process. Therefore, the phosphorylation level of MAPs would not depend on the presence of the $p34^{cdc2}$ and MAP kinase proteins, which could be associated in a permanent way, but on the presence of their kinase activity, which is turned on only at the time of nuclear envelope breakdown.

The association between $p34^{cdc2}$ and microtubules could be an important key of a feedback control allowing the proper succession of the two metaphase spindles which take place consecutively during meiotic maturation. In *Xenopus* oocytes, the $p34^{cdc2}$ kinase activity increases abruptly during the prophase-prometaphase I transition, then drops to a minimum level at the metaphase I/anaphase I transition and further increases again until reaching a maximum stable level at metaphase II (Huchon *et al.*, 1993). It has been well established that the exit from mitosis in somatic cells is controlled by the completion of spindle assembly (reviewed by Murray, 1992). It has recently been demonstrated that in mouse oocytes, the presence of an intact metaphase spindle is required for cyclin B degradation (Kubiak *et al.*, 1993). It is also possible that, in *Xenopus* oocyte, a feedback control system monitors microtubular spindle progression in order to allow the proper succession of the different phases (prophase / metaphase I / anaphase I / metaphase II) of the meiotic maturation. Microtubules of metaphase I spindle could be part of a feedback control that induces the drop of $p34^{cdc2}$ kinase activity during the metaphase I-anaphase I transition, and microtubules of the metaphase II spindle could be part of another feedback control that allows the stabilization of MPF in the unfertilized egg, i.e. CSF activity, of which MAP kinase is a component (Haccard *et al.*, 1993b). In a giant cell such as *Xenopus* oocyte, the efficiency of such feedback controls would be greatly enhanced by the colocalization of the monitored molecules (microtubules) and the effectors of the feedback control ($p34^{cdc2}$ or MAP kinase). Moreover, tubulin and MAPs have already been shown to be able to regulate enzymatic activities, such as the serine/threonine phosphatase 2A (Jessus *et al.*, 1989). It would be interesting to determine whether tubulin or MAPs could play an active role in the regulation of the enzymatic activities of $p34^{cdc2}$ and MAP kinase.

Materials and Methods

Oocyte preparation

Xenopus laevis prophase oocytes were prepared as described by Jessus *et al.* (1987). Oocytes were induced to mature by addition of 1 μM progesterone to the external medium. The criterion for maturation was the appearance of a white spot at the animal pole of the oocyte, and germinal vesicle breakdown was checked by dissecting oocytes after fixation in 10% TCA. Immature oocytes are referred to as "prophase oocytes" and progesterone-matured oocytes as "metaphase oocytes". Metaphase oocytes were activated *in vitro* by calcium as described in Jessus and Beach (1992), and are referred to as "activated eggs".

Microtubule preparation from Xenopus oocytes

Microtubule extracts were prepared by using the procedure described by Jessus *et al.* (1985). Oocytes were washed and homogenized at 4°C in Lysis Buffer (100 mM PIPES pH 6.6, 5 mM EGTA, 1 mM $MgSO_4$, 0.9 M glycerol, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 2 mM phenylmethyl sulfonylfluoride (PMSF), 0.5 mg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 0.2 mg/ml aprotinin) in the proportion of 700 oocytes per 3 ml. The homogenate was centrifuged for 30 min at 30,000xg at 4°C. The supernatant was then centrifuged for 90 min at 165,000xg at 4°C or equivalently for 20 min at 356,000xg at 4°C. The supernatant was referred to as "cytosol" and used for microtubule extraction. Microtubule polymerization was induced by addition of 20 μM taxol (gift of Dr. M. Suffness, NIH, USA) and 1 mM GTP. After a 15 min incubation at 37°C, the extract was centrifuged for 30 min at 30,000xg at 4°C. The supernatant, depleted of microtubules, was referred to as "microtubule supernatant" and the pellet as "microtubular pellet".

Microtubule protein preparation from rat brain

Microtubule proteins were prepared from Sprague-Dawley adult rat brains according to the procedure by Fellous *et al.* (1977). Pure MAP2 was prepared by subjecting MAPs to gel filtration on Ultrogel ACA 34 (LKB) and pure tubulin was purified as in Fellous *et al.* (1977).

Elution of $p34^{cdc2}$ from Xenopus microtubular pellet by NaCl

Microtubular pellet from metaphase oocytes was resuspended in Lysis Buffer containing 50 mM NaCl and incubated under constant agitation for 10 min at 4°C. After centrifugation at 30,000xg for 30 min at 4°C, the supernatant was saved and the same procedure was repeated with the pellet, using increasing NaCl concentrations, i.e. 0.1 M, 0.2 M, 0.5 M and 1 M NaCl. One aliquot of the control initial pellet, all the eluted

supernatants and aliquots of the pellets remaining after NaCl extraction were then assayed for histone H1 kinase activity.

Preparation of p34^{cdc2} and MAP kinase

p34^{cdc2} was prepared by conjugation on p13-beads. p13-sepharose was prepared by conjugating p13 from overproducing strain of *E. coli* to CNBr-activated sepharose 4B as described previously (Brizuela *et al.*, 1987). *Xenopus* extracts were prepared as described in Jessus *et al.* (1991). 50 µl of oocyte extracts (corresponding to approximately 40 oocytes) was added to 30 µl of packed p13-sepharose beads and 920 µl of Extraction Buffer (80 mM ß-glycerophosphate, pH 7.3, 15 mM $MgCl_2$, 20 mM EGTA, 1 mM PMSF, 0.02 T.I.U. aprotin/ml, 1 mM benzamidine, 10 µg/ml leupeptin, 0.5 mg/ml soybean trypsin inhibitor). The mixture was incubated for 90 min at 4°C under constant rotation. After 4 washes of the pellet in Kinase Buffer (50 mM Tris pH 7.4, 5 mM EGTA, 10 mM $MgCl_2$ and 1 mM dithiothreitol), p34^{cdc2} kinase activity bound to the p13-sepharose beads was assayed using various substrates. MAP kinase was purified from *Xenopus* metaphase II-blocked oocytes as described in Haccard *et al.* (1993a).

Kinase assays

Two kinases were used to phosphorylate MAP2 or tubulin, either p13-bound p34^{cdc2} or MAP kinase, both of them extracted from *Xenopus* metaphase II oocytes as described previously. Phosphorylation was assayed in 50 µl Kinase Buffer containing various amounts of either tubulin or MAP2 or 1 mg/ml histone H1 (Boehringer), 20 µM of PKI-synthetic peptide (Sigma), 1 µCi of $\gamma[^{32}P]$ ATP (111 TBq/mmol), 0.1 mM ATP, for 15 min at 30°C, either with 100 µl of p13-bound p34^{cdc2} or with 10 µl of MAP kinase. In some cases (Fig. 3), phosphorylated substrates which were bound or not bound to the p13-beads were separated by centrifugation. The phosphorylation reaction was stopped either by addition of electrophoresis sample buffer (Laemmli, 1970) and boiling for 5 min, or followed by a proteolysis reaction in the case of MAP2. After SDS-PAGE electrophoresis (Laemmli, 1970), phosphorylated proteins were visualized by autoradiography.

Proteolysis reaction

Proteolysis reaction was induced by 0.7 µg/ml α-chymotrypsine at 37°C for 8 min as described by Vallee (1980). The proteolytic reaction was stopped either by addition of electrophoresis sample buffer and boiling (Laemmli, 1970) or by 2 mM PMSF followed by an incubation at 37°C for 30 min of phosphorylated proteolytic MAP2 fragments with tubulin and non-labeled MAP2 to allow microtubule polymerization. Samples were then centrifuged at 105,000g for 30 min to separate the microtubule pellet from non-assembled proteins. Proteolytic products of MAP2 were subjected to 10% SDS-PAGE electrophoresis (Laemmli, 1970) and phosphorylated proteins were visualized by autoradiography.

Immunoblotting procedures

Western blots were performed as described in Jessus *et al.* (1991). A polyclonal rabbit antibody raised against the PSTAIRE sequence, EGVPSTAIRELLKE, highly conserved in p34^{cdc2} of all species studied until now was provided by Dr. M. Dorée (CRBM-CNRS, Montpellier, France). The polyclonal antibody raised against mammalian ERK1 was a gift of Dr. J. Pouyssegur (CNRS-UMR 134, Nice, France). Mouse anti-α- and anti-ß tubulin monoclonal antibodies were purchased from Amersham.

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