Binding of E-MAP-115 to Microtubules Is Regulated by Cell Cycle-dependent Phosphorylation

Danièle Masson and Thomas E. Kreis
Département de Biologie Cellulaire, Université de Genève, Sciences III, 30, quai Ernest-Ansermet, CH-1211 Geneva, Switzerland

Abstract. Expression levels of E-MAP-115, a microtubule-associated protein that stabilizes microtubules, increase with epithelial cell polarization and differentiation (Masson and Kreis, 1993). Although polarizing cells contain significant amounts of this protein, they can still divide and thus all stabilized microtubules must disassemble at the onset of mitosis to allow formation of the dynamic mitotic spindle. We show here that binding of E-MAP-115 to microtubules is regulated by phosphorylation during the cell cycle. Immunolabeling of HeLa cells for E-MAP-115 indicates that the protein is absent from microtubules during early prophase and progressively reassociates with microtubules after late prophase. A fraction of E-MAP-115 from HeLa cells released from a block at the G1/S boundary runs with higher apparent molecular weight on SDS-PAGE, with a peak correlating with the maximal number of cells in early stages of mitosis. E-MAP-115 from nocodazole-arrested mitotic cells, which can be obtained in larger amounts, displays identical modifications and was used for further biochemical characterization. The level of incorporation of $^{32}$P into mitotic E-MAP-115 is about 15-fold higher than into the interphase protein. Specific threonine phosphorylation occurs in mitosis, and the amount of phosphate associated with serine also increases. Hyperphosphorylated E-MAP-115 from mitotic cells cannot bind stably to microtubules in vitro. These results suggest that phosphorylation of E-MAP-115 is a prerequisite for increasing the dynamic properties of the interphase microtubules which leads to the assembly of the mitotic spindle at the onset of mitosis. Microtubule-associated proteins are thus most likely key targets for kinases which control changes in microtubule dynamic properties at the G2- to M-phase transition.

The organization of microtubules varies according to their different functions during the cell cycle. Interphase microtubules are involved in the positioning of organelles and in the movement of vesicles, whereas during mitosis the spindle microtubules ensure the accurate segregation of the chromosomes between the two daughter cells. Transition from the interphase to the mitotic organization requires the rapid rearrangement of the microtubule network (Karsenti, 1993) and is accompanied by changes in the dynamic properties of microtubules (Salmon et al., 1984; Saxton et al., 1984; Belmont et al., 1990).

Factors controlling these events have recently been identified. Katanin, an ATPase from sea urchin eggs (McNally and Vale, 1993), and a protein from Xenopus laevis eggs (Shiina et al., 1992b) can sever microtubules in vitro and may have a similar activity at the G2- to M-phase transition in vivo. cdc2 (Verde et al., 1990) and MAP kinase (Gotoh et al., 1991a, b) have been shown to induce changes in microtubule dynamics in vitro in Xenopus egg extracts. Since microtubule-associated proteins (MAPs) control microtubule dynamics, it has been proposed that they are the targets of these kinases. A 220-kD MAP from Xenopus has been demonstrated to detach from microtubules throughout M-phase when it is phosphorylated on cdc2 and MAP kinase sites (Shiina et al., 1992a). Recently, XMAP230, a protein from Xenopus eggs which is hyperphosphorylated during mitosis, was observed to dissociate from microtubules at the onset of prophase and to rebind to spindle microtubules during metaphase and anaphase (Andersen et al., 1994). Similar studies in mammalian cells have been difficult because most MAPs characterized so far are from neuronal origin, that is from nondividing cells. In vitro phosphorylation by cdc2 kinase of MAP4, the best studied nonneuronal MAP, suppresses its microtubule assembly-promoting activity (Aizawa et al., 1991). However, MAP4 has been observed to be associated with microtubules during mitosis and to copurify with spindles, although its phosphorylation state varies (Vandré et al., 1995).
Materials and Methods

Cell Culture and Synchronization

Attached HeLa cells were grown in MEM supplemented with 1% L-glutamine, 1% nonessential amino acids and 10% FCS. Cell synchronization was performed in two steps combining nocodazole arrest in M phase (Zieve et al., 1980) with a block at the G1/S boundary due to DNA synthesis inhibition by aphidicolin (Huberman, 1981). Sub-confluent HeLa cells were arrested in mitosis by culturing them for 10 h in the presence of 0.05 μM nocodazole. After washing of the culture dishes to remove cell debris, mitotic cells were shaken off and collected, plated on 6 cm dishes (400,000 cells/dish) and cultured for 22 h in medium containing 5 μg/ml aphidicolin to synchronize them at the G1/S boundary. BS-U incorporation was used to verify inhibition of DNA synthesis (not shown). Culture dishes were then washed with PBS to remove nonattached cells (only about 70% nocodazole-blocked cells divide and attach under these conditions) and remaining cells were allowed to progress through the cell cycle by culturing them in medium without inhibitor. Cells were analyzed by immunoblotting for E-MAP-115 with mAb D9C1 (Masson and Kreis, 1993), and by immunofluorescence staining for E-MAP-115 and tubulin in parallel with Hoechst staining for DNA at various time points after release. We noticed that cells had different division rates when cultured on glass coverslips or on plastic. Therefore, immunolabeling was performed on cells fixed on culture dishes. For both analyses medium was removed carefully to minimize lossing of weakly attached, rounded mitotic cells, and cells were either directly fixed for 4 min in methanol at -20°C and processed for immunofluorescence staining or solubilized in boiling sample buffer for SDS-PAGE and immunoblotting.

Synchronization in mitosis was performed according to Zieve et al. (1980) with slight modifications. 30% confluent attached HeLa cells were first cultured for 22 h in medium containing 5 μg/ml aphidicolin. 10 h after removal of the drug cells were accumulated in mitosis by incubation with 0.3 μM nocodazole for 2 h. Mitotic cells were harvested by mechanical release from their substrate, washed once, and resuspended in carbonate buffer (100 mM KH2PO4, 100 mM NaCl, pH 6.8 (PEM)) at 4°C. Cells were then washed twice in PEM containing 0.1 mM nocodazole, after which cells were resuspended in PEM containing 0.1 mM nocodazole and 0.1% Triton X-100, 100 μM NaCl, 100 μM MgCl2, and 50 mM NaF and then rinsed twice with 50 mM Tris, pH 7.4, 100 μM NaCl, 100 μM PMSF, 100 μM orthovanadate, and 50 mM NaF. Nontreated adherent cells were solubilized in this buffer after two washes in PBS on the dish. Triton X-100 was added at a final concentration of 2% to dilute the SDS and DNA was sheared by passage through a 21G hypodermic needle. The lysates were spun at 15,000 g for 15 min and the clarified supernatants were pre-adsorbed by incubation for 1 h with 40 μl of a 50% slurry of protein A-Sepharose at 4°C. After removal of the sepharose by centrifugation, the lysates were incubated for 4 h at 4°C with anti-E-MAP-115 mAb D9C1 (Masson and Kreis, 1993), and then with 5 μl of sheep anti-mouse IgG antibody (Dianova GmbH, Hamburg, Germany) and 40 μl of protein A-Sepharose for ~12 h at 4°C with gentle mixing. The beads were washed five times with 20 mM Tris, pH 7.4, 100 mM NaCl, 0.1% SDS, 1 mM PMSF, 100 μM orthovanadate, and 50 mM NaF and then rinsed twice with 50 mM Tris, pH 7.4, 100 μM orthovanadate and 50 mM NaF. Bound protein was solubilized in 50 μl gel sample buffer and separated by 6% SDS-PAGE and visualized by autoradiography. Immunoprecipitated protein was quantified on immunoblots and autoradiograms as described above.

Treatment of E-MAP-115 with Alkaline Phosphatase

HeLa cells were grown in 10 cm dishes for 12 h with or without 0.2 μM nocodazole. For labeling, cells were incubated for 5 h at 37°C in 4 ml of phosphate-free MEM buffered at pH 7.25 with 50 mM HEPES and supplemented with 5% non-dialyzed FCS with or without 0.2 μM nocodazole and with 200 μCi/ml [32P]orthophosphate (PBS1; Amersham Corp., Arlington Heights, IL). Cell dishes were washed once with ice-cold PBS. Mitotic cells were collected by repeated flushing of PBS on the dishes, spun and resuspended in 1 ml of 20 mM Tris, pH 7.4, 100 mM NaCl, 0.4% SDS, 1 mM PMSF, 100 μM orthovanadate, and 50 mM NaF. Nontreated adherent cells were solubilized in this buffer after two washes in PBS on the dish. Triton X-100 was added at a final concentration of 2% to dilute the SDS and DNA was sheared by passage through a 21G hypodermic needle. The lysates were spun at 15,000 g for 15 min and the clarified supernatants were pre-adsorbed by incubation for 1 h with 40 μl of a 50% slurry of protein A-Sepharose at 4°C. After removal of the sepharose by centrifugation, the lysates were incubated for 4 h at 4°C with anti-E-MAP-115 mAb D9C1 (Masson and Kreis, 1993), and then with 5 μl of sheep anti-mouse IgG antibody (Dianova GmbH, Hamburg, Germany) and 40 μl of protein A-Sepharose for ~12 h at 4°C with gentle mixing. The beads were washed five times with 20 mM Tris, pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 100 μM PMSF, 100 μM orthovanadate, and 50 mM NaF and then rinsed twice with 50 mM Tris, pH 7.4, 100 μM orthovanadate and 50 mM NaF. Bound protein was solubilized in 50 μl gel sample buffer and separated by 6% SDS-PAGE and visualized by autoradiography. Immunoprecipitated protein was quantified on immunoblots and autoradiograms as described above.

Tryptic Phosphopeptide Mapping

Tryptic phosphopeptide mapping was performed according to Ottaviano and Geraese (1985) with slight modifications. Following SDS-PAGE of 32P-labeled immunoprecipitated E-MAP-115, the gel was agitated in five successive changes of distilled water and dried under vacuum without pro-

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tein fixation. Labeled E-MAP-115 was detected by autoradiography and the corresponding bands were excised from the gel. The gel slices were swelled in 10 mM Tris, pH 8.0, 50 mM ammonium acetate (untritiated), 1 mM DTT and placed in 0.5 ml of the same buffer for trypsin digestion. 25 μl of 1 mg/ml TPCK-trypsin in the same buffer was added and E-MAP-115 digested during 6 h at 37°C on a shaker. A further 10 μl of the trypsin solution was added and the digestion continued for 12 h. Released peptides were removed and the gel slices were washed by two additions of 0.4 ml H2O and shaking for 25 min. The three supernatants were pooled and spun for 60 min at 150,000 g at 4°C. The resulting supernatant was lyophilized in a speed-vac. The dried peptides were resuspended in 20 μl electrophoresis buffer (7.8% acetic acid, 2.5% formic acid, and 93% H2O). The samples, with addition of trace amounts of phenol red, were spotted on cellulose-coated thin layer plates (G1440; Schleicher & Schuell, Keene, NH) and electrophoresed for 2 h at 700 volts at 4°C in the electrophoresis buffer. The peptides were separated in the second dimension by chromatography in 37.5% n-butanol, 25% pyridine, 7.5% acetic acid and 30% H2O for 5 h at room temperature. Following chromatography, labeled peptides were visualized by autoradiography.

**Phosphoaraino Acid Analysis**

Radiolabeled E-MAP-115 separated on SDS-PAGE was treated as above. After lyophilization, the peptides were resuspended in 1 ml 6 M HCl and hydrolyzed for 90 min in an oven at 110°C. After drying, the samples were solubilized in 10 μl of the first dimension electrophoresis buffer, mixed with 5 μg of standard phosphoamino acids and analyzed by two-dimensional electrophoresis on cellulose-coated thin layer plates. The first dimension was run at pH 1.9 in 7.8% acetic acid, 2.5% formic acid, and 89.7% H2O at 750 volts for 3 h 15 min and the second at pH 3.5 in 5% acetic acid, 0.5% pyridine, and 94.5% H2O at 500 volts for 2 h 30 min at 4°C. 32P-labeled amino acids were first detected by autoradiography and standard phosphoamino acids were then detected by ninhydrin staining.

**Immunofluorescence**

Cells grown on coverslips or on culture dishes were fixed for 4 min in methanol at −20°C with 0.05% Triton X-100, pH 6.8 as described (Kreis, 1987). Alternatively, cells were fixed in 3% paraformaldehyde in PBS for 20 min, quenched by 50 mM NH4Cl for 5 min, and permeabilized with 0.1% Triton X-100 in PBS for 4 min. Fixed cells were labeled with mAb 19D9, or rabbit polyclonal antibodies against a peptide deduced from E-MAP-115 cDNA sequence (Masson and Kreis, 1993) and with affinity-purified rabbit polyclonal antibodies (anti-T13) or a mAb (1A2) specific for tyrosinated tubulin (Kreis, 1987) followed by rhodamine- and fluorescein-labeled secondary antibodies. Epifluorescence microscopy was performed using a Zeiss 45x/1.4 Planapo oil immersion objective on a Zeiss Axioskop microscope. Images were recorded and processed as described (Pierre et al., 1994) and printed on Agfapan 25 film using a slidewriter

**Microtubule-binding Assays**

100 μl of cytosol at a protein concentration of ~8 mg/ml was incubated with 20 μM taxol to induce tubulin polymerization, or 20 μM nocodazole as a control, at 37°C for 30 min. The samples were loaded on a 580 μM 20% sucrose cushion in PEM. After 30 min centrifugation at 30,000 g in a TST 55.5 rotor (Kontron Instruments, Redwood City, CA), the supernatants on top of the sucrose cushions were collected and analyzed together with the pellets for the presence of E-MAP-115 by immunoblotting.

**Gel Electrophoresis and Immunoblottings**

SDS-PAGE was performed according to the procedure of Laemmli (1970) using a Mini-Protean II Dual Slab Cell system (Bio Rad Labs., München, Germany) for Figs. 4 B and 8, and using a Mini-Slab gel system (Ida Scientific Co., Minneapolis, MN) for Figs. 3, 4 A, and 5. IEF was performed using a Mini-Protean II 2-D Cell system (Bio Rad Labs.). For immunoblotting, transfer of proteins to nitrocellulose, and detection of proteins was performed as described (Rickard and Kreis, 1990). Immunoblots in Figs. 3, 4 A and B, 5 C, and 8 were developed using ECL (Amer sham Corp.).

**Results**

**Binding of E-MAP-115 to Microtubules Varies during the Cell Cycle**

E-MAP-115 is associated with subsets or subdomains of perinuclear microtubules in HeLa cells (see Masson and Kreis, 1993; and Fig. 2 B). In epithelial Caco-2 cells, however, E-MAP-115 is associated with longer segments of microtubules and more distinct labeling along the microtubules to the cell periphery can be observed (see Fig. 6, A and B in Masson and Kreis, 1993). However, a significant heterogeneity in intensity of immunolabeling for E-MAP-115 on microtubules within the same population of cells can be observed (Fig. 2 E) suggesting a cell cycle-dependent regulation of its distribution. Thus, the precise distribution of E-MAP-115 was investigated during mitosis. In early prophase, when the microtubule network is still intact (Fig. 1 A) but the chromatin condensing (Fig. 1 B), staining for E-MAP-115 on microtubules is dramatically decreased, with occasional diffuse labeling over the separated microtubule-organizing centers (MTOCs) (Fig. 1 C). Most likely E-MAP-115 has dissociated from the microtubules and been extracted with other cytosolic proteins during the fixation procedure. At later time points, when cells have rounded up (Fig. 1 D), E-MAP-115 staining increases near each spindle pole and some spindle microtubules appear labeled (Fig. 1 F). From this stage of mitosis onwards, E-MAP-115 progressively reassociates with spindle microtubules (Fig. 1 J). Microtubule-associated E-MAP-115 is most intense on the reforming interphase microtubule network in telophase (Fig. 1 I, lower cell) and cleaved cells with still visible midbodies (not shown). The same results are obtained independently of the method of cell fixation or the antibodies used. In cells fixed in paraformaldehyde instead of methanol and labeled with a mAb (not shown) or with polyclonal antibodies (Fig. 2, C–E), signals for E-MAP-115 are bright on a subset of perinuclear microtubules in interphase but relatively weak and diffuse in early prophase cells (Fig. 2 E, asterisk). These results suggest that E-MAP-115 dissociates from microtubules at the beginning of mitosis and progressively reassociates with microtubules as cell division progresses.

**Modification of E-MAP-115 in Mitosis**

Two explanations can be considered for the dramatic decrease in microtubule-bound E-MAP-115 in early mitosis. E-MAP-115 may dissociate from microtubules due to specific posttranslational modification and subsequently be extracted by the fixation procedures used, or alternatively, E-MAP-115 may be degraded at the onset of mitosis. Cell cycle-dependent degradation has in fact been observed, for example for the microtubule motor protein CENP-E (Yen et al., 1992) and for cyclins (Murray et al., 1989). However, we consider this second explanation unlikely, since E-MAP-115 reappears rapidly in metaphase cells. To distinguish between these two possibilities we followed E-MAP-115 during the cell cycle by immunoblotting of HeLa cells released from an aphidicolin block at the G1/S boundary (Fig. 3). DNA staining and immunofluorescence labeling for tubulin were performed in parallel to determine their stage in the cell cycle (Table I). Mitotic cells ap-
pear 11 h after release and are most abundant (~19%) at 12–14 h. During this period, a discrete band, with higher apparent molecular weight than interphase E-MAP-115, can be observed. This band is equally well detected by the mAb and polyclonal antibodies (not shown). Due to the large difference in amount of protein in the two bands and their minor separation on the gel, it has not been possible to quantify the precise ratio between these two forms of E-MAP-115. Clearly, however, the upper band contains less than 20% of the total protein, and thus cannot be present in all the mitotic cells, but rather only during a limited period during mitosis. Our results in fact indicate a correlation between the occurrence of this upper band and the maximal number of cells in prophase and metaphase (12 and 13 h).

To correlate more precisely a putative posttranslational modification of E-MAP-115 with the different stages of mitosis, we followed the protein by immunoblotting of extracts of HeLa cells released from a nocodazole block (Fig. 1). In parallel we classified the cells into the different stages of mitosis by DNA staining and phase contrast microscopy (Table II). Cells blocked in mitosis by nocodazole appear to be in a prophase-like stage and peak in metaphase 40–60 min after release from the block. By 90–120 min many cells (15–25%) have still not recovered from the nocodazole treatment and appear to degenerate. These are probably included in the 47% cells apparently still in a prophase-like stage 60 min after removal of nocodazole. All E-MAP-115 runs with slower electrophoretic mobility at the beginning of the release; during the subsequent two hours of chase, the apparent molecular weight of E-MAP-115 progressively shifts back to its lower interphase value. Quantification of the mitotic and interphase forms of E-MAP-115 confirms posttranslational modification of the protein during prophase and metaphase.

We compared E-MAP-115 from cells synchronized in mitosis by microtubule-active drugs or after release from a block at the G1/S boundary by two-dimensional gel elec-

Figure 1. Localization of E-MAP-115 in mitotic HeLa cells. HeLa cells were fixed in methanol after detergent preextraction and double immunofluorescence labeling was performed with anti-T13 against tubulin (A, D, and G) and mAb D9C1 against E-MAP-115 (B, E, and H), and followed by fluorescein and rhodamine-labeled secondary antibodies. DNA staining with Hoechst (B, E, and H) allowed the identification of the different stages of mitosis. Cells at the onset of prophase (A–C), later in prophase (D–F), in metaphase (G–I, upper cell) and telophase (G–I, lower cell) are shown. Note the decreased staining for E-MAP-115 on microtubules in early prophase (C). Bar, 50 μM.

Figure 2. The localization of E-MAP-115 is independent of the method of fixation. HeLa cells were fixed in methanol after detergent preextraction (A and B) or in paraformaldehyde (C–E) and labeled with anti-T13 against tubulin (A) and mAb D9C1 against E-MAP-115 (B) or with anti-tubulin mAb 1A2 (C) and polyclonal antibodies raised against a peptide deduced from the E-MAP-115 cDNA sequence (E) followed by fluorescein and rhodamine-labeled secondary antibodies. DNA was stained with Hoechst (D). E-MAP-115 localization is independent of the method of cell fixation (B and E), but microtubules are better fixed in methanol than in paraformaldehyde. A representative image of interphase HeLa cells shows labeling of E-MAP-115 on perinuclear sub-sets or subdomains of microtubules (B). As in cells fixed with methanol and stained with mAB D9C1 (see Fig. 1), staining for E-MAP-115 on microtubules is decreased in early prophase cells (E, asterisk). Bar, 50 μM.
from a Block at the G1/S Boundary

The block at the G1/S boundary induced by aphidicolin was released by transferring HeLa cells into normal medium. Mitotic cells were scored by immunofluorescence labeling for tubulin and Hoechst staining for DNA at different time points after the release. Immunoblotting was performed at the same time points in parallel cultures (see Fig. 3). ~ 700 cells were counted for each time point and classified into the different stages of mitosis and interphase (written as percent of total cells). Interestingly, there is only one main region of the protein, the “PAPA-box”, predicted to have hydrophobic properties (Masson and Kreis, 1993).

Table I. Quantitation of Mitotic HeLa Cells following Release from a Block at the G1/S Boundary

| Time after release (h) | Prophase | Metaphase | Anaphase | Telophase | Interphase |
|------------------------|----------|-----------|----------|-----------|------------|
| 0                      | 0        | 0         | 0        | 100       |            |
| 5                      | 0        | 0         | 0        | 100       |            |
| 9                      | 0        | 0         | 0        | 100       |            |
| 10                     | 0        | 0.2       | 0        | 99.6      |            |
| 11                     | 2.1      | 1.8       | 0.6      | 1.8       | 93.7       |
| 12                     | 5.8      | 4.6       | 0.3      | 8.4       | 80.9       |
| 13                     | 4.7      | 4.5       | 0.3      | 9.3       | 81.2       |
| 14                     | 2.2      | 5.3       | 0        | 10.2      | 82.3       |
| 15                     | 1.9      | 2.4       | 0.5      | 5.7       | 89.5       |
| 16                     | 0.5      | 0.9       | 0        | 3.8       | 94.8       |

The block at the G1/S boundary induced by aphidicolin was released by transferring HeLa cells into normal medium. Mitotic cells were scored by immunofluorescence labeling for tubulin and Hoechst staining for DNA at different time points after the release. Immunoblotting was performed at the same time points in parallel cultures (see Fig. 3). ~ 700 cells were counted for each time point and classified into the different stages of mitosis and interphase (written as percent of total cells). Cells were considered as prophase cells as soon as the chromatin had condensed and the MTOCs separated. Divided cells which were still linked by the midbody but with an interphase intracellular organization (e.g., nuclear organization, arrangement of microtubules) were counted as interphase cells.

Further biochemical analysis of phosphorylation of E-MAP-115 required higher amounts of mitotic cells. We decided to use nocodazole treatment to block cells in M phase, since E-MAP-115 from "mitotic" cells synchronized with microtubule-active drugs appears identical to modified E-MAP-115 from a population with cells enriched in mitosis after release from the aphidicolin block; we refer to the nocodazole-arrested cells as "mitotic" and to the non-synchronized cells as "interphase" cells.

E-MAP-115 was immunoprecipitated from metabolically 32P-labeled mitotic and interphase cells. Amounts of protein and level of phosphorylation were quantified by immunoblotting (Fig. 5 A) and autoradiography (Fig. 5 B), respectively. Interphase E-MAP-115 is phosphorylated. Incorporation of phosphate into E-MAP-115, however, is more than 15-fold increased in mitotic cells. This increased mitotic phosphorylation of novel sites on E-MAP-115 probably induces a conformational change of the protein, leading to decreased electrophoretic mobility. Indeed, the apparent molecular weight of mitotic E-MAP-115 shifts back to its lower interphase value upon treatment with alkaline phosphatase (Fig. 5 C). Phospho-amino acid analysis revealed only phosphoserine in the interphase protein (Fig. 6 A). In addition to increased serine phosphorylation, a strong phosphorylation of threonine was observed in the mitotic protein. Suggesting phosphorylation by a specific mitotic kinase (Fig. 6 B).

To examine further possible differences in phosphorylation sites in interphase and mitosis, E-MAP-115 phosphorylated both in nonsynchronized and synchronized cells was analyzed by tryptic phosphopeptide mapping (Fig. 7). Several peptides barely detectable in interphase cells are strongly labeled in mitosis (Fig. 7 C, arrows); other peptides appear less phosphorylated because identical amounts of total radioactivity were loaded for both samples. The actual amounts of phosphate associated with these latter peptides remained approximately the same. The most prominent mitotic phosphopeptide of E-MAP-115 shows significantly higher hydrophobicity than the others (Fig. 7 C, arrowhead). This peptide is hardly labeled in interphase E-MAP-115 (residual phosphate is most likely due to contamination with peptide from mitotic cells present in a normal population of cells). Interestingly, there is only one main region of the protein, the “PAPA-box”, predicted to have hydrophobic properties (Masson and Kreis, 1993).
Table II. Quantitation of Mitotic Cells and of Modified E-MAP-115 following Release from a Nocodazole Block

| Time after release (min) | Interphase cells | Prophase cells | Metaphase cells | Anaphase cells | Telophase cells | Degenerated cells | Mitotic E-MAP-115 | Interphase E-MAP-115 |
|-------------------------|-----------------|----------------|-----------------|---------------|----------------|------------------|------------------|-------------------|
|                         |                 |                |                 |               |                |                  |                  |                   |
| 5                       | 0               | 99             | 1               | 0             | 0              | 0                | 100              | 0                 |
| 20                      | 0               | 95             | 5               | 0             | 0              | 0                | 100              | 0                 |
| 40                      | 0               | 69             | 25              | 3             | 3              | 0                | 96               | 4                 |
| 60                      | 0               | 47             | 23              | 8             | 22             | 0                | 69               | 31                |
| 90                      | 6               | 6              | 12              | 3             | 47             | 26               | 29               | 71                |
| 120                     | 12              | 0              | 7               | 1             | 63             | 17               | 6                | 94                |

Cells synchronized in mitosis by nocodazole were collected by mechanical release from their substrate and allowed to proceed through mitosis in suspension in drug-free medium. Cells were plated on poly-L-lysine-treated dishes 5 min before the different time points, at which mitotic stages were quantified by observation of cells stained for DNA (written as percent of total cells). Immunoblotting was performed in parallel cultures (see Fig. 5A) and mitotic and interphase E-MAP-115 were quantified by scanning of the immunoblots (written as percent of total E-MAP-115 for each time point).

Analysis of the predicted amino acid sequence of E-MAP-115 for potential phosphorylation sites indicates numerous S/TP residues in the nonhelical domains of the protein, and a S/TPXR (aa 209-212, SPDR) sequence at the end of the microtubule-binding region (Masson and Kreis, 1993). Interestingly, a predicted tryptic peptide including the hydrophobic PAPA-box contains two TP motifs (aa 416-7 and 450-1). Such sequences are potential substrates for cdc2 kinase (Nigg, 1991). Thus, we investigated the effect of chicken cdc2-cyclin-B (kindly provided by E. Nigg, ISREC, Lausanne, Switzerland) on E-MAP-115. Preliminary results suggest, however, that E-MAP-115 is not a substrate of this kinase: the characteristic shift in apparent molecular weight of the protein is not induced by incubation with the mitotic kinase (not shown), only weak phospholabeling of E-MAP-115 is detected and tryptic phosphopeptide map analysis does not reveal any significant labeling of the peptides predominantly phosphorylated in mitotic cells (not shown). Clearly, further work will be necessary to identify the kinase involved in phosphorylating E-MAP-115.

E-MAP-115 from Mitotic Cells Does Not Bind to Microtubules In Vitro

To analyze the phosphorylation-dependent interaction of E-MAP-115 with microtubules, tubulin was polymerized by addition of taxol to cytosol from mitotic HeLa cells. Microtubules were then sedimented through a sucrose cushion and supernatant and pellet were analyzed by immunoblotting. Control cytosol from interphase cells was analyzed in

Figure 4. Comparison of E-MAP-115 from mitotic cells obtained by different synchronization procedures. (A) Analysis of E-MAP-115 in nocodazole-arrested cells at different time-points after removal of the drug. Mitotic HeLa cells were produced as described in Materials and Methods and E-MAP-115 separated on 6% SDS-PAGE and analyzed by immunoblotting with mAb D9C1. Note the modification of E-MAP-115 electrophoretic mobility coinciding with prophase and metaphase (see Table II for comparison). (B) 2-D gel analysis of E-MAP-115 from cultures enriched in mitotic cells by different synchronization procedures. HeLa cells were either arrested at the G1/S boundary with aphidicolin (see Materials and Methods) and analyzed 13 h after removal of the inhibitor (M), were synchronized in mitosis by 0.2 μM nocodazole (N-t, N-d, and N-a), or by 6 μM taxol (T-d). E-MAP-115 was immunoprecipitated from either a total cell lysate (M, N-t), from lysates of mitotic rounded-up cells (N-d and T-d) or from the adherent cells remaining after shaking off of the mitotic cells (N-a). Immunoprecipitates were analyzed on 2-D gels (the second dimension was 7% SDS-PAGE) followed by immunoblotting with mAb D9C1.

Figure 5. E-MAP-115 is hyperphosphorylated in mitosis. (A and B) HeLa cells were metabolically labeled with [32p]orthophosphate for 4 h after growth for 12 h with (mit.) or without (int.) 0.2 μM nocodazole. E-MAP-115 was immunoprecipitated from cell lysates, separated by SDS-PAGE and detected by immunoblotting with mAb D9C1 (A) after autoradiography of the filter (B). (C) The effect of alkaline phosphatase treatment (AP+) on immunoprecipitated interphase (int) and mitotic (mit) E-MAP-115 was followed by immunoblotting with anti-E-MAP-115 polyclonal antibodies. β-glycerophosphate was used as a control to inhibit alkaline phosphatase activity (AP−).
E-MAP-115 from mitotic cells still weakly interacts with microtubules. In fact, some E-MAP-115 is found on the spindle microtubules of cells released from a block in mitosis by nocodazole, when virtually all protein detected by immunoblotting appears to be still modified (not shown). We conclude that hyperphosphorylation during prophase and metaphase decreases microtubule-binding of E-MAP-115.

Discussion

E-MAP-115 was identified as a microtubule-associated protein which stabilizes interphase microtubules in polarizing epithelial cells (Masson and Kreis, 1993). We report here that E-MAP-115 dissociates from microtubules at the beginning of mitosis and progressively reassociates with microtubules from late prophase onwards. Synchronization of cells allowed the biochemical analysis of E-MAP-115 during mitosis. We observed hyperphosphorylation of E-MAP-115 coinciding with the maximal number of cells in prophase and metaphase. This hyperphosphorylation correlates with a decreased binding of E-MAP-115 to microtubules in vitro, and is consistent with the prediction that stabilizing proteins must be transiently released from microtubules to allow their dynamic reorganization at the onset of mitosis.

Since hyperphosphorylation of E-MAP-115 is transient, we arrested cells in early mitosis with nocodazole. Based on morphological observations, nocodazole has been reported to block cells either at the onset of mitosis (Zieve et al., 1980) or in metaphase (Jordan et al., 1992). Thus, it is not exactly known to which stage of mitosis this block corresponds and whether nocodazole-arrested cells can be fully compared to normal mitotic cells. Furthermore, the activity of factors controlling E-MAP-115 modification may depend on intact microtubules. The active form of
cdc2 kinase, for example, appears to be concentrated at the mitotic spindle (Tombes et al., 1991; Kubiak et al., 1993) probably via the interaction of cyclin B with MAP4 (Ookata et al., 1995), and regulation of its activity depends on microtubules (Andreassen and Margolis, 1994). Thus, we compared modified E-MAP-115 from a population of cells enriched in mitosis after release from the aphidicolin block to the G1/S boundary with E-MAP-115 from cells arrested early in mitosis by the microtubule-active drugs nocodazole and taxol. E-MAP-115 appeared identically modified in these different cells, excluding possible effects of the drugs and modification of microtubule dynamics on the phosphorylation of E-MAP-115. Synchronization of cells with aphidicolin or nocodazole both suggest that E-MAP-115 is hyperphosphorylated in prophase and in metaphase cells; however, we cannot exclude an effect of nocodazole treatment on the normal time course of E-MAP-115 phosphorylation/dephosphorylation during mitosis.

E-MAP-115 expression levels increase with epithelial cell polarization (Masson and Kreis, 1993), thus, we assume it is predominantly involved in microtubule stabilization in interphase. Release of E-MAP-115 from microtubules at the onset of mitosis may destabilize microtubules and may also render them accessible to severing factors (Shiina et al., 1992b; McNally and Vale, 1993), thus leading to the assembly of the dynamic mitotic spindle. Since the bulk of E-MAP-115 is hyperphosphorylated until about metaphase and only little associated with spindle microtubules until late in prophase, and furthermore, because only low levels of E-MAP-115 can be detected in fibroblasts, we conclude that E-MAP-115 does not play an important role in spindle formation. Yet, we cannot exclude that a fraction of E-MAP-115 may stabilize a subset of spindle microtubules. Indeed, not all spindle microtubules are dynamic, and significant labeling of the metaphase spindle with E-MAP-115 can be seen. Stabilization of a subset of spindle microtubules probably occurs to a large degree by capping of their plus ends at the kinetochore (Mitchison et al., 1986). Other MAPs may also regulate microtubule dynamics. A role for MAP4 in modulating spindle fiber assembly has been proposed since microinjection of an anti-MAP4 mAb into cells before anaphase induces spindle dissolution (Izant et al., 1983). Another candidate is the stable tubule only polypeptide (STOP) which has been localized on stable microtubules in mitotic spindles (Margolis et al., 1990) and a microtubule regulating function during mitosis has also been proposed for XMAP230 (Andersen et al., 1994). Alternatively, E-MAP-115 may interact with microtubules in late prophase and metaphase without stabilizing them. Indeed, different forms of E-MAP-115 may exist between early mitosis and interphase, in agreement with mitotic E-MAP-115 heterogeneity and the fact that trace amounts of the hyperphosphorylated mitotic protein co-sediment with microtubules in vitro. These forms would be defined by subtle changes in phosphorylation which would determine different affinities for microtubules. In this respect, it is interesting that microinjected MAP2 and MAP4 do bind to microtubules in mitotic cells. Their rates of exchange between microtubules and cytosol, however, increase (Olmsted et al., 1989), and, although MAP4 binds to microtubules, it does not stabilize them during mitosis (Ookata et al., 1995).

The kinase activity associated with the cdc2-cyclin-B complex plays a crucial role at the G2 to M phase transition. Since some cytoskeletal proteins are direct substrates of this kinase (lamin; Peter et al., 1990; vimentin; Chou et al., 1990), we investigated its possible activity on E-MAP-115. Our data suggest, however, that another kinase phosphorylates E-MAP-115 and is involved in modulating its function. Yet, we cannot unambiguously exclude the possibility that E-MAP-115 is not phosphorylated by cdc2-cyclin-B, because other factors (e.g., interactive proteins) may be missing in our in vitro system, or because the protein used as the substrate (affinity-purified or bacterially expressed E-MAP-115), although it binds to microtubules, is not properly folded. In addition it is also possible that the microtubule-binding activity of E-MAP-115 is regulated by cdc2-cyclin-B via a MAP kinase. Furthermore, although cdc2-cyclin-B kinase was initially believed to be the universal regulator of the cell cycle, a growing number of cdc2-related kinases and of different cyclins are being discovered (Nigg, 1993). Specific kinases may phosphorylate different MAPs and have different effects on these proteins. Alternatively, MAPs may be substrates of a unique kinase at the onset of mitosis and their phosphorylation state may then be controlled by (specific) phosphatases. The rates of removal of phosphate may vary between different MAPs depending on their properties and functions. In this respect, MAP4 (Olmsted et al., 1989; Vandré et al., 1991) and XMAP230 (Andersen et al., 1994) might be dephosphorylated rapidly, since they colocalize with spindle microtubules. In contrast to these proteins, the 220-kD MAP from Xenopus appears to remain cytosolic until late in mitosis (Shiina et al., 1992a). MAPs which are regulated in their function during the cell cycle will provide important tools for characterizing novel kinases and phosphatases and for the characterization of the recently discovered cdc2-related enzymes.

Interaction of MAPs with microtubules appears to be generally regulated via phosphorylation (see for example Pallas and Solomon, 1982; Lindwall and Cole, 1984; Díaz-Nido et al., 1988; Brugg and Matus, 1991; Rickard and Kreis, 1991). We have shown that E-MAP-115 interacts with microtubules via its basic NH2-terminus (Masson and Kreis, 1993); phosphorylation of sites in this part of the
protein would reduce the net positive charge of this domain and weaken its binding to the acidic surface of the tubulin polymer. Alternatively, phosphorylation in another region of the molecule might induce an alteration of the conformation of the protein. Such a conformational change could lead to the neutralization of the highly charged basic NH2-terminal part of E-MAP-115 by interaction with its acidic COOH terminus. These two regions are connected via the PAPA-box, a proline-rich region, which could play the role of a hinge. In fact, this proline-rich region contains the most hydrophobic polypeptide of the protein, and one of the peptides predominantly phosphorylated in mitosis is significantly much more hydrophobic than all other phosphopeptides. The precise characterization of the sites of E-MAP-115 which are phosphorylated during mitosis will thus be essential for the further analysis of the regulation of its microtubule-binding activity and may also allow identification of the specific kinase involved. In addition it will contribute to a better understanding of the domain organization of the protein; in particular, it will provide information on whether the role of the COOH-terminal half of the protein is to modulate the microtubule-binding activity of its NH2-terminal part or if it has other independent functions.

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