Regulation of Microtubule Organization during Interphase and M Phase

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ABSTRACT. Microtubule (MT) dynamics and organization change markedly during interphase-M phase transition of the cell cycle. This mini review focuses first on p220, a ubiquitous MT-associated protein of Xenopus. p220 is phosphorylated by p34cdc2 kinase and MAP kinase in M phase, and concomitantly loses its MT-binding and MT-stabilizing activities. A cDNA encoding p220 was cloned, which identified p220 as a Xenopus homolog of MAP4, and p220 was therefore termed XMAP4. To examine the physiological relevance of XMAP4 phosphorylation during mitosis, Xenopus A6 cells were transfected with cDNA encoding wild-type or various XMAP4 mutants fused with a green fluorescent protein (GFP). Mutations of serine and threonine within potential phosphorylation sites for p34cdc2 kinase to nonphosphorylatable alanine interfered with mitosis-associated reduction in MT-affinity of XMAP4 and their overexpression affected chromosome movement during anaphase A. These results indicated that phosphorylation of XMAP4 by p34cdc2 kinase is responsible for the decrease in its MT-binding and MT-stabilizing activities during mitosis which are important for chromosome movement during anaphase A. The second focus is on a novel monoclonal antibody W8C3, which recognizes α-tubulin. W8C3 stained spindle MTs but not interphase MTs of Xenopus A6 cells, although tubulin dimers in M phase and interphase were equally recognized by this antibody. The difference in MT staining pattern may be because the W8C3-recognition site on α-tubulin is sterically hidden in interphase MTs but not in spindle MTs.

Key words: XMAP4/p34cdc2 kinase/microtubule dynamics/mitosis/chromosome movement/tubulin

Microtubules (MTs) are dynamic polymers composed of 13 protofilaments of α- and β-tubulin heterodimers. The heterodimers assemble onto and disassemble from both ends of MTs and the MTs alternate between periods of growth and shrinkage (15). In interphase cells, the heterodimers constitute relatively stable and long MTs emanating from centrosomes. These interphase MT networks serve as tracks for intracellular transport of vesicles and as scaffolds for cellular organelle distribution. At the onset of mitosis, under the control of p34cdc2 kinase (11, 14, 19, 22), the frequencies of transition in MTs from growth to shrinkage (catastrophe) increase (5), resulting in the organization of the dynamic and unstable MTs into the mitotic spindle which is responsible for chromosome segregation (12, 21, 25, 26, 30).

In this mini review, we focus on I. p220, a ubiquitous MT-associated protein (MAP) of Xenopus, which is one of candidates for the factors responsible for regulation of MT dynamics, and II. W8C3, a novel monoclonal antibody which reacts differently to interphase and M phase MTs.

Phosphorylation of p220 (Xenopus MAP4) and chromosome movement during M phase

MAPs bind to and stabilize MTs both in vitro and in vivo, and are thought to regulate MT dynamics during the cell cycle. p220 was identified and purified as a ubiquitous MAP of Xenopus and shown to have MT-binding and MT-stabilizing activities in vitro (27). p220 is phosphorylated specifically in mitosis, which is associated with a decrease in its MT-binding and MT-stabilizing activities (27). Kinases responsible for p220 phosphorylation have been identified as p34cdc2 kinase and MAP kinase (27), both of which are known to be activated in mitosis (9, 16). XMAP230 has also been identi-
fied and purified as a Xenopus MAP and thought to be identical to p220 due to its similar characteristics (2). In mammalian tissues and cells, MAP4 (1, 6, 20, 23, 32) is known to be phosphorylated specifically at the onset of mitosis (29), and MAP4 phosphorylation by p34\(^{cdk2}\) kinase has been shown to decrease the ability of MAP4 to stabilize MTs in vitro (24). Phosphorylation of MAP4 by MARK has also been reported to markedly decrease its MT-stabilizing ability (8, 13).

**cDNA cloning of p220**

As the first step to examine the physiological relevance of p220 phosphorylation during mitosis, we cloned a cDNA encoding Xenopus p220 (28). The obtained cDNA encoded a protein of 1224 amino acids with a predicted molecular mass of 130 kD. The deduced amino acid sequence contained one of the internal peptide sequences directly determined from purified XMAP230 (3), indicating that XMAP230 is identical to p220.

A data base search revealed that p220 was a Xenopus homolog of MAP4, and p220 was designated as XMAP4. XMAP4 contained 6 potential phosphorylation sites for p34\(^{cdk2}\) kinase, 2 sites for MAP kinase and 2 sites for both kinases. Four potential phosphorylation sites for MARK were also contained in the sequence (28).

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**A nonphosphorylatable mutant XMAP4 affects chromosome movement during anaphase**

We constructed a mutated XMAP4, named CM10A, in which all of the 10 potential phosphorylation serine/threonine residues for p34\(^{cdk2}\) kinase and/or MAP kinase were mutated to alanine, and transfected as a green-fluorescent protein (GFP)-fusion protein into Xenopus cultured A6 cells. Compared to control cells expressing GFP-wild-type XMAP4 (GFP-WT) which show normal mitosis, the cells expressing GFP-CM10A showed abnormal mitosis; the cells entered into mitosis with normal mitotic spindle formation, but furrowing was initiated without spindle elongation, followed by the formation of multiple cleavage furrows. For example, Figure 1A shows a time-lapse of an A6 cell expressing GFP-CM10A observed by GFP-fluorescence which is localized to spindle MTs and phase contrast. The cell had two cleavage furrows, a and b, at the beginning of the observation. At 3 min, furrowing at b proceeded and the spindle was pushed leftward. Then, at 12 min, furrowing at a was triggered and the spindle was pushed rightward. We named this series of movement “back-and-forth peristaltic movement” (BFP movement). In some cases, multiple cleavage furrows were induced during BFP movement, which led to abnormal cytokinesis (Fig. 1B).

To examine the phenotype in more detail, chromosomes were visualized by DAPI staining. In control cells expressing GFP-WT, chromosomes were aligned
Fig. 2. Impaired anaphase chromosome separation in a living cell expressing GFP-CM10A. A living A6 cell expressing GFP-CM10A was observed by phase contrast, GFP fluorescence and DNA staining. Time after the beginning of observation is indicated in the top right of each image. The cell had been exhibiting BFP movement at the beginning of observation and arrows indicate the position of failed cytokinesis. Arrowheads denote another position of cytokinesis which proceeded at ~6–8 min without chromosome segregation.
at the spindle equator in metaphase, and separated toward spindle poles without spindle elongation in anaphase A, followed by spindle elongation in anaphase B and cytokinesis (28). In contrast, in cells expressing GFP-CM10A, spindle elongated without chromosome separation (Fig. 2), i.e. chromosomes failed to move toward spindle poles during anaphase A. Moreover, cytokinesis appeared to proceed without chromosome segregation (Fig. 2, 6–8 min). Finally, in some cases, cytokinesis failed to proceed and chromosomes began to decondense without segregation, and in other cases chromosomes were forced to segregate into two daughter cells (28).

Phosphorylation and MT-affinity of XMAP4 during mitosis

To confirm whether CM10A was not phosphorylated during mitosis, A6 cell extracts were immunoblotted with anti-XMAP4 antibody for band-shift assay (Fig. 3). In mitotic cells, GFP-WT as well as endogenous XMAP4 were shifted upward. When the extracts were treated with phosphatase, these upward shifts were completely suppressed, indicating that the upward-shifted XMAP4 was the phosphorylated form. In contrast to GFP-WT, GFP-CM10A did not show any mobility shift in mitotic cells, indicating that GFP-CM10A was nonphosphorylatable during mitosis.

Furthermore, MT-affinity of GFP-WT and GFP-CM10A was examined by a co-sedimentation assay with MTs (Fig. 4). GFP-WT and endogenous XMAP4 bound to and co-precipitated with MTs in large amounts in interphase, but in mitosis, they showed little binding to MTs. In contrast, most of the GFP-CM10A bound to and co-precipitated with MTs even in mitosis. These results indicated that GFP-WT reduced its MT-binding ability in mitosis, but GFP-CM10A did not.

*p34^cdc2 kinase-specific phosphorylation sites on XMAP4 are responsible for mitotic regulation of XMAP4

We constructed three more XMAP4 mutants, C6A, in which 6 potential phosphorylation residues specific for p34^cdc2 kinase were mutated to alanine, CM8A, in which 2 residues for both p34^cdc2 kinase and MAP kinase were mutated to alanine in addition to the above 6 residues, and R4A, in which 4 residues for MARK were mutated to alanine (28). As summarized in Table

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**Fig. 3.** Phosphorylation of wild-type and mutant XMAP4. Interphase (I) and mitotic (M) extracts of A6 cells expressing GFP-WT or GFP-CM10A were immunoblotted with anti-XMAP4 antibody. Note that the bands of GFP-WT as well as endogenous XMAP4 were shifted upward up to the top arrow and arrowheads in mitosis, whereas the band of GFP-CM10A did not show any mobility shift. When the extracts were treated with phosphatase, the upward shifts in mitosis were completely suppressed.
MT-binding and MT-stabilizing abilities. Then, chromosomes move toward the spindle poles by depolymerization of MTs at the plus ends. If nonphosphorylatable XMAP4 is abundant along spindle MTs, these mutant XMAP4 molecules would hyper-stabilize the spindle MTs, which would affect the MT depolymerization-driven chromosome movement. Of course, this model does not exclude the possible involvement of motor proteins in anaphase chromosome movement (7, 10, 17, 18, 31).

It has been generally thought that phosphorylation of MAP4 during mitosis may be involved in reorganization of MTs at the interphase-M phase transition, i.e. the elimination of interphase MTs prior to formation.

I, phosphorylation and MT-affinity of GFP-C6A and GFP-CM8A in mitosis were the same as those of GFP-CM10A, and mitotic phenotype of the cells expressing GFP-C6A or GFP-CM8A was also the same as that of the cells expressing GFP-CM10A. In contrast, the effects of GFP-R4A were the same as those of GFP-WT. These results indicated that XMAP4 phosphorylation at p34cdc2 kinase-specific phosphorylation sites was primarily responsible for its regulation of MT-affinity which is important for anaphase chromosome movement.

Possible role of XMAP4 phosphorylation in M phase

A possible model for the role of XMAP4 phosphorylation in anaphase chromosome movement is shown in Figure 5. During mitosis, XMAP4 is phosphorylated by p34cdc2 kinase, followed by reduction in its

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Fig. 5. A possible model for the role of XMAP4 phosphorylation in anaphase chromosome movement. During mitosis, XMAP4 is phosphorylated by p34cdc2 kinase and concomitantly reduces its MT-binding and MT-stabilizing abilities (A). If nonphosphorylatable XMAP4 is abundant on MTs, the MTs would be hyper-stabilized, which would affect MT depolymerization-driven chromosome movement (B).
of spindle MTs. Expression of nonphosphorylatable mutant XMAP4 affected anaphase chromosome movement, but did not affect the elimination of interphase MT networks. To determine the regulatory mechanism of MT dynamics and organization, further analyses on other MT regulatory factors may be required.

**Different reaction on interphase and M phase MTs of W8C3 monoclonal antibody that recognizes α-tubulin**

**W8C3 stains spindle MTs but not interphase MT networks**

We produced monoclonal antibodies against pericentriolar material (PCM) from *Xenopus* eggs. One of the obtained monoclonal antibodies, W8C3, stained centrosomes of cultured *Xenopus* A6 cells throughout the cell cycle (Fig. 6). This centrosome staining revealed to be attributed to PCM-1 (4). Interestingly, in addition to the centrosomes, W8C3 stained spindle MTs but not interphase MTs of A6 cells (Fig. 6). The staining of MTs by W8C3 appeared at prometaphase, lasted during mitosis and disappeared at late telophase. Interphase MTs were not stained even when A6 cells were treated with purified W8C3 immunoglobulin at a higher concentration (2 mg/ml). As PCM-1 was never localized to spindle MTs, another antigen was considered to be localized to spindle but not to interphase MTs.

**W8C3 recognizes α-tubulin**

The staining of spindle MTs with W8C3 remained even after the cells were treated with 0.5 M KCl, suggesting that the antigen firmly binds to spindle MTs or is tubulin itself. To identify the antigen, MTs with their associated proteins were prepared from *Xenopus* M phase egg extracts, subjected to column chromatography, and blotted with W8C3. Peak fractions recognized by W8C3 eluted coincidently with tubulin dimers, which suggested that W8C3 recognizes tubulin. Tubulin dimers purified to near homogeneity from *Xenopus* eggs or pig brains were also recognized by W8C3. Furthermore, recombinant *Xenopus* α-tubulin and β-tubulin fused with GST were expressed in *E. coli*, purified by glutathione-Sepharose chromatography, and blotted with W8C3. GST-α-tubulin, but not GST-β-tubulin, was recognized by W8C3, indicating that W8C3 recognizes α-tubulin. In addition, immunoprecipitation from A6 cells revealed that tubulin dimers were the major and specific proteins precipitated with W8C3. These lines of evidence indicated that W8C3 recognizes α-tubulin and that the antigen which is localized specifically to spindle MTs is α-tubulin.

This raises the question why W8C3 stains spindle MTs but not interphase MTs. W8C3 may recognize some differences in post-translational modifications of α-tubulin, but the observation that interphase and M phase tubulin of *Xenopus* eggs were equally recognized by W8C3 excluded this possibility. The possibility, which our observations favor, is that W8C3-binding to α-tubulin is sterically inhibited in interphase MTs, but not significantly in M phase MTs. Further studies, e.g. identification of W8C3-binding sites on α-tubulin and reaction of W8C3 on MT formation in the *Xenopus* egg cell-free system, will elucidate the mechanism of different reactions of W8C3 on interphase and M phase MTs.

**References**

Microtubule Organization in Interphase and M Phase


