

Microtubules in Mitosis

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The roles of the microtubule cytoskeleton in a variety of cellular functions have long been discussed as among some of the most fascinating problems in cell biology. Many works have been focused on the molecular functions of microtubules in mitosis, including the microtubule-organizing function of the centrosomes, organization of mitotic spindles and kinetochore microtubules, and spindle motors. Numerous Hypotheses have been proposed for the mechanism of mitosis during the past decades, and it appears that we are now reaching the threshold leading to the analyses of the molecular mechanism of mitosis. This minireview discusses recent progress in the study of mitosis, focusing on microtubules in mitosis.

1. Centrosomes

No one would deny that the centrosome is the organelle that has long been discussed most frequently in connection with the cellular functions relevant to mitosis (1–3). It has been established that the centrosome is the organelle that is inevitable for the formation of the bipolar spindle (4). Since Gould and Borisy (5) demonstrated that it is not the centriole but pericentriolar materials that support the microtubule-nucleating ability of the centrosome, many attempts have been carried out to identify the protein components in the pericentriolar materials necessary for the nucleation of microtubule assembly (for a review, see ref. 6). A recent review by Kalt and Schliwa (7) describes that more than thirty components are involved in the centrosome. However, few proteins have been identified that function to nucleate microtubule assembly *in vitro* as well as *in vivo*.

Relevant to the microtubule-nucleating ability of the centrosomes, the interrelationship between microtubule dynamics and the activity of cdc2 kinase has been suggested first by Verde *et al.* (8). Incubation of the centrosomes isolated from KE37 lymphocytes with the extracts of *Xenopus* egg at interphase caused marked growth of microtubules from the centrosome at a velocity of 18 $\mu\text{m}/\text{min}$. Using mitotic extracts, the growth rate was 3.8 $\mu\text{m}/\text{min}$ slower than that observed using interphase extracts. Namely, cdc2 kinase, which is known

to be activated at mitotic phase, seemed to cause phosphorylation of MAPs leading to an enhancement of the dynamic instability of microtubules, since the addition of 6-dimethylaminopurine totally suppressed enhancement of the dynamic instability of microtubules caused by cdc2 kinase.

The treatment of the isolated centrosome with 2 M urea was found to destroy the ability of the centrosome to nucleate microtubules. Then, the addition of *Xenopus* egg extract to the inactivated centrosomes was found to recover the activity of the centrosome (9). This kind of "maturation" (10) of the centrosome was expected to occur at the onset of mitosis. Therefore, they examined the effect of cyclins on the recovery of the microtubule-nucleating ability of the centrosome. Both cyclin A and B induced the activation of H1 kinase in the extract. However, cyclin A-associated kinase alone resumed the ability of the centrosome to nucleate microtubules. It is not known why cyclin B does not work for the recovery.

Independent of Karsenti's group, Ohta *et al.* (11) demonstrated that the phosphorylation of the pericentriolar materials by MPF is necessary for the maturation of the centrosome. They isolated the centrosomes from L517Y cells. Inactivation of the centrosomes was carried out by treating the isolated centrosomes with 1 M KCl. Recovery of the microtubule-nucleating activity of the KCl-treated centrosomes by *Xenopus* egg extract was shown to be accompanied by marked accumulation of pericentriolar materials as demonstrated by using anti- γ tubulin antibody, anti-centrosome antibody, and by electron microscopy. It was shown that during the recovery process the number of microtubules per centrosome was found to increase with time. The maturation of the centrosome was supposed to be induced by MPF which is known to be activated at the G₂/M phase. Therefore, they added K252a or Staurosporine to inhibit the kinase activity. As expected, these inhibitors suppressed the formation of asters initiated from the KCl-treated centrosomes.

Comparing the ability of both the interphasic and mitotic extracts to resume the activity of the KCl-treated

centrosomes, the latter was shown to have 3.5 to 5 times higher activity than the former. However, an important finding was that the amount of the accumulated pericentriolar materials was almost the same for both cases. Therefore, the difference in the activity to nucleate microtubules was attributed to the difference in the degree of phosphorylation of some centrosomal components by MPF. Then, the addition of cdc2/cyclin B complex was demonstrated to enhance the ability of the KCl-treated centrosome to nucleate microtubules. In contrast, MAP kinase, which was recently demonstrated to be situated downstream in the MPF signaling pathway for the G₂/M transition (12), revealed little activity to complement the inactivated centrosomes. The regulation of the activity of the centrosome to nucleate microtubules has previously been discussed (10). It remains to be solved what kind of protein components in the pericentriolar materials are responsible for the substrate of MPF and for the nucleation of microtubules.

A recent paper by Maniotis and Schliwa (13) using BSC-1 cells originated from African green monkey kidney presented an exciting concept on the role of the centrosome. They made cytoplasts and karyoplasts by microdissection. Although the karyoplast had no centrosomes, it formed a big aster without the centrioles after 20 to 30 hrs, which was supposed to be a microtubule network characteristic of interphase. The karyoplast seemed to replicate its genome, but the condensation of chromatin and the breakdown of the nuclear envelope did not occur. This phenomenon suggests that MPF would not be activated in the karyoplast because of the absence of the centrosome. This experimental result gives us a very important concept that the centrosome may govern the activity of MPF which in turn matures the centrosome.

2. Formation of mitotic spindle

It is the mitotic spindle that has a long history of study and is still a mysterious organelle body. Many hypotheses including the dynamic equilibrium theory (14) have been proposed for this mass of microtubules embedded in the membranous matrix.

As soon as the nuclear envelope breaks down at prometaphase, the centrosome quickly initiates spindle microtubules (15) which soon capture the kinetochore facing to the pole in bivalent chromosomes. This causes rapid movement of the chromosomes to the pole (16). However, microtubules initiated from the opposite centrosome capture the counterpart kinetochore in the bivalent, which results in the congression of chromosomes until metaphase is established. Therefore, the inward force in the spindle will be exerted to the poles. This may be due to minus-end directed motors working at the kinetochores. McNeil and Berns (17) clearly showed that when one of the opposite kinetochores is irradiated

by a laser microbeam at prometaphase or metaphase, the bivalent moves to the pole facing the unirradiated kinetochore. This happens before anaphase, indicating that the kinetochores are already competent to produce forces before anaphase. Unless a counterforce, that is an outward force, is given in the spindle produced by plus end-directed motors, the spindle will be collapsed (18). This force may be produced by kinesin-like proteins, possibly associated with intercalating microtubules at the equator region of the spindle.

It is a great contribution to the study of mitosis that Lohka and Maller (19) have established a way to form the spindle *in vitro*. This was confirmed by Mitchison's group (20) who found that the spindle is formed *in vitro* in two ways. The extract was prepared from *Xenopus* eggs after Lohka and Maller (19) and spindles were allowed to be formed in the extract. In the mitotic extract, the spindle was formed by the fusion of a pair of half spindles. In contrast, when the sperm nuclei were exposed to the interphase extract, DNA was replicated and bipolar spindles were formed from the beginning. These results strongly raise the possibility that in the near future the chromosome movement will be analyzed using spindles formed *in vitro*.

3. Microtubule dynamics in the spindle

Metaphase is characterized by equilibrated microtubule dynamics in the spindle. The geometric array of the bivalents on the equator seems to be an apparent steady state in the balance of counterforces between the plus and minus end-directed motors. Once the connection between daughter chromosomes in each bivalent is released, the minus end-directed motors will transport daughter chromosomes to each pole.

Fluoresceine-labeled tubulin was shown to be incorporated into mitotic spindles within 30 seconds when injected into live mitotic sea urchin eggs (21, 22). In the case of the maturation division of oocytes, exogenous tubulin was incorporated and equilibrated in the meiotic spindle within 15 min (23). Photobleaching of this spindle revealed that 80% of the fluorescence were recovered within 4 min, indicating that rapid turnover of tubulin is one of characteristics of the spindles.

It has been established that tubulin flux is seen in the spindle at metaphase, being incorporated at the kinetochore and depolymerizing at the poles (24). This flux was observed at metaphase but not at anaphase (25). The rate of the flux [0.39 $\mu\text{m}/\text{min}$ (26)] was close to that of treadmilling *in vitro* (27). However, the rate of microtubule flux in the spindle produced *in vitro* [3 $\mu\text{m}/\text{min}$ (28)] was shown to be much faster than the rate of tubulin flux mentioned above, which may indicate that some sort of motor protein is involved in the microtubule flux.

An elegant work done by Spurck *et al.* (29) showed

that in PtK₁ cells irradiated by a laser microbeam for 3 seconds, the polar side of the irradiated region depolymerized quickly with a rate of $0.33 \pm 0.01 \mu\text{m}/\text{sec}$. However, the minus end of the irradiated microtubules did not change at least for 3 min, time enough to detect any change if tubulin flux had actually occurred. This result is consistent with those reported by Walker *et al.* (30) in *in vitro* experiments on dynamic instability of polymerized microtubules, but contradicts the data convincing the flux of tubulin or microtubules in the spindle.

4. Kinetochore microtubules

It has been established that the kinetochore captures the plus end of microtubules polymerized from the poles (16). This experimental result leads one to examine the possibility that the kinetochore of isolated chromosomes is capable of capturing microtubules *in vitro*. This kind of work was previously carried out by Huitorel and Kirschner using chromosomes isolated from CHO cells (31). Both the minus and plus ends of reconstituted microtubules were captured by the kinetochore of the isolated chromosomes. The rate of the capture reaction was the same for both ends. However, the minus end was shown to dissociate from the kinetochore 7 times faster than the plus end. This result is consistent with the behavior of microtubules grown from the centrosome at prometaphase.

Microtubules have been believed to be stabilized when the plus and minus ends are capped. However, the situation is somewhat different in the spindle as clearly shown by Hyman and Mitchison (32), although we have no evidence indicating that the attachment of microtubules to the kinetochore is really a capping of the plus end. When the plus end of microtubules is attached to the kinetochore, the dynamic instability of the microtubules is enhanced, accompanied by frequent shortening of the microtubules. This may explain how kinetochore microtubules are depolymerized at the kinetochore after anaphase (24, 33). If one explains the dynamic instability of microtubules by the GTP cap hypothesis (34, 35), non-hydrolyzable analogues of GTP will calm the microtubule dynamics. In fact, GMP-PNP suppresses depolymerization of microtubules from the kinetochore by stabilizing the microtubule plus ends (32).

That microtubules depolymerize at the kinetochore at anaphase is due to the interaction between the kinetochore and the microtubule plus end. This kind of interaction has been shown to be affected by tubulin concentration around the spindle. Shelden and Wadsworth (36) injected biotin-labeled tubulin into PtK₁ cells at anaphase to observe the incorporation of the labeled tubulin into the spindle. The fluorescence was observed at the basal region of the kinetochore within 15 seconds. The tubulin incorporation was observed during anaphase A, but not observed during anaphase B. When

the kinetochore incorporated tubulin, the chromosome movement toward the pole was reversed, the chromosomes temporarily moving back toward the equator. This is an important observation, that is, even anaphase cells incorporate tubulin at the kinetochore provided the tubulin concentration is much increased in cells, causing an elongation of kinetochore microtubules and the movement of chromosomes toward the plus end of microtubules, namely, toward the equator. When injected tubulin concentration was lowered to 0.3 mg/ml, no reverse movement was observed. This may indicate that after anaphase the tubulin content around the kinetochores should be maintained at a lower level to support the movement of the chromosomes toward the poles.

5. Motor proteins in the spindle

No review of microtubules in mitosis would be complete without at least a brief discussion of spindle motor proteins in connection with microtubules. The microtubule-based motors transduce chemical energy of ATP to the unidirectional movement along microtubules as railroads. Therefore, the polarity of the microtubules would have allowed to exist two kinds of motor proteins, one being the dynein family as plus end-directed motors and the other the kinesin family as minus end-directed motors. Both the motor proteins seem to share various motile roles in cells. Cytoplasmic dynein was first purified from sea urchin eggs (37). Four years later, MAP 1C was identified as a cytoplasmic dynein and demonstrated to be a retrograde translocator (38).

After kinesin was identified as a plus-end directed motor protein capable of transporting vesicles or organelles along microtubules (39), many proteins involved in kinesin superfamily have now been identified (for reviews, see refs. 18, 40 and 41). The heavy chains of proteins involved in the kinesin family are homologous to each other, but other regions are not. Kinesin heavy chain contains a globular domain that binds ATP and microtubules. The head domain alone is sufficient for transportation of vesicles (42). A group called bimC subfamily was classified into kinesin superfamily including the products encoded by the *bimC* gene of *A. nudulans* (43), the *cut 7* gene of the fission yeast (44), the *CIN8* and *KIP1* genes of the budding yeast (45) and the *Eg5* gene of *Xenopus laevis* (46).

One of the cellular functions of microtubule-based motor proteins is attributed to the activity in mitosis as biomolecules capable of producing forces driving various movements in the spindle. Recent papers on this field appear to focus on the role of motor proteins in mitosis. Evidence has been presented that the mutation of genes that encodes *bimC* subfamily results in the failure of the separation of the spindle poles (43–45, 47). Therefore, it is plausible that these plus end-directed motors

may exert forces against the inward forces produced by the minus end-directed motors, since the disruption of *KAR3* which seems to be a minus end-directed motor partially suppresses the phenotype produced by mutation of *cin8* and *kip1* genes (48). Although the direction of the movement induced by *KAR3* has not yet been determined, it is close to *ncd* protein from *Drosophila* in the primary structure with the motor domain at the carboxyl terminal for both the proteins (49, 50).

Concerning the kinetochore motors, it is already three years ago when cytoplasmic dynein was identified to be localized to the kinetochore (51, 52). Furthermore, a kinesin-like motor protein, CENP-E, was also found to be localized to the kinetochore at prometaphase, moved to the midbody at anaphase and disappeared at telophase (53). This motor was assumed to play roles in the movement of the chromosomes and the elongation of the spindle. Therefore, when the progress of the cell cycle was arrested by treatment with Colcemid, CENP-E was not degraded.

Prior to the above report, Hyman and Mitchison (54) made an important contribution to the study of kinetochore motors. When they made the kinetochore capture microtubules *in vitro*, the addition of ATP induced the movement of the isolated chromosomes toward the minus end of microtubules. When ATP- γ S was included in the capture reaction, some components of the kinetochore were thiophosphorylated. What was surprising was that the direction of the movement of the chromosome was reversed, moving back toward the plus end. The velocity of the plus end-directed movement was $2.9 \pm 1.5 \mu\text{m}/\text{min}$. This result suggests two possibilities. One is that the phosphorylation of a motor protein alters the direction of the movement. Another is that the kinetochore has two motor proteins and phosphorylation induces inhibition of the minus end-directed motor with activation of the plus end-directed motor. In this connection, a dynein-like motor protein in a fresh water amoeba is known to drive the movement for both directions (55). The regulation of the directionality of the intracellular movement is an intriguing problem to be solved and the phosphorylation of the motor proteins is one of the candidates that modulate their activity like kinase cascades as seen in the signaling pathway in the cell cycle (56).

The CDE III region of the 125 base pairs DNA sequence of the centromere in the budding yeast (CEN sequence) is known to be a major region that controls the chromosome segregation. A protein complex capable of associating with this CDE III region was affinity purified and called CBF3 (57). This complex was composed of 110 kDa, 64 kDa, and 58 kDa, having activities to bind microtubules to DNA as well as to translocate microtubules as a minus end-directed motor protein with a velocity of $4.1 \pm 1.6 \mu\text{m}/\text{min}$ (58), almost the same vel-

ocity as that of chromosome movement. Although the molecular weight of neural cytoplasmic dynein (MAP 1C) is larger than that of the subunit of CBF3, there may be smaller cytoplasmic dynein in non-neural cells. Whether this motor can be categorized into cytoplasmic dynein remains to be solved.

CONCLUSION

The maturation of the centrosome in terms of its acquisition of the ability to nucleate microtubules has recently been demonstrated *in vitro* using isolated centrosomes from mammalian cells and cell-free extracts of *Xenopus* oocyte, in which phosphorylation of some components of the centrosome by *cdc2* kinase was shown to be involved. An elegant experiment performed by Maniotis and Schliwa (13) brought forth an important idea that the centrosome may govern the activation of MPF. Evidence has accumulated suggesting that the maturation of the centrosome is followed by the collaboration of the plus and minus end-directed spindle motor proteins in the formation of the mitotic spindle. An exciting subject in the study of mitosis has been identification and characterization of spindle motors for the transport of daughter chromosomes toward the poles, and they may be clarified in the near future with further analysis of the regulation of the directionality of the chromosome movement induced by spindle motors. Moreover, the establishment of *in vitro* assembly of spindles originally developed by Lohka and Maller (19) may favor analyses of the mechanism of spindle formation and chromosome movement.

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