The spindle checkpoint in *Xenopus Laevis*

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1. ABSTRACT

The spindle checkpoint ensures accurate chromosome segregation by delaying anaphase onset until all kinetochores have properly established bipolar attachment to spindle microtubules. This mechanism is important for all eukaryotic cells and is evolutionarily conserved. Much of our understanding of the molecular and biochemical mechanisms of the spindle checkpoint has been gained from parallel studies in various experimental systems. In particular, the cytoplasmic extract from the eggs of *Xenopus laevis* provides an unsurpassable system for biochemical analysis of the spindle checkpoint and has made important contributions to the field. This article reviews the progress of the spindle checkpoint studies in *Xenopus laevis* with a focus on the regulation by phosphorylation.

2. EGG EXTRACTS OF THE *XENOPUS LAEVIS*

Xenopus oocytes and eggs have played crucial roles in advancing our understanding of the cell cycle control in general. One of the major advantages of the system arises from the synchronous cell cycle arrests during oocyte development. The oocyte first arrests at G2/M transition of the first meiotic division. Upon hormone stimulation, the oocyte completes meiosis I and becomes arrested at the metaphase-to-anaphase transition of meiosis II by the cytostatic factor activity (CSF) (review in 1), a process termed maturation. After fertilization, the mature egg enters anaphase II and completes meiosis. Thus, the naturally arrested oocytes are excellent systems for investigating the controls of both G2/M and metaphase/anaphase transitions. Metaphase arrests induced by both the spindle checkpoint and CSF result in the

inhibition of cyclin B degradation by the Anaphase Promoting Complex (APC), an E3 ubiquitin protein ligase that triggers sister chromatid separation and exit from mitosis. The CSF-induced arrest employs c-Mos/MEK/ERK pathway and is independent of chromosomes (2), whereas the spindle checkpoint arrest is mediated by unattached kinetochores. Some of the spindle checkpoint proteins are shown to be involved in the CSF arrest in frog (3-5), but not in mouse oocytes (6).

In addition to the advantage of synchronous cell cycle arrests, *Xenopus* egg extract allows biochemical dissection and reconstitution of complex processes in a test tube and has greatly facilitated cell cycle studies since its first establishment in 1984 by Lohka and Masui (7). This is made possible by the large size of the eggs (~1 mm in diameter) in comparison with somatic cells. The eggs can thus be broken open to separate the cellular contents simply by centrifugation without the addition of any buffer or detergent. The extracts prepared in this manner are essentially like undiluted cytoplasm and behave similarly to intact cells. The extracts derived from oocytes arrested at G2 also remain at interphase, whereas CSF-arrested eggs give rise to metaphase extracts. These extracts are able to reproduce many cellular events in vitro, including the spindle checkpoint.

3. ACTIVATION OF THE SPINDLE CHECKPOINT IN EGG EXTRACTS

The spindle checkpoint signal is generated from kinetochores that are not attached to spindle microtubules or not under tension that is normally generated from bipolar attachment. Likewise, the checkpoint can be reproduced in egg extracts by the addition of frog sperm nuclei and the microtubule depolymerizing agent nocodazole to create unattached kinetochores. It is generally believed that unattached kinetochores recruit checkpoint proteins and promote the formation of a complex between Cdc20 and spindle checkpoint proteins Mad2, BubR1 (Mad3 in yeast), and Bub3. In this way, Cdc20 is sequestered by the checkpoint proteins and cannot function as an activator and substrate adaptor for the APC (8). Even a single unattached kinetochore in the cell is able to inhibit anaphase onset (9). In egg extracts, it is necessary to add the sperm nuclei to a high enough density (~9000 nuclei per μl extract) in order to produce a sufficient amount of the checkpoint complex to completely inhibit Cdc20 (10). The checkpoint is generally reproduced in extracts prepared from mature eggs that are arrested at metaphase II by CSF. Once the checkpoint is activated, the extracts remain at M phase and cannot be released into anaphase upon the addition of calcium that normally inactivates CSF (11).

The ability of egg extracts to reproduce spindle checkpoint permits in vitro biochemical manipulation of the system. By immunodepleting the protein of interest, one may reveal the role of the protein in the spindle checkpoint. In addition, the effect of specific mutations on the checkpoint can be examined by adding back proteins carrying the mutations. These biochemical knock-out and knock-in have greatly facilitated the studies of spindle checkpoint in vertebrates before RNAi approach in cultured cells becomes a routine. The knock-in approach is traditionally performed with recombinant proteins purified from bacterial or baculoviral expression system which may be time consuming and may produce insoluble proteins. To overcome these problems, we have established a new expression method to produce the protein of interest directly in egg extracts (12). It uses in vitro transcription to generate desired mRNA that is then added into immunodepleted egg extracts for the extracts to synthesize the proteins. This method allows production of several proteins at the same time. In addition, the synthesis of proteins in the natural system ensures that the proteins contain the physiological modification and function. Besides immunodepletion, the neutralizing antibody or dominant-negative mutants can be used to determine when the protein is required for the checkpoint (13). Adding the antibody or mutant proteins to egg extracts before incubation with nuclei and nocodazole answers the question of whether the protein is required for the establishment of the spindle checkpoint. On the other hand, the requirement for the maintenance of the spindle checkpoint is determined by adding the antibody or mutants to extracts that have been first incubated with nuclei and nocodazole to activate the checkpoint.

4. ORDERED ASSEMBLY OF SPINDLE CHECKPOINT PROTEINS AT THE KINETOCHORE

Systematic immunodepletion of individual checkpoint proteins has been used to examine the kinetochore localization of various checkpoint proteins and the order in their assembly to kinetochores in *Xenopus* egg extracts. It has been demonstrated that there is a hierarchy as well as mutual dependency among the spindle checkpoint proteins. Kinetochore association of Mps1, Bub1-Bub3, and BubR1-Bub3 is necessary for Mad1 and Mad2 to localize to these loci, but not vice versa (12, 14, 15). Mps1, Bub1, and BubR1 are mutually dependent in kinetochore localization (15). In addition, Mad1 associates with Mad2 and the interaction is required for Mad1 to recruit Mad2 to kinetochores (16). It appears that these proteins act in a complex network, rather than in a simple pathway. These results are generally consistent with studies in other model systems, but there are a few exceptions. For example, BubR1 is not required for Bub1 localization to kinetochores in human cells (17). In addition, Mps1 is not necessary for kinetochore localization of human Bub1 and BubR1 (18). The inconsistency may reflect variation between different species or cell types, a limitation in the detection method, or a different extent of the protein knockdown.

Besides their central roles in the spindle checkpoint, some of the spindle checkpoint proteins have other functions. Bub1 not only recruits outer kinetochore components, but is also required for the assembly of the inner centromeric region (19), suggesting that Bub1 plays an additional role in the structural integrity of the kinetochore. Consistent with this notion, RNAi study shows that Bub1 is important for chromosome congression
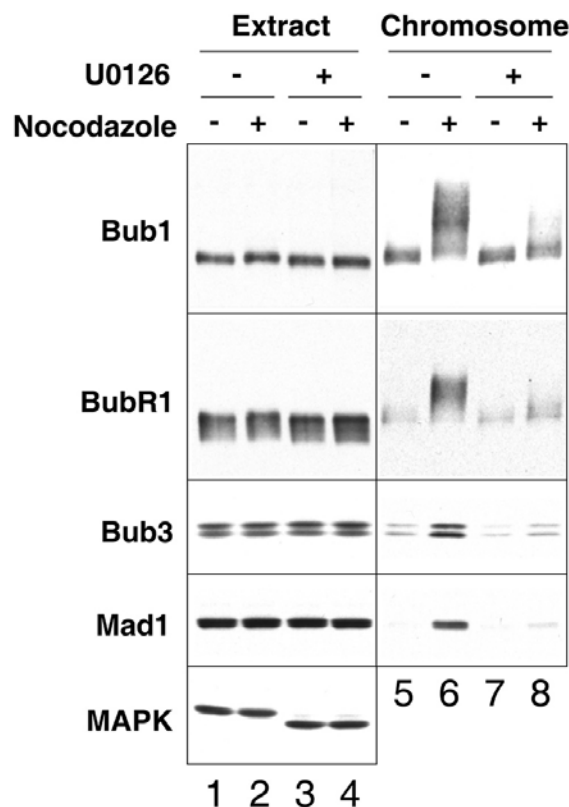
Figure 1. Hyperphosphorylation of Bub1 and BubR1 at unattached kinetochores. Total proteins (lanes 1-4) and chromosomal fractions (lanes 5-8) from metaphase samples (odd lanes) and samples treated with nocodazole (even lanes) in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of MEK inhibitor U0126 were immunoblotted for proteins indicated on the left. (Reproduced with permission from 23)

(17). Furthermore, Mad1 and Mad2 localize to nuclear envelope during interphase in *Xenopus* and human cultured cells and in budding yeast (13, 20, 21). These proteins associate with the nuclear pore complex and are involved in nuclear transport (22).

5. REGULATION OF BUB1 BY PHOSPHORYLATION

Unattached kinetochore is where the spindle checkpoint signal is generated through interactions of the checkpoint proteins. However, it has been very difficult to examine the biochemical properties of these proteins at kinetochores due to the relatively low level of kinetochore proteins and the technical limitation of purifying a sufficient amount of chromosomes in most model systems. With the ability to reproduce the spindle checkpoint in egg extracts, we have developed a simple protocol to purify mitotic chromosomes through a sucrose cushion (16). This approach has enhanced the biochemical studies of kinetochore-bound proteins and provided new information about the regulation of these proteins.

By Western blot analysis, it is shown that unattached chromosomes associate with a higher level of Bub1, BubR1, and Bub3, in comparison with attached chromosomes (Figure 1) (14, 23). In addition, Mad1 and Mad2 are present only on unattached, but not attached chromosomes (16). These results are consistent with immunofluorescence studies, indicating that the chromosome purification protocol indeed separates the chromosomes from the cytosol. They also suggest that microtubule attachment displaces Mad1 and Mad2 from kinetochores, and reduces the amount of Bub1, BubR1, and Bub3 at these loci.

In addition to easy quantification of protein levels associated with chromosomes, Western blot of chromosomal fractions prepared from egg extracts also reveal important posttranslational modifications of the spindle checkpoint proteins. Notably, Bub1 and BubR1 are found to be highly phosphorylated on unattached, but not attached kinetochores (Figure 1) (23). Hyperphosphorylation of Bub1 enhances its kinase activity, because the protein eluted from unattached kinetochores has a higher autophosphorylation activity than that from unattached kinetochores (23). Hyperphosphorylation and activation of Bub1 are not necessary for the spindle checkpoint. However, titration experiments using a range of nuclear density or nocodazole concentration shows that phosphorylation and activation of the kinase activity are important for the spindle checkpoint under a sub-optimal condition without a full-flag checkpoint signal. It indicates that phosphorylation facilitates the spindle checkpoint by efficiently recruiting other spindle checkpoint proteins to unattached kinetochores. This study suggests that activation of Bub1 at kinetochores enhances the generation of the spindle checkpoint signal and is likely important for maintaining the checkpoint towards late prometaphase when the cell contains only a few or even a single unattached kinetochore. Because a similar manipulation of the proteins or the number of unattached kinetochores cannot be easily achieved in other experimental systems, it is not known whether the same mechanism also operates in other vertebrates. The study in other vertebrates awaits the generation of specific anti-phosphoprotein antibodies in the future.

The functional significance of BubR1 hyperphosphorylation at unattached kinetochores in egg extracts remains to be addressed. BubR1 contains a Mad3 domain at the N-terminal and a kinase domain at the C-terminal regions, both of which are necessary for the spindle checkpoint. The kinase domain binds kinetochore motor protein CENP-E (24). Using purified components, it has been shown that CENP-E binding to BubR1 activates the kinase activity of BubR1, and that microtubule binding to CENP-E inactivates BubR1 and silences the spindle checkpoint (24). Thus, CENP-E appears to be involved in both activation and inactivation of BubR1. It remains a possibility that CENP-E regulates the phosphorylation state of BubR1 at kinetochores.

MAP kinase (MAPK) contributes to Bub1 hyperphosphorylation at unattached kinetochores (Figure
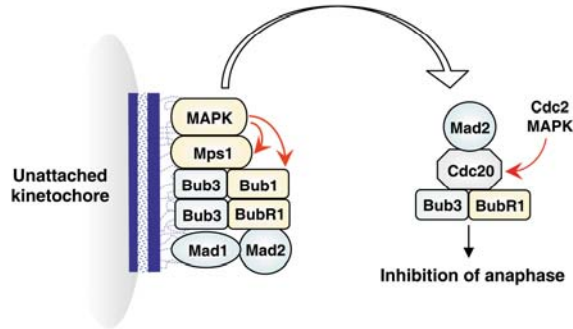
Figure 2. The function of MAPK in the spindle checkpoint. The unattached kinetochore recruits spindle checkpoint proteins and promotes the formation of Cdc20, Mad2, BubR1, and Bub3 complex. Molecules shaded in yellow are kinases. MAPK phosphorylates Bub1, Mps1, and Cdc20 in *Xenopus* egg extracts. The effect of the phosphorylation is described in the text.

1)(23), indicating that MAPK protein or its activity may be regulated by microtubule attachment. Alternatively, a kinetochore-associated protein phosphatase may be regulated by microtubules. In addition to MAPK, Mad1 is also important for Bub1 hyperphosphorylation. This finding reveals an additional function for Mad1 in the spindle checkpoint, besides recruiting Mad2 to kinetochores (16). It is possible that Mad1 stabilizes or enhances the activity of MAPK at kinetochores, or that Mad1 blocks the access of a phosphatase to Bub1. These important questions remain to be addressed in the future.

The downstream events of Bub1 are not well characterized. Bub1 in budding yeast phosphorylates Bub3 (25), but it is not known whether the phosphorylation is important for the spindle checkpoint. Similar to yeast, Bub1 and Bub3 also form stable complex in metazoans, but it is not clear whether Bub1 also phosphorylates Bub3 in organisms other than yeast. In human cells, Bub1 is shown to phosphorylate and inhibit Cdc20 in vitro (26). Cdc20 mutated at the phosphorylation sites is partially defective in response to the spindle checkpoint, even though the mutant protein can still bind Mad2 and BubR1. It remains to be determined how the phosphorylation controls the checkpoint response and whether the same phosphorylation occurs in other organisms.

6. ROLE OF MAPK IN THE SPINDLE CHECKPOINT

The role of MAPK in the spindle checkpoint was first demonstrated in *Xenopus* egg extracts. Immunodepletion of MAPK or the addition of specific MAPK phosphatase (MKP) or MAPK kinase inhibitor PD98059 to egg extracts abolishes the spindle checkpoint (10, 27, 28). In *Xenopus* tissue cultured cells, microinjection of MAPK antibodies or MKP also perturbs the checkpoint (29). Identifying the substrates for MAPK is necessary for understanding how MAPK functions in the spindle checkpoint. Besides Bub1 described above, MAPK has been shown to phosphorylate Cdc20 and Mps1 in the spindle checkpoint. Cdc20 is phosphorylated at multiple

sites in both human and *Xenopus* egg extracts by Cdk1 and MAPK (30-33). The phosphorylation is not required for Cdc20 to activate APC (31, 32). However, phosphorylation at all Cdk1 and MAPK sites is necessary for Cdc20 to be bound with and inhibited by the spindle checkpoint proteins (30). This study suggests that spindle checkpoint can only target fully phosphorylated Cdc20. The finding also has an implication in the termination of the spindle checkpoint. It is possible that inactivation of MAPK or Cdk1 during anaphase onset may result in the initial dephosphorylation of Cdc20, leading to the disassembly of existing checkpoint complex and partial activation of APC. The APC-Cdc20 then further degrades cyclin B to allow complete dephosphorylation of Cdc20 and full activation of APC-Cdc20. Thus, dephosphorylation of Cdc20 may provide a positive feedback mechanism to quickly silence the checkpoint and trigger the anaphase. This model is yet to be determined.

MAPK also targets Mps1 in *Xenopus* egg extracts. The kinase activity of Mps1 is required for the spindle checkpoint (34) and for kinetochore localization of Bub1, Bub3, Mad1, and Mad2 (34, 35). Phosphorylation of Mps1 by MAPK is not necessary for the kinase activity of Mps1, but is required for the spindle checkpoint (15). Blocking the phosphorylation disrupts kinetochore association of Mps1, Bub1, BubR1, Mad1, and Mad2 (15). The study suggests that phosphorylation of Mps1 by MAPK may create a phosphoepitope that allows Mps1 to interact with kinetochores. It also demonstrates for the first time that active Mps1 must localize to kinetochores in order to execute its checkpoint function.

These studies in *Xenopus* egg extracts demonstrate that MAPK plays a pivotal role in the spindle checkpoint and that MAPK activity is required for the major biochemical events of the checkpoint, indicating that MAPK likely lies on the top of the spindle checkpoint pathway (Figure 2). However, the function of MAPK in spindle checkpoint in mammalian somatic cells remains controversial. In mouse cells, it has been shown that active MAPK is enriched at unattached kinetochores (36), consistent with a role in spindle checkpoint signaling. However, other study fails to find MAPK at kinetochores (37). Moreover, a recent live cell imaging study shows that MAPK is not involved in the spindle checkpoint in either normal or transformed mammalian somatic cells (38). It is not known whether the difference in the immunofluorescence studies is due to different sensitivity of various anti-phosphoMAPK antibodies. It remains a possibility that MAPK may be present at a low level at kinetochores or dynamically interact with kinetochores, so that it is hard to detect the protein. In addition, the kinetochore-bound MAPK may not be accessible to a phosphatase, so that inhibiting MAPK kinase has little effect on the small fraction of active MAPK on kinetochores. Furthermore, MAPK family includes extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), c-Jun N-terminal kinase (JNK), and p38. The major form of MAPK in *Xenopus* eggs is p42 ERK2 that is responsible for the spindle checkpoint. On the other hand, it has been shown that p38 is important for the spindle

checkpoint in cultured mouse fibroblasts (39), suggesting that different members of the MAPK family may be involved in the spindle checkpoint in different cell types.

7. AURORA B

Aurora family kinases are involved in several mitotic processes, including G2/M transition, mitotic spindle assembly, chromosome segregation, and cytokinesis (review in 40). Aurora A localizes to centrosomes and microtubules near the spindle poles during mitosis. Aurora B is a subunit of the chromosome passenger protein complex that resides at inter centromeres at prometaphase and metaphase, moved to anaphase spindle midzones after anaphase, and to midbody during cytokinesis. Aurora B plays a role in proper microtubule-kinetochore attachment by phosphorylating the motor protein MCAK (mitotic centromere-associated kinesin), a microtubule depolymerase that is required for the changes in microtubule dynamics during mitotic spindle formation (41, 42). Aurora B regulates the activity and localization of MCAK at the centromere, and this regulation is important for correcting syntelic microtubule-kinetochore attachment (42-45). In addition, Aurora B is important for the spindle checkpoint in response to microtubule disruption in mammalian cells and frog egg extracts (35, 46-48), and is also involved in the arrest induced by lack of tension across kinetochores in mammalian cells (46). Aurora B appears to regulate the early step of spindle checkpoint pathway, because it controls the kinetochore localization of Mps1, Bub1, Bub3, and CENP-E (35). As described above, MAPK also lies on the top of the spindle checkpoint signaling in egg extracts. It is of interest to determine whether Aurora B and MAPK act in the same pathway and the order of their actions. In fact, there is a crosstalk between Aurora B and Raf/MEK/ERK pathway. It is recently shown that Raf Kinase Inhibitory Protein (RKIP) is localized to centrosomes and kinetochores, and RKIP depletion causes a decrease in the phosphorylation and activity of Aurora B as well as disruption of mitotic arrest induced by nocodazole or taxol (49). This study indicates that Aurora B is regulated by Raf/MEK/ERK signaling pathway in mammalian cells. It also suggests that spindle checkpoint requires a balance of Raf/MEK/ERK activity. How this pathway is finely controlled and how it regulates Aurora B await future studies.

8. SUMMARY

Xenopus egg extract is able to reproduce the spindle checkpoint and is an excellent system for biochemical studies of the spindle checkpoint. Immunodepletion coupled with add-back of mutant proteins in the system provides an efficient way to dissect the functional role of spindle checkpoint proteins and the effect of specific mutations. In addition, the ability to easily fractionate chromosomes from the cytosol allows examination of chromosomal proteins and has provided information about biochemical properties of kinetochore-bound spindle checkpoint proteins. Despite these advantages, the egg extract has some limitations in the checkpoint study. For one thing, the spindle checkpoint

cannot be easily turned off in the extract once it is activated by the addition of nuclei and nocodazole, because there is no way to remove or inactivate nocodazole. Thus, the system is not suitable for studying the process of spindle checkpoint termination upon kinetochore attachment to microtubules. In addition, the microtubule-stabilizing agent taxol induces a checkpoint-dependent mitotic arrest by creating untensed kinetochores in the cell. However, taxol is unable to arrest the egg extracts at metaphase, so that the system has not been used for studying tension-sensitive checkpoint mechanism. Furthermore, it has been shown that MAPK plays a crucial role in the spindle checkpoint in *Xenopus* egg extracts and tissue cultured cells, whereas similar results have not been reproduced in other vertebrate systems. Nevertheless, *Xenopus* system has been and shall continue to be valuable in studying the biochemical and molecular controls of the spindle checkpoint.

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