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- Review -

Delay in the Cell Cycle by a Single Unattached Kinetochore

Taekyung Kim*

Department of Biology Education, Pusan National University, Busan 46241, Korea

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Mitosis is a process in which a replicated genome is distributed to two daughter cells, and it is necessary for cell survival and organismal development. During mitosis, the spindle assembly checkpoint (SAC) ensures faithful chromosome segregation by monitoring the kinetochore attachment to the mitotic spindle. Although the SAC mechanism has been extensively studied over the last 30 years, the mechanism by which a single unattached kinetochore activates the SAC remains unclear. The key components of the SAC are Mad1, Mad2, Mad3 (BubR1 in higher eukaryotes), Bub1, Bub3, and Cdc20, which are all required for SAC activation. An essential step for SAC activation is the formation of the Mad2–Cdc20 complex in the unattached kinetochore, which is kinetically disfavored. Although the mechanism by which Mad2 and Cdc20 are recruited to unattached kinetochores is well-known, it is not clear how they form a complex. Recently, a key mechanism for the formation of the Mad2–Cdc20 complex has been identified, which is catalyzed by an unattached kinetochore. This supports the evidence that a single unattached kinetochore can activate the SAC signaling. Herein, we discuss the known key mechanism for SAC activation, review the recent studies on SAC, and conclude how their discoveries improved the understanding of mitosis.

Key words : Cdc20, kinetochore, Mad2, mitosis, spindle assembly checkpoint

Introduction

The accurate distribution of the replicated genome during cell division is necessary for cell survival and organismal development. Errors in this process are not tolerated and can lead to birth defects and aneuploidy, which is a hallmark of cancer cells [11, 39]. At the core of this process is the kinetochore, a large multiprotein complex that couples chromosomes to spindle microtubule ends, and serves as a signaling platform to monitor errors in attachment and direct the progression through the cell cycle. During mitosis, once all kinetochores are attached to the spindle microtubules, anaphase promoting complex (APC/C or cyclosome), a key component of anaphase progression, is activated. This leads the progression of the cell cycle to the anaphase and the mitotic exit. The interaction between the kinetochores and the spindle microtubules is the key step for faithful chromosome segregation [30]. These interactions are stochastic, whereby not every kinetochore attaches to the spindle microtubules at the same time; some interact with spindle microtubules later than the others [37].

The spindle assembly checkpoint (SAC) delays the onset of anaphase until all kinetochores are stably attached to the spindle microtubules. The main effector of the SAC is the mitotic checkpoint complex (MCC), which consists of four proteins including Mad2 and Cdc20. The key to step to SAC activation is the formation of the Mad2-Cdc20 complex, which ultimately allows for MCC complex that formation that inhibits APC/C [23, 30].

However, how a single unattached kinetochore can activate the SAC remains unclear. Here, we review the two recent studies with different experimental approaches that identify the mechanism of the formation of Mad2-Cdc20 complex at unattached kinetochore, which is the key step for SAC activation [21, 34].

Overview of the Spindle Assembly Checkpoint

SAC genes were discovered through the genetic screens performed in budding yeast. They screened the cells that survived after treatment with benzimidazole that disrupts spindle microtubules, and found SAC genes including the mitotic-arrest deficient (Mad) genes Mad1, Mad2, Mad3 (termed BubR1 in higher eukaryotes), and Bub1 and Bub3,

***Corresponding author**
Tel : +82-51-510-2693, Fax : +82-51-514-8576
E-mail : taekim@pusan.ac.kr

and Mps1 [15, 24, 45]. Since the discovery of these genes, SAC has been extensively studied, and here we discuss key steps for the SAC activation [22, 23, 29, 30].

During mitosis, the APC/C, a E3 ubiquitin ligase, is activated by interacting with the essential co-activator Cdc20. This leads to anaphase progression by ubiquitinating anaphase inhibitors securin and CyclinB, resulting in activation of separase and inactivation of the mitotic kinase Cdk1, respectively (Fig. 1A). The SAC halts the anaphase progression by inhibiting APC/C activation via the MCC. The MCC, which consists of Bub3, Mad2, BubR1, and Cdc20, interacts with APC/C to inhibit its activation (Fig. 1B) [23, 30].

The formation of MCC requires several steps. First, the SAC components need to be recruited to unattached kinetochores. Bub1, which functions as a scaffold to recruit other spindle checkpoint components, is recruited to the kinetochore protein KNL-1 though the phosphorylation by Mps1 kinase (or alternatively Plk1) (Fig. 2) [9, 16, 27, 41, 46]. Bub1 contains several conserved motifs that interact with Bub3, BubR1, Mad1, Cdc20, and the Plk1 kinase. Some of these interactions may be mediated by the phosphorylation. It has been shown that interaction of the Bub1 with Mad1 is mediated by the phosphorylation of the CM domain of Bub1 by Mps1 kinase, with an exception in *Caenorhabditis elegans* [19, 21, 26]. Bub1 also recruits BubR1 to the kinetochore [17, 33]; however, in *C. elegans* and budding yeast, Mad3, the homolog of BubR1, is not recruited to the kinetochore [10, 12]. This indicates that kinetochore recruitment of BubR1 may not be essential for SAC activation. Additionally, Bub1 contains the ABBA motif that interact with Cdc20 [5, 6, 18]. Moreover, it has been suggested that BubR1 contains the ABBA motif [5, 6, 25], and this motif in both Bub1 and BubR1 contribute to the Cdc20 interaction [5, 6, 18, 25]; however, this remains to be clarified.

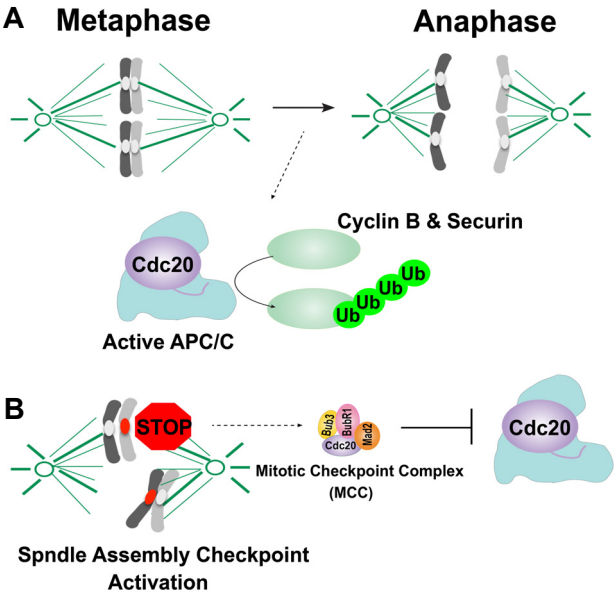

Fig. 1. Anaphase progression in mitosis and function of Spindle assembly checkpoint (SAC) in inhibition of the APC/C. A. Schematic of the function of APC/C in anaphase progression in mitosis. B. The role of SAC in delaying anaphase progression by inhibition of APC/C at unattached kinetochores.

Secondly, Mad2, the core component of the SAC activation, is recruited to the kinetochore by interacting with Mad1, which resides at the unattached kinetochore [22]. In addition, RZZ complex contribute to the kinetochore localization of Mad1-Mad2 in metazoans [4, 20, 42]. Whether there is a direct relationship between Mad1 and RZZ is needs to be identified. Mad2 exists in the two forms, open-free form (O-Mad2) and the closed- ligand bound form (C-Mad2) [28]. The current model suggests that formation of the Mad1-C-Mad2 complex recruits another O-Mad2 from the cytoplasm through dimerization of the Mad2, which leads the subsequent interaction with the Cdc20 and forms the C-Mad2-Cdc20 complex [2, 47].

Finally, the formation of the C-Mad2-Cdc20 complex allows sequential interaction of BubR1 and Bub3 to form the MCC complex that inhibits APC/C [13, 32].

Thus, Mad2-Cdc20 formation is thought to be mechanism that amplifies the SAC signal. However, the interaction of the Mad2 with Cdc20 is kinetically unfavorable and requires catalysis. Therefore, it is important to understand how unattached kinetochores catalyze the Mad2-Cdc20 formation.

Cell Biological approaches that identify the mechanism of the Mad2-Cdc20 formation

Lara-Gonzalez et al. and *Piano et al.* uncovered the mechanism by which unattached kinetochores catalyze the Mad2-Cdc20 formation using an *in vivo* and *in vitro* approaches [22, 34].

First, Lara-Gonzalez et al. generated a C-terminal MAD-2::GFP fusion transgene in *C. elegans*, which was the key tool for identifying the mechanism. Although MAD-2::GFP was not functional in spindle checkpoint signaling, it was able to localize to the kinetochore, which allowed for the monitoring of MAD-2::GFP in the cytosolic pool. MAD-2::GFP was unable to localize to the kinetochore when it is the only

source of the MAD-2, indicating that MAD-2::GFP localized to the kinetochore through dimerization with untagged MAD-2 bound to MAD-1 at the kinetochore.

Next, they tested where the C-Mad2-Cdc20 forms. The MAD-2::GFP mutant that cannot convert to the C-MAD-2 did not localize to the kinetochore, and the mutants of BUB-1 that eliminate CDC-20 to the kinetochore abolished the MAD-2::GFP localization at the kinetochore. This suggests that C-Mad2-Cdc20 forms at the kinetochore. However, bringing CDC-20 to the kinetochore and the conversion of the O-Mad2 to C-Mad2 at the kinetochore was not sufficient for the formation of the C-Mad2-Cdc20. Moreover, the Bub1 CM region, which is phosphorylated by Mps1 (or PLK-1 in *C. elegans*), was required for the MAD-2::GFP kinetochore localization. This suggested that phosphorylation of the Bub1 CM region and its interaction with MAD-1 are also required for the formation of the C-Mad2-Cdc20. But why does C-Mad2-Cdc20 preferentially form at the kinetochore? They proposed a model: when Cdc20 is in the cytoplasm, the motif in the Cdc20 N-terminus that interacts with Mad2 is masked; however, when Cdc20 is recruited to the kinetochore, phosphorylated Mad1 at the kinetochore may expose this motif (Fig. 2). Consistent with their hypothesis, when they mutated MAD-1's phosphorylation region, it abolished the spindle checkpoint signaling, and also eliminated the formation of the MAD-2::CDC-20 complex at the kinetochore.

In conclusion, these results suggested that Cdc20 has to interact with Bub1 and Mad1 simultaneously for the formation of the Mad2-Cdc20, and phosphorylation of Mad1 is essential for this process, which can only occur at unattached kinetochores.

Biochemical approaches that identify the mechanism of the Mad2-Cdc20 formation

Piano et al. approached the same question using biochemical assays based on reconstitution of the spindle checkpoint protein [34]. Using the several fluorescence-based *in vitro* sensors, they measured the association rate of the Cdc20 and Mad2 and monitored the MCC assembly. It has been suggested that once O-Mad2 is converted into C-Mad2, by docking to the Mad1:C-Mad2, it rapidly interacts with CDC20. However, they found that C-MAD2 alone is not associated well with full-length CDC20; instead, when Bub1-Bub3 complex was added, MCC assembly was significantly increased, suggesting that other MCC component was required for its assembly. In addition, adding the CDC20 fragment, $CDC20^{MIM}$, that interacts with Mad2 alone, showed much lower association rates compared to those when adding the full-length CDC20. This indicated that CDC20 region other than the MIM motif is required for their assembly (Fig. 2).

Then, they tested which region of CDC20 is required for

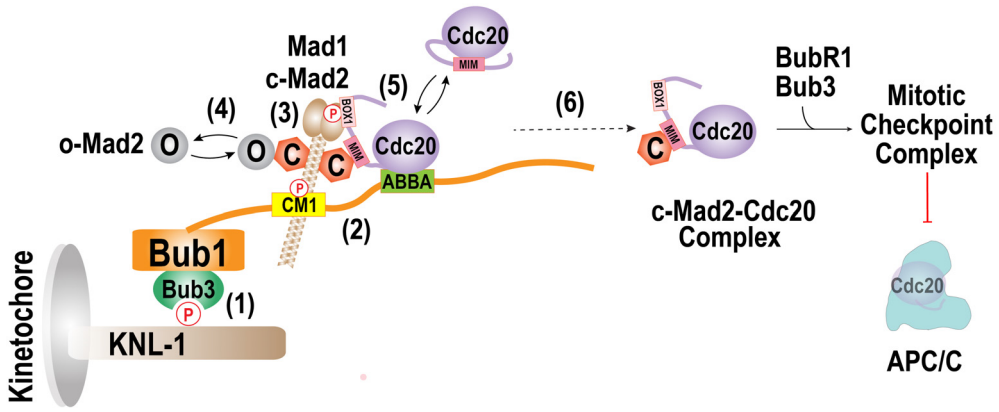

Fig. 2. Key steps in activating the SAC. Schematic of the spindle assembly checkpoint activation at unattached kinetochore. It requires several steps; (1) Bub1/Bub3 complex is recruited to the kinetochore through the phosphorylated region of KNL1, (2) Bub1 recruits Mad1 and Cdc20 through the interaction with CM1 motif and the ABBA motif, respectively. CM1 motif is phosphorylated by the Mps1 kinase and the Plk1 kinase, (3) Mad1 recruits O-Mad2 in the cytosolic pool, and O-Mad2 is changed to the C-Mad2, (4) C-Mad2 recruits O-Mad in the cytosolic pool through dimerization, (5) Phosphorylation of the $Mad1^{CTD}$ interact with $Cdc20^{BOX1}$. This interaction also unfurls the Cdc20 in the cytosolic pool, (6) $Cdc20^{MIM}$ form a complex with C-Mad2, which allows the sequential recruitment of other components of the MCC, Bub3 and BubR1, and MCC inhibit APC/C activation.

the catalysis, and found that both C-terminal beta-propeller and the N-terminal extension regions are required. It has been suggested that the C-terminal region of MAD1 interacts with N-terminal region of CDC20 via Mps1 phosphorylation. Using cross-linking mass spectroscopy, they found that the BOX1 motif in the N-terminal region of CDC20 interacts with C-terminal domain of MAD1 ($MAD1^{CTD}$), and the RWD domain and the RLK motif in the $MAD1^{CTD}$ are needed for the MCC assembly.

Mad1 and Cdc20 interact with Bub1 via its CM domain and ABBA motif, respectively; removing the Bub1 CM domain or mutation of the ABBA motif disrupted MCC assembly. Additionally, the minimal Bub1 constructs encompassing the CM1 and KEN-ABBA sites allowed for MCC formation, suggesting that simultaneous interaction of BUB1 with MAD1 and CDC20 promotes MCC assembly (Fig. 2).

In addition, by observing the Mad1:Mad2 complex by low-angle metal shadowing electron microscopy (EM), they found that a 10 nm~12 nm distance between the BOX1 and MIM motifs of CDC20 is needed for the interaction of Cdc20 and the C-Mad2, and the $MAD1^{CTD}$. Consistently, mutating the residues between the BOX1 and MIM motifs of CDC20 resulted in normal MCC assembly, which suggested that a minimal distance, not a specific sequence, between them is required for the MCC assembly.

Collectively, they demonstrated that Mad2 and Cdc20 must bind concomitantly with the catalytic complex for catalytic MCC assembly, which is fully consistent with the results of *Lara-Gonzalez et al* [21].

Conclusion

Here, we have discussed the key known mechanism for the SAC, and the reviewed the recent studies that identified mechanism of the Mad2-Cdc20 formation, which the key process for SAC activation. These studies identified that Bub1 functions as a scaffold that recruits Mad1, Mad2, and Cdc20 to the unattached kinetochore and bring them to a proximal distance to facilitate Mad2-Cdc20 formation. In addition, they showed that phosphorylation of the Mad1 at unattached kinetochore plays as a key role in Mad2-Cdc20 formation. Importantly, these findings suggest that post-translational regulation is critical for SAC signaling. Many kinases, including Cdk1, Mps1, Aurora B, PLK1, and phosphatases such as PP1, PP2A-B56, have been shown to be important for regulating mitosis [1, 3, 7-9, 14, 16, 27, 31, 35, 36, 38, 40, 41, 43, 44, 46]. Future research should investigate how phosphatase precisely regulate SAC via dephosphorylation of Mad1 and examine other phosphorylation events of spindle checkpoint proteins, which will provide insight into how cells continue to maintain their genome integrity.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 방추사와 연결되지 않은 단 하나의 키네토코어가 세포분열의 속도를 늦추는 기전

김태경*
(부산대학교 생물교육과)

세포의 유사분열 과정에서 Spindle Assembly Checkpoint (SAC)는 키네토코어와 방추체의 미세소관의 연결을 확인하여 오류 없이 염색체 분열이 진행되도록 돕는 역할을 한다. SAC는 30년이 넘는 오랜 기간 동안 많은 연구자들에 의해 연구되었다. 하지만 단 하나의 연결되지 않은 키네토코어가 SAC를 어떻게 활성화시킬 수 있는지에 대해서는 그 기작이 명확히 밝혀지지 않았다. SAC의 핵심 단백질은 Mad1, Mad2. Mad3 (상위 진핵세포에서는 BubR1), Bub1, Bub3, Cdc20를 포함하는데, 이 단백질 모두 SAC의 활성화에 필요하다. SAC의 활성화에 핵심적인 단계는 미세소관과 연결되지 않은 키네토코어에서 Mad2과 Cdc20가 결합하여 복합체를 만드는 것인데, 이 과정은 화학반응에서 쉽게 일어나지 않는 반응이다. Mad2와 Cdc20가 어떻게 키네토코어로 갈 수 있는지에 대해서는 잘 알려져 있었지만, 어떻게 Mad2과 Cdc20가 결합하여 복합체 만들 수 있는지에 대해서는 알려지지 않았다. 최근 다른 실험 방법을 이용한 두 개의 다른 논문들이 어떻게 미세소관과 연결되지 않은 키네토코어에서 Mad2-Cdc20 복합체를 형성하는 지에 대한 핵심적인 기작을 밝혔다. 이 연구들은 단 하나의 연결되지 않은 키네토코어가 SAC 활성화시킬 수 있다는 것에 대한 가설을 뒷받침하고 있다. 본 논문에서는 SAC 활성화에 중요한 주요 기작들을 정리하고, SAC에 과한 최신 연구들을 자세히 살펴본 후, 이 결과들이 세포 분열 연구 분야에 있어서 어떻게 기여했는지 논의할 것이다.