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Invited Mini Review

PP2A function toward mitotic kinases and substrates during the cell cycle

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To maintain cellular homeostasis against the demands of the extracellular environment, a precise regulation of kinases and phosphatases is essential. In cell cycle regulation mechanisms, activation of the cyclin-dependent kinase (CDK1) and cyclin B complex (CDK1:cyclin B) causes a remarkable change in protein phosphorylation. Activation of CDK1:cyclin B is regulated by two auto-amplification loops-CDK1:cyclin B activates Cdc25, its own activating phosphatase, and inhibits Wee1, its own inhibiting kinase. Recent biological evidence has revealed that the inhibition of its counteracting phosphatase activity also occurs, and it is parallel to CDK1:cyclin B activation during mitosis. Phosphatase regulation of mitotic kinases and their substrates is essential to ensure that the progression of the cell cycle is ordered. Outlining how the mutual control of kinases and phosphatases governs the localization and timing of cell division will give us a new understanding about cell cycle regulation. [BMB Reports 2013; 46(6): 289-294]

INTRODUCTION

Animal cells undergo massive structural reorganizations when they enter mitosis, such as cell rounding (1), nuclear envelope breakdown (2), chromosome condensation (3) and the assembly of the mitotic spindle (4). These changes make possible the attachment of cytoplasmic microtubules to the kinetochores and movement of the sister chromatids to the cell's opposite poles. In addition, intracellular organelles, such as the endoplasmic reticulum (5) and the Golgi apparatus (6), are also reorganized to lessen their partial interference with the mitotic spindle and to facilitate their partitioning into the two daughter cells during cytokinesis (7).

These cellular reorganization events are mediated at the interphase-to-mitosis transition point, primarily by members of the Aurora and Polo-like kinase (PLK) families as well as protein complex CDK1:cyclin B, which is also known as a maturation-promoting factor. These key players are mitotic Ser/Thr protein kinases, and they enable cellular reorganization through a pattern of phosphorylation that is both location and time specific, as well as through a broad range of mitosis-specific phosphorylation events on a large number of substrates (8). The CDK1:cyclin B complex triggers mitosis by phosphorylating many downstream mitotic proteins, including other protein kinases such as Aurora and PLK, in all eukaryotic cells (9). We believe that this huge increase in protein phosphorylation at the start of mitosis is responsible for causing all of the structural changes associated with mitosis. Many other studies have identified hundreds of mitotic phosphoproteins, and most of them are likely phosphorylated by CDKs (10-12). However, much is still unclear about how these phosphorylation events are regulated and coordinated to ensure an ordered cell cycle progression.

High CDK1:cyclin B activity is sustained until all of the chromosomes are aligned at the metaphase plate. In these early stages of mitosis, CDK1 prepares for its own inactivation by phosphorylating APC/C (anaphase-promoting complex, also known as the cyclosome). The phosphorylation of APC/C enables it to bind its co-activator CDC20 and form an E3 ubiquitin ligase, which later attaches ubiquitin to many mitotic proteins, including cyclin B, targeting them for degradation by the 26S proteasome (13, 14). However, APC/C^{CDC20} is kept inactive by the spindle assembly checkpoint until all of the chromosomes are attached to microtubules from opposite spindle poles (15). Once microtubule attachment is accomplished, the inhibitory signal from the spindle assembly checkpoint is relieved, committing the cell to exit mitosis. All of the events that occur after 'satisfaction' of the spindle assembly checkpoint are part of the mitotic exit transition point, including chromosome segregation, cytokinesis and the reassembly of interphase cell structures. Each step mentioned above is regulated by the degradation of mitotic factors and the elimination of phosphates from mitotic substrates. APC/C^{CDC20} activity makes the degradation of those mitotic determinants possible. APC/C^{CDC20}-induced proteasomal destruction of cyclin B inactivates mitotic CDK1 (16), and the subsequently low CDK1 activity allows APC/C to bind to a second co-activator, CDC20 homologue 1 (CDH1). The binding of CDH1 broadens APC/C's substrate specificity to substitute CDC20 for Aurora kinases

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and PLK1 (17-21).

In this review, we aim to discuss recent advances in the identification and characterization of the phosphatases with an emphasis on PP2A, which counteracts mitotic kinases including CDK1, Aurora kinases and PLK1. Discussing how the mutual control of kinases and phosphatases regulates cell cycle progression will give us a new understanding about the cell cycle and its timing.

Phosphatases counteracting CDK1:cyclin B

Regulation at the kinase and phosphatase levels during the cell cycle results in a shift of CDK1-dependent substrate phosphorylation. It has been known that phosphatase regulation governs CDK1 activation during mitotic entry in yeast and animal cells (22-24). Enzymes of the PP1 and PP2A families have emerged as CDK1counteracting phosphatases in animal cells through several recent experiments.

CDK1:cyclin B activity is regulated by transcriptional control, phosphorylation and intracellular localization. First, the formation of CDK1:cyclin B complexes begins during interphase, through an increase in cyclin B synthesis. However, before the start of mitosis, the Wee1 and myelin transcription factor 1 (Myt1) kinases obstruct the activity of CDK1:cyclin B through inhibitory phosphorylation at Thr14 and Tyr15 on CDK1. When the cell enters into mitosis, the conserved dual-specificity phosphatase Cdc25 removes these phosphates, facilitated by the inhibition of Wee1 and Myt1. Cdc25, which in mammals has the three potentially redundant isoforms Cdc25A, Cdc25B and Cdc25C, is itself regulated by 14-3-3 proteins and PP2A. During interphase, Cdc25 is kept inactive by associating with 14-3-3 proteins (25, 26) and PP2A, which is bound to the regulatory subunit B56 (27). At mitosis, the activation of CDK2 partially enhances Cdc25 activity, which promotes its dissociation from 14-3-3 proteins. Further Cdc25 activation occurs through PP1's removal of inhibitory phosphates (28, 29). Partially active CDK1 contributes to not only the activation of CDC25 but also the inhibition of Wee1 and Myt1 by direct phosphorylation, thus forming two amplification loops that establish a switch-like and retained activation of CDK1:cyclin B (30, 31).

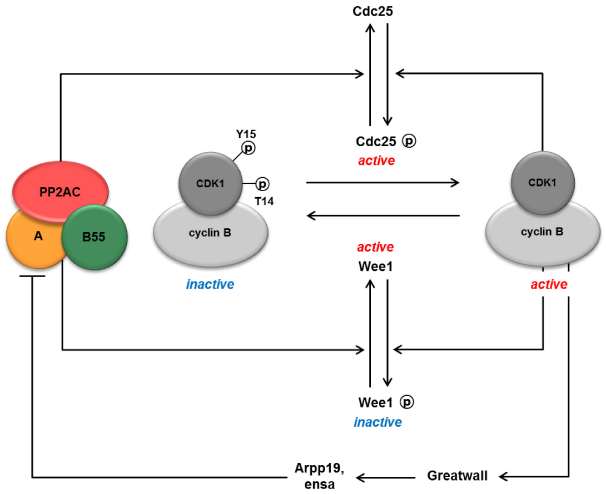

Fig. 1. A model of mitotic CDK1:cyclin B regulation.

Focusing on PP2A activity more deeply, it is active in interphase to reverse the small amount of CDK1-dependent phosphorylation that usually occurs (32). An inhibition of CDK1-counteracting phosphatases, especially PP2A, empowers the phosphorylation of CDK1 target proteins, which can occur even if CDK1 activities are low. Moreover, PP2A inhibition generates CDK1 activation by affecting the feedback loops involving Wee1 and Cdc25 because Wee1 and Cdc25 are also CDK1 targets. Thus, inhibition of PP2A can allow these proteins to stay in their phosphorylated forms, which leads to Cdc25 activation, Wee1 inhibition, and eventually the full activation of CDK1:cyclin B dimers, even at low cyclin B levels (Fig. 1).

Although PP2A has a broad spectrum of target proteins, PP2A can target specific substrates at particular times, depending on the binding of regulatory subunits (33). PP2A enzymatic activity was measured at mitosis using cells synchronized by the double thymidine block and release method and compared with asynchronized cells. PP2A activity was found to be increased greatly at the mitotic phase when compared to the normal phase (unpublished data). Further work identified a PP2A holoenzyme, consisting of a B55δ regulatory subunit, that plays a role as a specific and regulated CDK1-counteracting phosphatase (34). A depletion of B55δ from cycling Xenopus egg extracts advanced mitosis entry and compromised the exit from mitosis, via cyclin B degradation. The mitotic advancement was caused by a premature activation of CDK1 and the subsequent phosphorylation of mitotic substrates. By contrast, the addition of extra, purified PP2A-B55δ complexes delayed and blocked CDK1 activation and slowed mitosis entry in a dose-dependent manner, due to the Wee1-dependent phosphorylation of CDK1 (34). Similar to the addition of OA, the simultaneous effects of promoting CDK1 inhibitory phosphorylation and the dephosphorylation of CDK1 substrates in interphase suggested that PP2A-B55δ acts on both the downstream CDK1 substrates and the CDK1 auto-activation loops (i.e., on Wee1 and Cdc25). Other recent studies have implicated the B55α regulatory subunit of PP2A in the regulation of mitosis, also suggesting that it acts as a CDK-counteracting phosphatase in different experimental systems (35). Therefore, we refer to the CDK1-counteracting phosphatase as PP2A-B55, implying that several B55 isoforms might act as CDK1-counteracting phosphatases in different cells or in different conditions.

A key player regulating PP2A-B55 is Greatwall kinase (which is also known as microtubule-associated Ser/Thr kinase-like (MASTL)) (36). Experiments that used mutant Greatwall or depleted Greatwall through RNAi in *D. melanogaster* cells showed defects in prophase chromosome condensation, nu-

clear envelope breakdown, chromosome congression and spindle morphology. These results raise the possibility that Greatwall promotes mitotic entry (37). Subsequently, this hypothesis was confirmed in *X. laevis* embryonic extracts, which indicated that Greatwall is essential to enter mitosis and to maintain the mitotic state (38). Greatwall phosphorylates two small regulatory proteins, α-endosulfine (ensa) and cyclic AMP-regulated phosphoprotein 19 (arpp19), which then bind to PP2A-B55 and inhibit it (39). Considering this property, we can conclude that Greatwall indirectly regulates PP2A-B55. Inhibition of PP2A-B55 promotes the mitotic state in two ways. First, it increases the net phosphorylation on numerous CDK1 substrates by reducing PP2A-B55's counteracting dephosphorylation (40). Second, Greatwall removes the inhibitory Tyr14 and Thr15 phosphorylation from CDK1 and, consequently, activates CDK1 as part of a regulatory feedback loop (38). In this autoregulatory loop, the Greatwall-induced inhibition of PP2A-B55 by ensa and arpp19 may increase the number of activating phosphorylation events on Cdc25 phosphatase, as well as the number of inhibitory phosphorylation events on Wee1 and Myt1. This model was verified in human and mouse cells, suggesting that the CDK1-Greatwall-PP2A-B55 network is evolutionarily conserved in mammalian cells (36).

The two molecules that inhibit PP2A-B55, ensa and arpp19, do not inhibit any PP2A complexes that contain regulatory subunits from other subfamilies (39), indicating that Greatwall specifically regulates CDK-counteracting PP2A-B55 complexes, rather than generally inactivating PP2A. This allows other PP2A complexes to perform their mitotic functions even in the presence of high Greatwall activity; for example PP2A-B56, which protects centromeric cohesion until anaphase onset, would be unaffected (41).

Other mechanisms regulating PP2A-B55 are emerging, including post-translational modifications and associations with other subunits. One newly discovered mechanism is the phosphorylation of B55α, which was revealed by mass-spectrometric analysis from human tissue culture cells. Phosphorylation of Ser167 on B55α was found to be particularly abundant during mitosis. A phosphomimicking mutant Ser167Glu of B55α binds less efficiently to the PP2A core dimer (the catalytic and scaffold subunits), indicating that phosphorylation of the regulatory B55α subunit may control the formation of a functional heterotrimeric PP2A complex. Interestingly, Ser167 is part of a CDK1 substrate motif (Ser-Pro-X-Arg), which signifies potential feedback between CDK1 and PP2A-B55α. However, this hypothesis and its functional relevance for cell cycle progression have not yet been studied. Additionally, the scaffold subunit of PP2A physically and functionally interacts with the nuclear import factor importin β1 during mitosis, which could also be part of another unidentified PP2A regulatory mechanism (42).

Phosphatases counteracting Aurora B kinase

Aurora B kinase is an enzymatically active subunit of the chromosome passenger complex (CPC), which includes three other non-enzymatic subunits: INCENP, survivin and borealin. These non-enzymatic subunits regulate the activity and specificity of Aurora B's intracellular localization, as well as its functions. Specifically, the functions of Aurora B are controlling the mitotic chromosome structure and mitotic spindle assembly, correcting erroneous kinetochore-microtubule attachments, and regulating cleavage furrow ingression and cytokinetic abscission (15).

Several mechanisms contribute to the regulation of the CPC. Some of them, including phosphorylation on the Tloop of Aurora B and clustering of the CPC on chromatin, are essential for its kinase activity. Dephosphorylation of INCENP manages the translocation of the CPC from the centromeres to the central spindle at anaphase onset (43). Furthermore, after Aurora B ubiquitylation by the E3 ubiquitin ligase cullin 3, the CDC48 (also known as p97) system interacts with the kinesin MKLP2 and triggers the removal of the CPC from anaphase chromosomes. Eventually, the APC/C^{CDH1}-proteasome pathway degrades Aurora B to inactivate it after mitosis (20).

Collaboration between Aurora B and PLK1 appears to keep the attachment/detachment cycle active in prometaphase, and the coupling of Aurora B and PLK1 activities appears logical. This model can be explained as follows below. A gradient in Aurora B activity is concentrated at the centromeres and becomes less effective to phosphorylate its substrates, once a kinetochore is stretched away from its centromere. If kinetochores are attached incorrectly, they detach due to the effect of Aurora B, and the kinetochores require PLK1 activity to be re-attached correctly. Such a cycle becomes possible through phosphatase activity. Recently, the PP2A complex with its B56 regulatory subunit (PP2A-B56) has been shown to antagonize both Aurora B and PLK1 activities during prometaphase. A balance between Aurora B, PLK1 and PP2A-B56 is required for proper chromosome attachment and congression in prometaphase (44).

The phosphatase action counteracting Aurora B kinase occurs differently in anaphase. Recent studies using fluorescence resonance energy transfer (FRET)-based phosphorylation biosensors revealed that the dephosphorylation of Aurora B substrates on anaphase chromosomes proceeds in a remarkable spatiotemporal pattern (45). Chromosome separation away from Aurora B at the central spindle occurs simultaneously with the removal of Aurora B-added phosphates on the chromatin substrates. Dephosphorylation presents as a gradient, in which high phosphorylation is observed on chromatin regions close to the central spindle midzone and lower phosphorylation exists on chromatin towards the cell cortex. There may be a diffusible component or a spatial gradient of phosphatase activity that counteracts the Aurora B kinase activity. PP1, especially PP1γ (three isoforms, α, β/δ and γ are expressed in mammalian cells) translocates dramatically from the kinetochores and cytoplasmic regions to the area of the cleavage furrow and midbody. This indicates that PP1 could be a good candidate

phosphatase to work against Aurora B (46).

Phosphatases reversing PLK1 phosphorylation

PLK1 is another key mitotic kinase, and its localization dynamically changes from the kinetochores, to the centrosomes and finally to the central spindle during cell division. It controls entry into mitosis, centrosome maturation, sister chromatid cohesion, the activation of the APC/C, and cytokinesis (47).

The phosphorylation of PLK1, which was primed by either CDK1 or by PLK1 itself, determines the binding affinity between its substrates and its Polo-box domain. Therefore, the regulation of PLK1's binding affinity to its substrate can be directly controlled by phosphatases that counteract PLK1 at either the substrate site or the primed phosphorylation site. The regulation of centromeric cohesion during prometaphase could occur through the classic case of the opposing activities of PLK1 and PP2A; however, no phosphatases counteracting PLK1 during mitotic exit have yet been identified.

PLK1 phosphorylates the cohesin subunit SA2 during prometaphase and induces the dissociation of cohesin from chromosome arms in mammalian cells (48). The protein shugoshin 1 (SGO1 or SGOL1) recruits PP2A-B56 at centromeric regions to protect SA2 against PLK1-mediated phosphorylation, thus enabling the centromere to maintain cohesion constantly (41). As well as regulating centromere-localized PP2A-B56 to prevent a premature loss of cohesion, PP1 broadly restrains the activity of PLK1 (49). It is important to establish a balance between kinase and phosphatase activities to maintain chromosomal patterns of cohesion throughout the metaphase chromosome axis. In contrast to Aurora B, the dephosphorylation of PLK1 substrates does not occur along a gradient, despite the fact that Aurora B and PLK1 are similar in their localization to the anaphase central spindle (45). This difference could be explained two ways: either by the different characteristics of the two kinases or by the distinct phosphatases that dephosphorylate Aurora B and PLK1 substrates.

CONCLUSION

In summary, the activities of phosphatases and kinases are elaborately regulated throughout the progression of the cell cycle. Among those enzymes, we primarily focused on the phosphatases Cdc25, PP2A and PP1 and the kinases CDK:cyclin B, Aurora B, and PLK. There is now firm evidence that PP2A-B55 plays a crucial role in dephosphorylating CDK1 substrates during the mitotic stages and that PP2A-B56 dephosphorylates PLK1 substrates. In addition to the B55 and B56 regulatory subunits of PP2A, many phosphorylation events caused by various PP2A regulatory subunits during cell cycle progression have been elucidated (50-52). In the near future, the reason for some noted phosphorylations on various PP2A regulatory subunits may be understood in terms of cell cycle regulation. This finding would help to reveal the more comprehensive cell cycle progression woven by kinases and phosphatases. In addition, more phosphatases, including PP1 and PP6, may emerge as cell cycle regulators, and the mechanisms governing their temporally and spatially ordered dephosphorylations may be uncovered.

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