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Article

A Role for Cdc2- and PP2A-Mediated Regulation of Emi2 in the Maintenance of CSF Arrest

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Summary

Background: Vertebrate oocytes are arrested in metaphase II of meiosis prior to fertilization by cytostatic factor (CSF). CSF enforces a cell-cycle arrest by inhibiting the anaphase-promoting complex (APC), an E3 ubiquitin ligase that targets Cyclin B for degradation. Although Cyclin B synthesis is ongoing during CSF arrest, constant Cyclin B levels are maintained. To achieve this, oocytes allow continuous slow Cyclin B degradation, without eliminating the bulk of Cyclin B, which would induce release from CSF arrest. However, the mechanism that controls this continuous degradation is not understood.
Results: We report here the molecular details of a negative feedback loop wherein Cyclin B promotes its own destruction through Cdc2/Cyclin B-mediated phosphorylation and inhibition of the APC inhibitor Emi2. Emi2 bound to the core APC, and this binding was disrupted by Cdc2/Cyclin B, without affecting Emi2 protein stability. Cdc2-mediated phosphorylation of Emi2 was antagonized by PP2A, which could bind to Emi2 and promote Emi2-APC interactions.
Conclusions: Constant Cyclin B levels are maintained during a CSF arrest through the regulation of Emi2 activity. A balance between Cdc2 and PP2A controls Emi2 phosphorylation, which in turn controls the ability of Emi2 to bind to and inhibit the APC. This balance allows proper maintenance of Cyclin B levels and Cdc2 kinase activity during CSF arrest.

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Introduction

Vertebrate oocytes remain arrested for prolonged periods in metaphase II of meiosis through the action of cytostatic factor (CSF), an activity defined on the basis of the ability of injected cytoplasm from unfertilized *Xenopus* eggs to block blastomere cleavage [1]. Fertilization triggers release from this arrest, promoting entry into the early embryonic cell cycles. The nature of CSF has not been fully defined, but Mos, the apical kinase in a MAPK cascade, is critical to the establishment of the CSF arrest [2–6]. Cdk2/Cyclin E has also been implicated as a CSF component [7].

A defining feature of the CSF arrest is maintenance of high Cdc2/Cyclin B activity. CSF inhibits the APC, an E3 ubiquitin ligase that promotes destruction of Cyclin B and other proteins whose degradation is important for M phase exit. Cdc20 or Cdh1 promotes APC activation, depending upon the precise cell-cycle stage [8–10]. CSF-arrest establishment has been reported to rely on factors that enforce APC inhibition by the spindle assembly checkpoint (e.g., Mad2) [11, 12]. However, Mad2 is not necessary for maintaining the CSF arrest, suggesting that CSF might rely upon factors other than spindle-checkpoint components to maintain APC inhibition [13].

Previously, it was reported that a protein known as Emi1 could bind Cdc20 to inhibit the APC [14]. Immunodepletion of Emi1 from CSF-arrested *Xenopus* egg extracts promoted release from CSF arrest. Moreover, recombinant Emi1 promoted a CSF-like arrest in egg extracts. Despite its ability to inhibit the APC, the importance of Emi1 in the CSF arrest was called into question by observations suggesting that Emi1 was present at insufficient levels in CSF-arrested eggs and extracts to be responsible for the arrest [15]. Moreover, Emi1 stabilized both A type and B type cyclins, whereas the endogenous arrest promoted stabilization of only Cyclin B [16]. Recently, an Emi1-related protein, Emi2, has been implicated in the CSF arrest. This APC inhibitor, which also contains a putative Cdc20 binding domain, can promote a CSF-like arrest and is sufficiently abundant in CSF extracts to potentially contribute to the arrest. Moreover, its depletion resulted in spontaneous APC activation and release from the CSF arrest [17, 18]. Importantly, the free-intracellular-calcium increase that accompanies fertilization and triggers release from the CSF arrest leads to Emi2 degradation, thereby alleviating APC inhibition and promoting CSF release [19, 20]. Thus, it seems likely that Emi2, inadvertently depleted by antibodies prepared against Emi1, is the more important mediator of the CSF arrest.

Calcium-induced Emi2 degradation is triggered by the CaMKII-mediated phosphorylation of Emi2 at Thr 195 (of *Xenopus* Emi2) [20, 21], providing a docking site for the Polo-like kinase, Plx1, whose phosphorylation of Emi2 creates a phosphodegron recognized by the β-TrCP E3 ubiquitin ligase. Together, CaMKII and Plx1 promote Emi2 degradation.

Although Cyclin B degradation occurs primarily at exit from CSF arrest (and with each subsequent M phase exit), there is evidence to suggest that Cyclin B degradation occurs at continuous low levels during a CSF arrest to counterbalance ongoing Cyclin B synthesis [22, 23]. Without this, Cyclin B levels would rise unabated throughout the arrest, resulting in a gradual, rather than a precipitous, exit from the CSF arrest upon fertilization (due to the presence of a large bolus of Cyclin B to degrade). This ongoing Cyclin B degradation appears to be triggered by Cdc2/Cyclin B kinase activity itself, such that Cyclin B synthesis leads to higher Cdc2 kinase activity and results in compensatory Cyclin B degradation [24]. Although this regulatory loop has been well-documented, the molecular mechanisms linking Cdc2/Cyclin B kinase activity and APC activation during a CSF arrest have not been reported. We show here that Emi2 is phosphorylated and inhibited by Cdc2/Cyclin B at sites distinct from the CaMKII/Plx1 sites, though Cdc2 phosphorylation does not promote Emi2 degradation. Rather, Emi2 can bind and inhibit the full APC (rather than Cdc20 alone) and Emi2-APC interactions are inhibited by Cdc2 phosphorylation of Emi2. Thus, Cdc2-mediated Emi2 phosphorylation relieves APC inhibition, allowing Cyclin B degradation. In addition, Cdc2′s effects on Emi2 are antagonized by PP2A-mediated Emi2 dephosphorylation, which facilitates the Emi2-APC interaction. These findings establish an auto-regulatory loop comprised of Cdc2/Cyclin B, PP2A, Emi2, and the APC that controls Cyclin B levels to properly maintain the CSF arrest and allow for rapid M phase exit upon fertilization.

Results

Cdc2/Cyclin B Targets Emi2 to Regulate Cyclin B

As reported by Kishimoto and colleagues [24], Cyclin B synthesis continues during a cytostatic factor (CSF) arrest, a finding we have repeated with similar results (Figure 1A, top). Continued protein synthesis would be expected to result in a steady increase in Cyclin B levels during a CSF arrest, but this steady increase is not observed. Rather, we observe constant levels of Cyclin B throughout CSF arrest (Figure 1A, bottom). Thus, for maintaining constant Cdc2/Cyclin B activity during the arrest, a continual low level of Cyclin B degradation is required, as demonstrated by the degradation of radiolabeled Cyclin B in a CSF-arrested extract (Figure 1B, left). This degradation is prevented by the proteasome inhibitor MG132 (Figure 1B, right). Additionally, immunodepletion of the APC using Cdc27 antibodies resulted in a gradual increase in Cyclin B, accompanied by increased Cyclin B/Cdc2 kinase activity (Figures 1C and 1D). We note that Histone H1 kinase activity eventually plateaued, even in the absence of the APC. This raises the interesting possibility that Cdc2/Cyclin B1 kinase activity not only might promote Cyclin B degradation, but might also limit its translation. Confirming previously reported results, we also found that addition of excess Cyclin B to CSF extracts to activate latent Cdc2 enhanced APC-mediated Cyclin B degradation, consistent with a role for Cdc2/Cyclin B activity in triggering Cyclin B destruction (Figure 1E). Moreover, Cdc2/Cyclin B kinase activity increased only transiently upon addition of recombinant Cyclin B, consistent with the induction of compensatory degradation (Figure 1F). As shown in Figure 1G, Cyclin B addition also induced the degradation of another APC substrate (Securin) in a dose-dependent manner.

With the discovery that Emi2 protein plays a key role in maintaining CSF arrest, we hypothesized that Cdc2/Cyclin B might control its own degradation by modulating Emi2 function. CaMKII-mediated phosphorylation of Emi2 at fertilization promotes Emi2 degradation, relieving APC inhibition. However, Cdc2/Cyclin B-induced Cyclin B degradation was not inhibited by CaMKII inhibitors at concentrations that prevented Ca^{2+}-induced Cyclin B degradation (Figure 1H, [25]). Moreover, addition of 40 nM Cyclin B to extracts did not promote Emi2 destruction (Figure 1I); similar results were obtained when 80 nM Cyclin B was added, although some Emi2 degradation could be observed at later time points, consistent with previous reports that very high Cdc2 kinase activity induces Emi2 degradation [21]. Therefore, we have restricted our use of recombinant Cyclin B1 to levels that do not induce Emi2 degradation. Interestingly, adding excess Emi2 to CSF extracts blocked acceleration of Cyclin B degradation by exogenous Cyclin B protein (Figure 1J). In that excess Emi2 could circumvent the ability of Cdc2/Cyclin B to increase Cyclin B degradation, these data suggested that Cdc2/Cyclin B might control Cyclin B levels by antagonizing Emi2.

Cdc2/Cyclin B Disrupts the Association of Emi2 and APC/Cdc20

Consistent with the hypothesis that Cdc2/Cyclin B could modulate Emi2, we found that Cdc2 could phosphorylate Emi2 in vitro (Figure 2A). To determine whether there were consequences of this phosphorylation, we analyzed Emi2-APC interactions. The Emi2 relative, Emi1, interacts with the APC activator Cdc20 to disrupt APC-Cdc20 interactions [26]. In examining Emi2-APC interactions, we found that Emi2 could coprecipitate with the APC-core component Cdc27 (Figure 2B). To see whether Cdc20 was required for this interaction, we generated a Cdc20-depleted extract and found that Emi2 could still coprecipitate with Cdc27, even in an egg extract immunodepleted of ~80% of Cdc20 (Figure 2C). Consistent with this, Emi2 was detected in Cdc27 immunoprecipitates, but was absent from Cdc20 immunoprecipitates (Figure 2D). We next separated CSF extracts on 5%–30% sucrose gradients and identified APC-containing fractions by immunoblotting for Cdc27 and APC2 (fractions 14–18; Figure 2E and data not shown). That the Cdc27 coprecipitation reflected a true interaction of Emi2 with the APC is suggested by the fact that a portion of Emi2 cosedimented with the intact APC (Emi2 antibody recognizes three bands, as discussed in Figure 2H). Although high levels of GST-Emi2 associated with and precipitated small amounts of Cdc20 from extracts even after APC immunodepletion (data not shown), we could not detect independent interaction between Emi2 and Cdc20 when Cdc20 was immunoprecipitated from gradient fractions (6 and 7) containing both Emi2 and Cdc20, but lacking other APC components (Figure 2F, left). In contrast, Emi2 was detected in anti-Cdc20 immunoprecipitates formed from gradient fractions containing the full APC (fractions 15 and 16), although there was considerably less Emi2 present