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APC/C–Cdc20-mediated degradation of cyclin B participates in CSF arrest in unfertilized *Xenopus* eggs

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Abstract

In vertebrates, unfertilized eggs are arrested at meiotic metaphase II (meta-II) by cytostatic factor (CSF), with Cdc2 activity maintained at a constant, high level. CSF is thought to suppress cyclin B degradation through the inhibition of the anaphase-promoting complex/cyclosome (APC/C)-Cdc20 while cyclin B synthesis continues in unfertilized eggs. Thus, it is a mystery how Cdc2 activity is kept constant during CSF arrest. Here, we show that the APC/C–Cdc20 can mediate cyclin B degradation in CSF-arrested *Xenopus* eggs and extracts, in such a way that when Cdc2 activity is elevated beyond a critical level, APC/C–Cdc20-dependent cyclin B degradation is activated and Cdc2 activity consequently declines to the critical level. This feedback control of Cdc2 activity is shown to be required for keeping Cdc2 activity constant during meta-II arrest. We have also shown that Mos/MAPK pathway is essential for preventing the cyclin B degradation from inactivating Cdc2 below the critical level required to sustain meta-II arrest. Our results indicate that under CSF arrest, Mos/MAPK activity suppresses cyclin B degradation, preventing Cdc2 activity from falling below normal meta-II levels, whereas activation of APC/C–Cdc20-mediated cyclin B degradation at elevated levels of Cdc2 activity prevents Cdc2 activity from reaching excessively high levels.

Keywords: Cytostatic factor; Metaphase II arrest; *Xenopus* egg; Cyclin B degradation; The anaphase-promoting complex/cyclosome; Cdc20; Mos; MAP kinase

Introduction

In vertebrates, mature eggs wait for fertilization with the meiotic cycle being arrested at metaphase of meiosis II (meta-II). The arrest at meta-II, which can last for a few days, is caused by the action of cytostatic factor (CSF), which was first identified in frog eggs (Masui and Markert, 1971). CSF arrest is established in meiosis II (MII) by the Mos/MAPK cascade (Kishimoto, 2003; Masui, 2000; Sagata, 1996) and is terminated through the activation of calcium/calmodulin kinase II (CaMKII) induced by a transient increase in Ca^{2+} concentration in the egg cytoplasm upon fertilization (Lorca et al., 1993; Morin et al., 1994). In CSF-arrested eggs, metaphase is maintained through keeping the cyclin B-Cdc2 kinase activity at a high level, which is achieved by maintaining the levels of cyclin B. In contrast, when eggs are released from CSF arrest by fertilization, cyclin B is rapidly degraded to undetectable levels, resulting in Cdc2 inactivation and hence exit from MII. It has been postulated that cyclin B degradation is suppressed during CSF arrest in unfertilized vertebrate eggs to sustain a prolonged metaphase prior to fertilization (Abrieu et al., 2001; Tunquist and Maller, 2003).

Cyclin B degradation, which is catalyzed by the 26S proteasome, is regulated through polyubiquitination by the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, and its activating factor Cdc20 (for review, see Harper et al., 2002; Peters, 2002). The APC/C is inactive in interphase and activated in M-phase through phosphorylation (for review, see Peters, 2002). The APC/C–Cdc20 is known to be suppressed in the metaphase arrest caused by the spindle assembly checkpoint (for review, see Yu, 2002), and the checkpoint proteins Bub1, Mad1 and Mad2 were shown to be involved in CSF arrest (Tunquist et al., 2002,

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2003). Thus, it has been widely accepted that in CSF-arrested eggs, cyclin B degradation is suppressed through prevention of APC/C–Cdc20 (for review, see Abrieu et al., 2001; Tunquist and Maller, 2003). On the other hand, there is evidence that considerable amounts of cyclin B are continuously synthesized in unfertilized eggs of the mouse and *Xenopus* (Kubiak et al., 1993; Thibier et al., 1997). Furthermore, cyclin B synthesis has been reported to increase in the presence of Mos/MAPK activity during the period from meiosis I (MI) to MII (Gross et al., 2000). Thus, the suppression of cyclin B degradation and continuous cyclin B synthesis in unfertilized eggs would be expected to lead to ever increasing Cdc2 activity, rather than the constant level seen during the long-lasting CSF arrest.

To address this issue, we have investigated the maintenance of Cdc2 activity at a constant level during meta-II arrest, using CSF-arrested *Xenopus* eggs and their extracts (CSF extracts) (Lohka and Maller, 1985; Murray et al., 1989). Our results show that, contrary to the widely-accepted model, the APC/C–Cdc20 can mediate cyclin B degradation in CSF-arrested eggs and extracts, in such a way that when Cdc2 activity is elevated beyond a critical level, APC/C–Cdc20-dependent cyclin B degradation is activated and Cdc2 activity consequently declines to the critical level. This feedback control of Cdc2 activity is shown to be required for keeping Cdc2 activity constant during meta-II arrest. We have also shown that Mos/MAPK pathway is critical in preventing the cyclin B degradation from inactivating Cdc2 below the critical level required to sustain an MII metaphase arrest.

Materials and methods

Recombinant proteins

Full-length *Xenopus* cyclin B1 cDNA (gift from T. Hunt) was cloned into the pTrc-His2A plasmid vector (Invitrogen) and transformed into *Escherichia coli* strain BL21. Myc/His6-tagged recombinant protein was expressed at 22°C for 14 h with 1 mM IPTG and purified using His-Bind Resin (Novagen). GST-ΔN85 cyclin B2 (ΔN cyclin B2; Iwabuchi et al., 2000) was expressed at 25°C for 4 h with 1mM IPTG in *E. coli* strain BL21 and purified according to the manufacturer's protocol (Amersham). The kinase inactive complex of Cdc2 and GST-ΔN cyclin B2 was produced as described previously (Iwabuchi et al., 2000). The construct for GST-Ste11ΔN (Gotoh et al., 1994) was kindly provided from E. Nishida. Protein was expressed in *E. coli* strain XLIBlue and purified according to the manufacturer's protocol (Amersham).

All the purified proteins were dialyzed against extraction buffer (EB: 100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES-KOH, pH 7.5) supplemented with 1 mM DTT, concentrated with microcon (Millipore) or vivaspin (Vivascience), frozen in liquid nitrogen and stored at −80°C.

Oocytes, eggs and extracts

Fully-grown oocytes and mature eggs of *Xenopus* were obtained as described (Ohsumi et al., 1994). CSF extracts of unfertilized eggs were prepared by the method of Murray (Murray, 1991) with modifications. Briefly, unfertilized eggs of *Xenopus* were dejellied with 2.5% thioglycolic acid–NaOH (pH 8.2), washed 5 times with EGTA-extraction buffer (EEB: 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, 20 mM HEPES-KOH, pH 7.4) and packed into a 1.5- or 2-ml plastic tube filled with EEB containing 50 μg/ml cytochalasin B. After a brief centrifugation (750 g, 10 s, 2°C), excess buffer was removed, and eggs were lysed by centrifugation at 15,000 × *g* for 10 min at 4°C. The cytoplasmic fraction between lipid cap and sedimented yolk was removed and centrifuged again at 15,000 × *g* for 20 min at 4°C to remove contaminating lipid. Interphase egg extracts were prepared by adding 0.4 mM $CaCl_2$ to CSF extracts at 22°C. Fully-grown oocytes were treated with 10 μg/ml progesterone to induce the resumption of meiosis, and extracts were prepared from oocytes at metaphase of meiosis I as described (Iwabuchi et al., 2000). Egg and oocyte extracts were kept on ice and used within 2 h of preparation. All incubations of extracts were done at 22°C.

In some experiments, oocytes matured in vitro were injected with 14 nl of 10 mM EGTA either with or without 6 μM of undegradable Emi1 (Ohsumi et al., 2004), and then injected with 9 nl of 3.3 μM His-cyclin B1.

Antibodies, immunodepletion and immunoblot analysis

Full-length *Xenopus* Cdc20 cDNA (Lorca et al., 1998) was isolated by PCR and cloned into pGEX4T-3 plasmid vector (Amersham). GST-Cdc20 fusion protein expressed in *E. coli* strain BL21 and contained in inclusion bodies was suspended into 6 M urea and diluted with the equal volume of PBS, by which most GST-Cdc20 protein was precipitated. Precipitated protein was then dissolved into SDS sample buffer (SSB: 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromophenol blue, 60 mM Tris–HCl, pH6.8). After separation by SDS–PAGE, GST-Cdc20 protein was electroeluted from the acrylamide gel, dialysed against PBS and used to immunize rabbits. Full-length *Xenopus* Mos cDNA (gift from N. Sagata) was cloned into pGEX plasmid vector (gift from K. Tachibana). GST-Mos fusion protein was expressed in *E. coli* strain BL21, purified according to manufacturer's protocol (Amersham) and used to immunize rabbits. Antisera raised against Cdc20 were pretreated with GST-beads, and Cdc20 antibodies were affinity-purified from the pretreated sera with nitrocellulose membranes onto which GST-Cdc20 protein had been transferred. Mos antibodies were affinity-purified from antisera with GST-Mos covalently conjugated to CNBr-coupling gel (Amersham). Purified antibodies were dialyzed against EB, concentrated with vivaspin (Vivascience) and stored at −80°C.

For immunodepletion of Mos and the APC/C from CSF extracts, purified antibodies for *Xenopus* Mos and human Cdc27 (mAB AF3; Yamano et al., 98) were conjugated to Protein G–Sepharose beads (sigma), and CSF extracts were treated twice with 10% extract volume of each antibody-beads. For mock-depletion, Protein G-beads conjugated with rabbit IgG were used. For immunoblot analysis, extracts were mixed with SSB, boiled for 2 min, run on SDS–polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 10% skimmed milk, membranes were incubated with primary antibodies for 2 h at room temperature or for 12 h at 4°C. Antibodies used were anti-*Xenopus* Mos, anti-*Xenopus* cyclin B1 and cyclin B2 (gift from J. L. Maller), anti-Cdc27 (MBL; TL-C40920) and anti-MAPK (Upstate; 06-182). After incubation with alkaline phosphatase-conjugated secondary antibodies, membranes were processed for visualizing signals by the BCIP/NBL phosphatase substrate system (KPL).

Protein synthesis measurement

For labeling proteins synthesized in egg extracts, 8.6 μCi of ^{35}S-methionine and cysteine (Pro mix L-[^{35}S] in vitro cell labeling mix: Amersham) was added to 10 μl of extracts either with or without 100 μg/ml CHX and incubated at 22°C. After a 30-min incubation, 3 μl of extracts were mixed with 17 μl of SSB. For precipitation with Protein G-beads conjugated with *Xenopus* cyclin B1 and B2 antibodies, 7 μl of extracts were diluted with 35 μl of cold kinase buffer (80 mM β-glycerophosphate, 20 mM EGTA, 5 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.5) and treated with 10 μl of either bead as described previously (Ohsumi et al., 1994) and beads-bound proteins were eluted with SSB. After boiling for 3 min, samples were subjected to SDS–PAGE, and the gel was stained with Coomassie Blue (CBB) and dried onto filter paper. ^{35}S incorporation into proteins separated by SDS–PAGE was analyzed with Bio-imaging analyzer (Fuji Photo Film).

Histone H1 kinase activity assay

For histone H1 kinase assay, extracts were quickly frozen in liquid nitrogen and stored at −80°C. Frozen extracts were thawed by adding 9 volumes of ice-cold kinase buffer. As for oocytes, five oocytes were crashed in 20 μl of kinase buffer and after a centrifugation (10,000 × *g*, 1 min, 2°C), 10 μl of cytoplasmic fraction was collected. Ten microliters of thawed extracts or oocyte cytoplasmic extracts was mixed with 20 μl of reaction buffer (80 mM β-glycerophosphate, pH 7.4, 20 mM $MgCl_2$, 0.6 mM ATP, 30 μg/ml leupeptin, 30 μg/ml aprotinin) containing 0.6 ng/ml histone H1 and 1 μCi [γ-^{32}P] ATP, and incubated for 30 min at 25°C. Reactions were stopped by the addition of SSB and boiling for 2 min. Histone H1 was separated by SDS–PAGE and stained with CBB and the band was excised. ^{32}P incorporation into the gel slice was quantified by the Cerenkov method.

Results

Cyclin B synthesis continues during meta-II arrest in CSF extracts

Unfertilized *Xenopus* eggs arrested at meta-II are known to continue the synthesis of cyclin B proteins (Thibier et al., 1997). We first examined whether cyclin B is also synthesized in CSF extracts. When CSF extracts were incubated in the presence of ^{35}S-methionine and ^{35}S-cysteine, considerable amounts of the label were incorporated into proteins (Fig. 1A). Quantitative analysis after a 30-min labeling revealed that protein synthesis in CSF

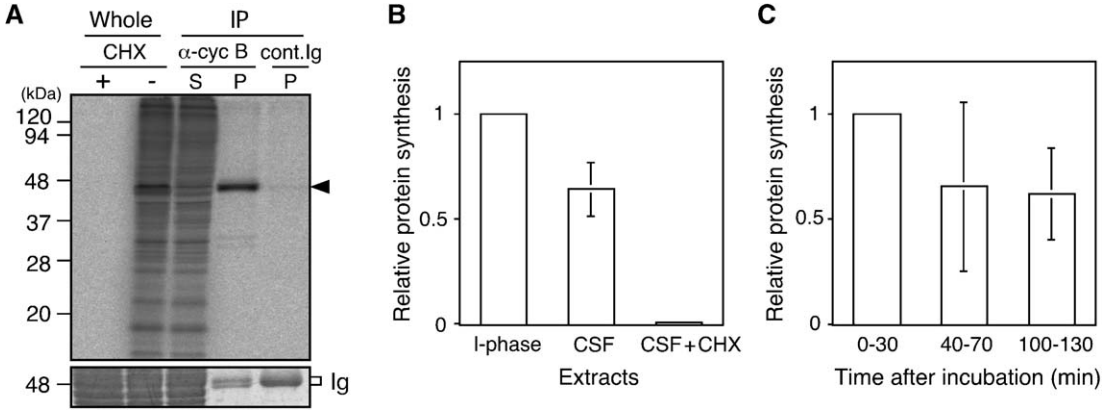

Fig. 1. Cyclin B synthesis continues in CSF extracts. (A) CSF extracts with or without cycloheximide were incubated in the presence of ^{35}S-labeled methionine and cysteine for 30 min at 22°C (Whole), and then treated with *Xenopus* cyclin B antibody-beads (IP). Whole extracts after the incubation (S) and proteins bound by the antibody beads (P) were separated SDS–PAGE and autoradiographed (upper) or stained with CBB to support that equal levels of proteins were applied (lower). Arrowhead indicates the position of cyclin B. (B, C) The rate of bulk protein synthesis in CSF extracts was measured at various times after incubation by 30-min pulse labeling with ^{35}S-methionine and cysteine. Error bars represent the standard deviation of three measurements. (B) The protein synthesis rate in CSF extracts is compared with that in activated extracts (I-phase). The synthesis rate in I-phase extracts is taken as 1. (C) The protein synthesis rate in CSF extracts was measured various times after incubation. The synthesis rate at the beginning of incubation is taken as 1.

extracts was 64% of that measured in activated, interphase (I-phase) extracts, which mimic fertilized eggs undergoing active protein synthesis (Fig. 1B). CSF extracts retained more than 60% of the initial protein synthesis levels when examined 2 h later (Fig. 1C). As seen in Fig. 1A, the major proteins synthesized in CSF extracts were those at 45 kDa (arrowhead), which were B-type cyclins that could be precipitated with *Xenopus* cyclin B antibodies. The amount of cyclin B synthesis in CSF extracts was as much as 52% of that in activated extracts (unpublished data), consistent with the difference in amount of radio-label incorporated into total proteins in the two types of extracts. This result was surprising since in activated extracts, newly synthesized cyclin B accumulates to an amount sufficient to generate a metaphase level of Cdc2 activity within a 2-h incubation. Therefore, cyclin B synthesized in CSF extracts would be expected to increase significantly during a 2-h incubation, resulting in an accompanying increase in Cdc2 activity. However, we observed that both the cyclin B amount (see Fig. 3G) and the Cdc2 activity level were unchanged in CSF extracts during the 2-h incubation period (see Figs. 3E, F). These observations suggested that a considerable amount of cyclin B degradation, equal to the amount of new synthesis, must take place during meta-II arrest in CSF extracts.

Increase in Cdc2 activity induces rapid cyclin B degradation in CSF extracts

If cyclin B degradation is balanced with cyclin B synthesis in CSF extracts to maintain Cdc2 activity at a constant level, it must be precisely regulated. To obtain a clue to the regulation of cyclin B degradation during meta-II arrest, we added various amounts of histidine-tagged full-length *Xenopus* cyclin B1 (His-cyclin B1) to CSF extracts and examined the changes in Cdc2 activity in the extracts. As shown in Fig. 2A, when His-cyclin B1 was first added to CSF extracts, the rapid increases in Cdc2 activity were proportional to the amount of His-cyclin B1 added. Subsequently, the Cdc2 activity dropped to the initial levels within 50 min of His-cyclin B1-addition, regardless of the amount of His-cyclin B1 added and the initial level of Cdc2 activity was maintained thereafter. Immunoblot analysis of cyclin B revealed that the rapid decline of Cdc2 activity in the extracts was caused by cyclin B degradation (Fig. 2B). However, the exogenously added cyclin B was chiefly degraded while endogenous proteins were not so much: cyclin B synthesis continued in CSF extracts added with His-cyclin B1 but at rather lower rates (data not shown). This result might indicate that the exogenous cyclin B was more sensitive to degradation than endogenous cyclin B. To test this possibility, we added ΔN-cyclin B2 to CSF extracts, which can generate Cdc2 activity but is indestructible because of the lack of the destruction box (Holloway et al., 1993), and examined the changes in cyclin B in the extracts. Consistent with a previous study (Minshull et al., 1994), the result showed that in CSF extracts to which ΔN-cyclin B2 was added, the degradation of endogenous cyclin B was proportional to the amount of ΔN-cyclin B2 added (Fig. 2C). A similar result was obtained with ΔN-cyclin B1 (data not shown). Thus, endogenous cyclin B in CSF extracts is capable of being degraded, but when exogenous cyclin B is added, the exogenous cyclin B is more likely to be degraded than endogenous one. To know whether cyclin B degradation is

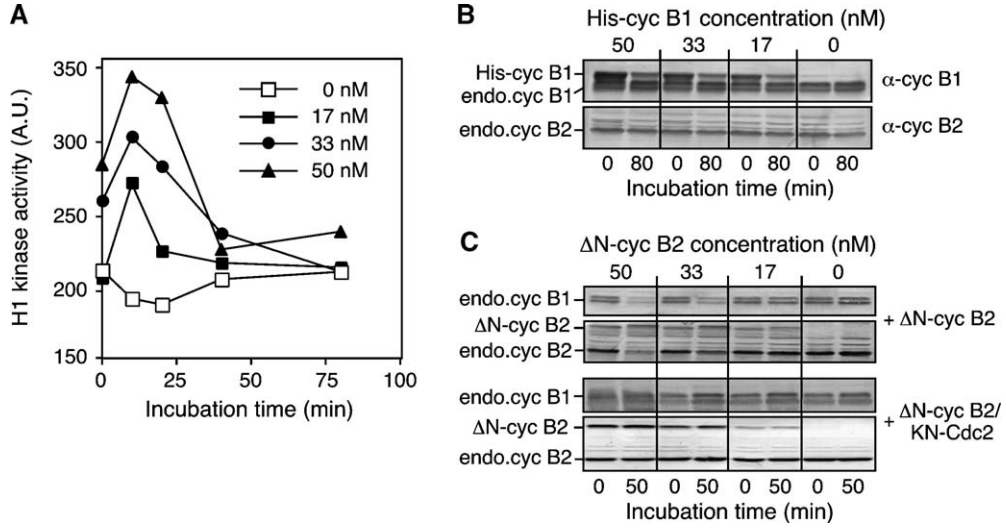

Fig. 2. Cyclin B degradation is induced by elevated Cdc2 activity in CSF extracts. (A) Cdc2 activity in CSF extracts together with various amounts of full-length cyclin B1 protein is expressed as a function of time. Cyclin B1 was added to CSF extracts at the beginning of incubation. (B) The amounts of endogenous cyclin B proteins and exogenously added cyclin B1 were examined by immunoblotting with cyclin B1 and B2 antibodies. (C) The endogenous cyclin B2 amount in CSF-arrested extracts incubated with various amounts of either indestructible ΔN-cyclin B2 or ΔN-cyclin B2 in a complex with kinase negative (KN)-Cdc2 was examined by immunoblotting.

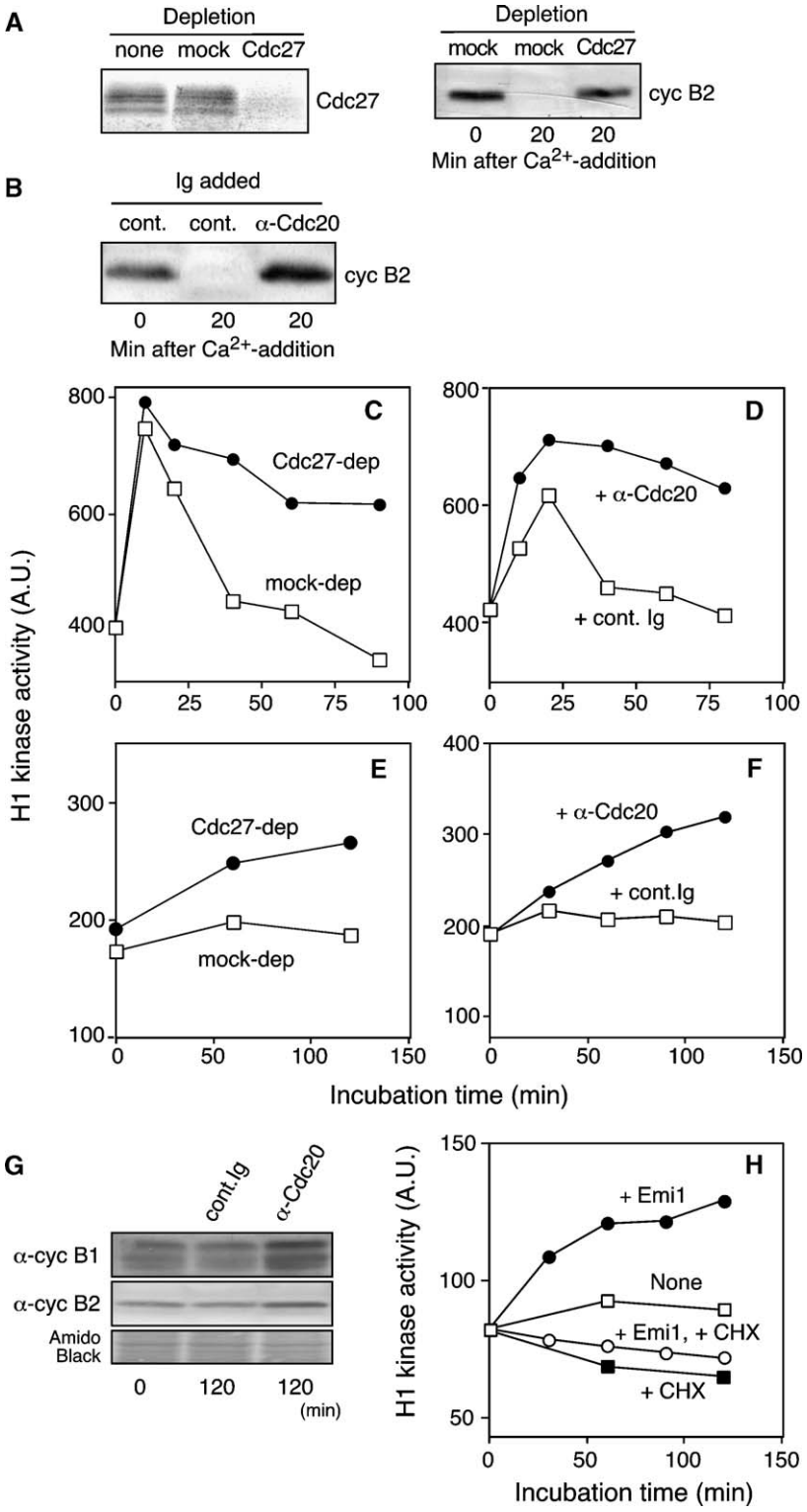

Fig. 3. APC/C–Cdc20 activity is required for cyclin B degradation induced by elevated Cdc2 activity in CSF extracts. CSF extracts were either immunodepleted of the APC/C with a Cdc27 antibody or mock-depleted (A, left, C, E) or incubated with either a Cdc20-inhibiting antibody or a nonspecific IgG (B, D, F). In some experiments, CSF extracts were added with an undegradable mutant of Emi1 to inhibited Cdc20 and treated with CHX to inhibit protein synthesis (H). Samples were assayed for cyclin B degradation after Ca^{2+}-addition by immunoblotting with a cyclin B2 antibody (A, right, B) and for Cdc2 activity after the addition of cyclin B1 (50 nM) (C, D) or without cyclin B addition (E, F, H). CSF extracts at 120 min after incubation with either the Cdc20-inibiting antibody or the control IgG were immunoblotted with *Xenopus* cyclin B1 and B2 antibodies (G). A part of the same transferred membrane was stained with Amido Black to support that equal levels of proteins were applied (G, bottom). Similar results were obtained in two or three independent experiments (C–F, H).

due to the increase in the Cdc2 activity or in the cyclin B amount, we added ΔN-cyclin B2 in complex with a kinase negative mutant of Cdc2 (KN-Cdc2), which does not generate Cdc2 activity when added to CSF extracts. In CSF extracts to which the complex of ΔN-cyclin B2 and KN-Cdc2 was added, the degradation of endogenous cyclin B was not induced (Fig. 2C). These results indicate that in CSF extracts, the elevation of Cdc2 activity causes cyclin B degradation that continues until Cdc2 activity returns to the meta-II level.

Cyclin B degradation under CSF arrest depends on APC/C–Cdc20

We next examined whether APC/C–Cdc20 is involved in the cyclin B degradation induced by elevated Cdc2 activity in CSF extracts. To do this, we ablated APC/C–Cdc20 activity from CSF extracts by either immunodepletion of the APC/C with a Cdc27 antibody or suppression of Cdc20 with a Cdc20-inhibiting antibody. As shown in Figs. 3A and B, either method effectively impaired cyclin B degradation induced by Ca^{2+}-addition to CSF extracts, demonstrating that APC/C–Cdc20 activity was successfully ablated. When His-cyclin B1 was added to CSF extracts deprived of APC/C–Cdc20 activity, Cdc2 activity was quickly elevated but with further incubation did not decline to the starting level, as was seen in control extracts with normal APC/C–Cdc20 activity (Figs. 3C, D). Thus, in CSF extracts, APC/C–Cdc20 activity is required for the cyclin B degradation that antagonizes the elevated Cdc2 activity caused by exogenous cyclin B addition. To confirm that APC/C–Cdc20-mediated cyclin B degradation is also ongoing during meta-II arrest in CSF extracts without exogenous cyclin B, we examined Cdc2 activity in CSF extracts deprived of APC/C–Cdc20 activity. The results showed that Cdc2 activity was gradually elevated with the cyclin B amount being increased in such extracts (Figs. 3E–G), indicating that during meta-II arrest, APC/C–Cdc20-mediated degradation of cyclin B is required to keep Cdc2 activity constant at the meta-II level in the presence of continuous cyclin B synthesis.

It should be noted that when protein synthesis in CSF extracts was inhibited by the addition of cycloheximide (CHX), Cdc2 activity was gradually declined (approximately 20–30% of the meta-II level of activity in a 2-h incubation; Fig. 3H). The decrease in Cdc2 activity induced by CHX-addition was mostly independent of APC/C–Cdc20 since, in CSF-extracts whose Cdc20 was inhibited by the addition of an undegradable mutant of Emi1 (Ohsumi et al., 2004), a similar extent of decrease in Cdc2 activity was induced by CHX-addition (Fig. 3H). This result suggests that a small portion of cyclin B is continuously degraded during meta-II arrest, independently of APC/C–Cdc20 activity. The APC/C–Cdc20-independent cyclin B degradation is insufficient to keep Cdc2 activity from increasing, since, in the absence of APC/C–Cdc20 activity, Cdc2 activity is increased to 160% of the meta-II level of activity in a 2-h incubation (Figs. 3E, F, H).

Regulation of cyclin B degradation in CSF-arrested eggs

We also examined whether or not the regulation of Cdc2 activity levels by APC/C–Cdc20-mediated cyclin B degradation operates in intact CSF-arrested eggs. When mature eggs were injected with His-cyclin B1 along with EGTA, which inhibited egg activation induced by injection, the Cdc2 activity level was transiently elevated but settled at the initial, meta-II level afterward (Fig. 4A). The return of

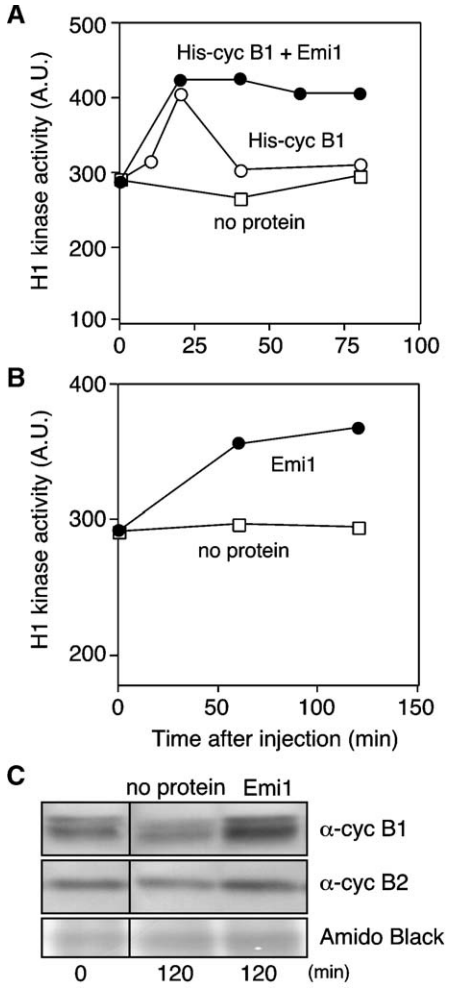

Fig. 4. The regulation of Cdc2 activity through APC/C–Cdc20-mediated cyclin B degradation operates in CSF-arrested eggs. (A, B) Cdc2 activity in CSF-arrested eggs injected with cyclin B1 and/or Emi1 is expressed as a function of time. CSF-arrested eggs were first injected with EGTA to inhibit activation either with or without undegradable Emi1 to inhibit Cdc20 (A, B), and then given a second injection of cyclin B1 (A). Injected eggs were examined for Cdc2 activity at various times after cyclin B1 injection (A) or Emi1 injection (B). (C) CSF-arrested eggs at 120 min after injection of either Emi1 or buffer (no protein) were examined for the cyclin B amount by immunoblotting. A part of the same transferred membrane was stained with Amido Black to support that equal levels of proteins were applied (C, bottom). Similar results were obtained in three independent experiments (A, B).

elevated Cdc2 activity to the meta-II level was dependent on APC/C–Cdc20, as it was prevented by inhibition of Cdc20 with the undegradable Emi1 (Fig. 4A). Immunoblot analysis revealed that cyclin B degradation was responsible for the decline of elevated Cdc2 activity (unpublished data). Furthermore, Cdc2 activity gradually increased accompanying the increase in the cyclin B amounts in mature eggs whose Cdc20 was inhibited with the Emi1 mutant (Figs. 4B, C). Thus, the feedback control of Cdc2 activity levels through the activation of APC/C–Cdc20-mediated cyclin B degradation operates during meta-II arrest in CSF-arrested eggs.

Mos/MAPK activity is required to maintain Cdc2 activity at the meta-II level

It was noteworthy that following the peak of Cdc2 activity seen after the addition of His-cyclin B1 to CSF extracts, the Cdc2 activity level never declined below the initial, meta-II level (Fig. 2A). This result suggested that under CSF arrest there is a threshold of Cdc2 activity below which cyclin B degradation is turned off, thereby maintaining the meta-II arrest. To investigate whether Mos is involved in the turning off of cyclin B degradation under CSF arrest, we ablated Mos activity from CSF extracts using Mos antibodies prior to the addition of His-cyclin B1. To do this, CSF extracts were first immunodepleted of Mos (Fig. 5A) and then a neutralizing antibody against Mos was added. Under our experimental conditions, both treatments were necessary for the complete ablation of Mos activity from CSF extracts; the ablation of Mos activity was confirmed by the complete inactivation of MAPK in the extracts (Fig. 5B). When His-cyclin B1 was added to Mos-ablated extracts, both endogenous and exogenous cyclin B proteins were degraded to undetectable amounts, accompanying Cdc2 inactivation to an I-phase level (Fig. 5C). In mock-treated extracts, cyclin B persisted, with the meta-II level of Cdc2 activity maintained (Figs. 5B, C). Thus, the Mos/MAPK pathway is involved in the turning off of cyclin B degradation once the meta-II level of Cdc2 activity is restored.

We found that in the Mos-ablated extracts, nuclear formation occurred after Cdc2 inactivation (data not shown). The result presented in Fig. 5C, therefore, suggested that the Mos-dependent suppression of cyclin B degradation is required to maintain meta-II arrest. Supporting this notion, when Mos was ablated from CSF extracts, endogenous cyclin B was degraded to an undetectable amount, without a requirement for exogenous His-cyclin B. Correspondingly, Cdc2 activity decreased to an I-phase level (Figs. 5D, E) and nuclear formation occurred in the extracts with inactivated Cdc2 (data not shown). The meta-II level of cyclin B and Cdc2 activity was restored by the addition of Ste11ΔN, a constitutive active form of a yeast MAPKK kinase (Gotoh et al., 1994). When MAPK activity was partially restored by the addition of smaller amounts of

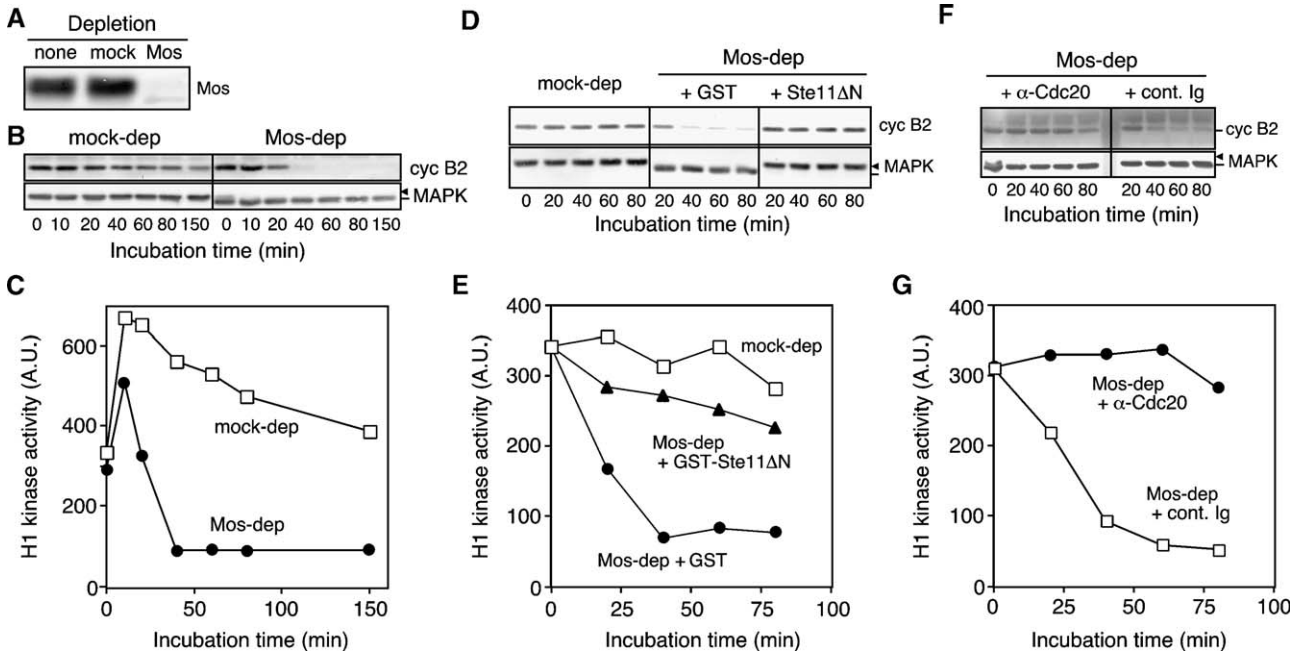

Fig. 5. Mos/MAPK activity is required for the turn off of cyclin B degradation at the metaphase level of Cdc2 activity in CSF extracts. CSF extracts were immunodepleted or mock-depleted of Mos (A, upper), mixed with either a Mos-inhibiting antibody (Mos-dep) or nonspecific IgG (mock-dep) and examined for the changes in cyclin B and MAPK (B, D, F) by immunoblotting and for the changes in Cdc2 activity (C, E, G). Samples were taken after the addition of cyclin B1 (50 nM) (B, C), either GST-Ste11ΔN fusion protein or GST alone (D, E), and either a Cdc20-inhibiting antibody or nonspecific IgG (F, G). The positions of active and inactive MAPK are indicated by arrowheads and bars, respectively. Similar results were obtained in two or three independent experiments (C, E, G).

Ste11ΔN, the meta-II level of cyclin B and Cdc2 activity was similarly restored (data not shown). Thus, the turning off of cyclin B degradation at the meta-II level of Cdc2 activity is dependent on Mos/MAPK activity and a partial activation of MAPK is sufficient for this regulation. To examine whether APC/C–Cdc20 activity is required for cyclin B degradation induced by Mos-ablation, we added the Cdc20-inhibiting antibody to CSF extracts that had been deprived of Mos activity. As seen in Fig. 5G, when APC/C–Cdc20 was suppressed with the Cdc20-inhibiting antibody in the absence of Mos activity, cyclin B persisted and Cdc2 activity was maintained at the meta-II level (Figs. 5F, G). These results indicate that Mos/MAPK activity is required to turn off APC/C–Cdc20-mediated cyclin B degradation at the meta-II level of Cdc2 activity, supporting the notion that Mos is essential for the maintenance of CSF arrest.

Mos/MAPK activity is required to suppress precocious cyclin B degradation in MII

Although Mos/MAPK activity is essential for establishing meta-II arrest, its role in the process of CSF establishment in MII is not clear. To investigate this, we utilized the meiotic cycling extract prepared from metaphase I (meta-I) oocytes, which performs the meiotic progression from meta-I to meta-II (Iwabuchi et al., 2000). In the meiotic extract, Cdc2 activity is high at the beginning, corresponding to the meta-I state, gradually declines with incubation time, but subsequently increases. After reaching the meta-II level, the high level of Cdc2 activity is maintained, corresponding to meta-II arrest. This change in Cdc2 activity occurs in parallel with the changes in the cyclin B amounts owing to the absence of inhibitory phosphorylation of Cdc2 during the MI–MII transition period (Iwabuchi et al., 2000, see also Fig. 6). To examine the role of Mos/MAPK activity in the establishment of meta-II arrest, we added U0126, an inhibitor of MEK, to meiotic extracts. Immunoblot analysis revealed that in U0126-treated meiotic extracts, MAPK was gradually inactivated, accompanied by a decline in Cdc2 activity, and remained completely inactive afterwards (Fig. 6A). In the absence of MAPK activity, the decline in Cdc2 activity was initially reversed but soon after declined again, instead of continuing to increase until the meta-II level was reached (Fig. 6B). By the addition of Ste11ΔN to U0126-treated meiotic extracts, a small fraction of MAPK remained activated, and Cdc2 activity was elevated to the meta-II level (Figs. 6A, B). Immunoblot analysis confirmed that the precocious decline of Cdc2 activity in the absence of MAPK activity was caused by a decrease in the amount of cyclin B. When APC/C–Cdc20 was suppressed with the Cdc20-inhibiting antibody, Cdc2 activity was elevated to a high level in the absence of Mos/MAPK activity (Figs. 6C, D). Thus, in the MAPK-ablated meiotic extracts, the precocious decline of Cdc2 activity was caused by APC/C–Cdc20-mediated cyclin B degradation. These results strongly suggest the role of Mos/MAPK activity in the establishment

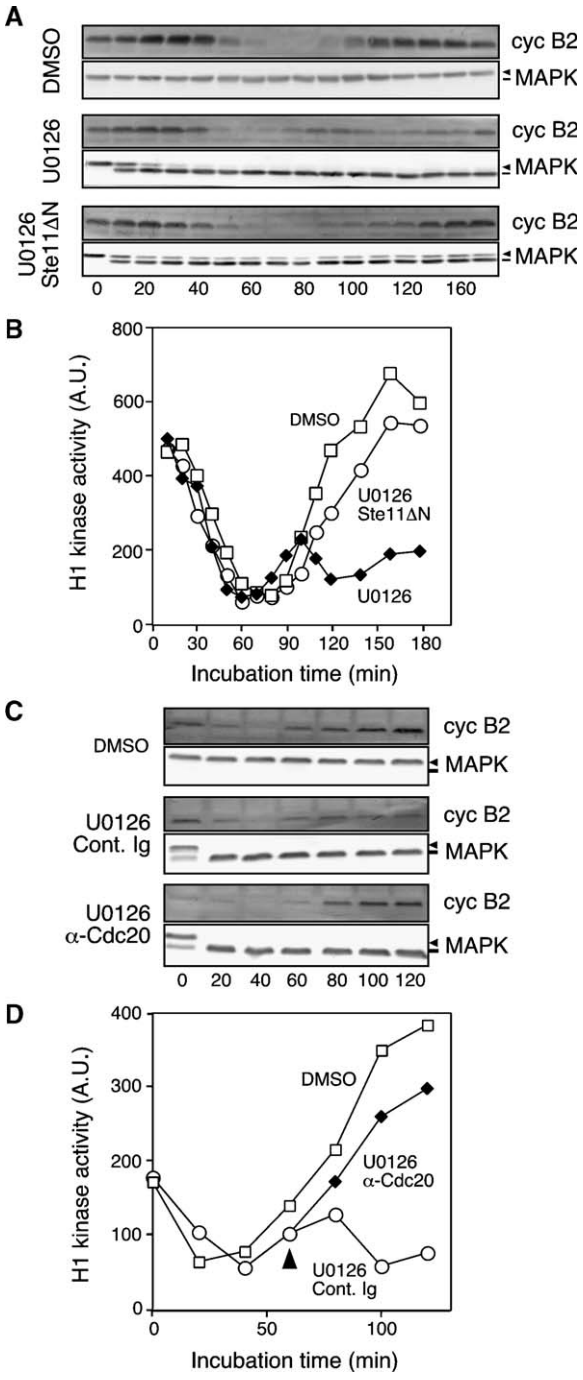

Fig. 6. Mos/MAPK activity is required for suppressing APC/C–Cdc20-mediated cyclin B degradation in MII at lower than the metaphase level of Cdc2 activity. Meiotic cycling extracts were incubated with DMSO, U0126 or U0126 in combination with either GST-Ste11ΔN (A, B), a Cdc20-inhibiting antibody (α-Cdc20) or nonspecific IgG (cont. Ig) (C, D). Samples were examined for the changes in cyclin B2 and MAPK by immunoblotting (A, C) and Cdc2 activity (B, D) by histone H1 kinase assays. The Cdc20-inhibiting antibody and nonspecific IgG were added to extracts at the indicated time (arrowhead in panel D). The positions of active and inactive MAPK are indicated by arrowheads and bars, respectively. Similar results were obtained in two independent experiments (B, D).

of the threshold, below which APC/C–Cdc20-mediated cyclin B degradation is prevented during the CSF arrest.

Discussion

In this study, we have investigated the regulatory mechanism for the Cdc2 activity level in CSF-arrested eggs and extracts. Our results have demonstrated that under CSF arrest, Cdc2 activity is kept constant at the meta-II level as follows; (i) cyclin B is continuously synthesized and, correspondingly, the Cdc2 activity level is elevated, (ii) when the Cdc2 activity level is elevated above the meta-II level, cyclin B degradation dependent on APC/C–Cdc20 is activated and the initial meta-II level of Cdc2 activity level is restored, and (iii) when Cdc2 activity is decreased to the meta-II level, cyclin B degradation is suppressed, depending on Mos/MAPK activity (Fig. 7). Thus, under CSF arrest, Cdc2 activity is maintained at the meta-II level through the dynamic feedback control based on APC/C–Cdc20-mediated cyclin B degradation (red arrows in Fig. 7), rather than by cessation of cyclin B metabolism or a coincidental equilibrium between the synthesis and degradation rates of cyclin B.

Regulation of cyclin B degradation under CSF arrest

The notion has been accepted that cyclin B degradation is suppressed through inhibition of APC/C–Cdc20 under CSF arrest (for review, see Tunquist and Maller, 2003). Our data have shown, however, that cyclin B degradation mediated by APC/C–Cdc20 occurs and even is required to keep the Cdc2 activity level constant in CSF-arrested eggs and extracts. This evokes the question of how APC/C–Cdc20-mediated cyclin B degradation is activated under CSF arrest. Our results indicate that cyclin B degradation is activated when Cdc2 activity is elevated beyond the meta-II level, and depends upon both the APC/C and Cdc20 (Figs. 2–4). Similar activation of cyclin B degradation is also induced when CSF extracts are treated with microcystin (Lorca et al., 1991) or okadaic acid (Lorca et al., 1998), potent inhibitors of protein phosphatase 2A; this phosphatase is thought to catalyze protein dephosphorylation that antagonizes protein phosphorylation by Cdc2. The cyclin B degradation induced by okadaic acid-treatment is also dependent on APC/C–Cdc20 (Lorca et al., 1998). Thus, it is very likely that the under CSF arrest, cyclin B degradation is activated in the presence of high Cdc2 activity, probably through the enhanced phosphorylation of protein substrates; this phosphorylation is distinct from that induced by CaMKII activity, which activates cyclin B degradation that induces exit from MII (Lorca et al., 1993). It is noteworthy in this context that separase, a protease responsible for sister chromatid separation, is inhibited by specific phosphorylation induced by Cdc2 activity higher than the meta-II level (Stemmann et al., 2001).

The most likely targets for high Cdc2 activity are APC/C components, particularly Apc1, Cdc27 and Cdc16, which are massively phosphorylated in M-phase (Kraft et al., 2003). We have confirmed that Cdc27 is hyperphosphorylated in CSF extracts with Cdc2 activity higher than the meta-II level. The APC/C is known to be activated by phosphorylation in M-phase, and recent studies have

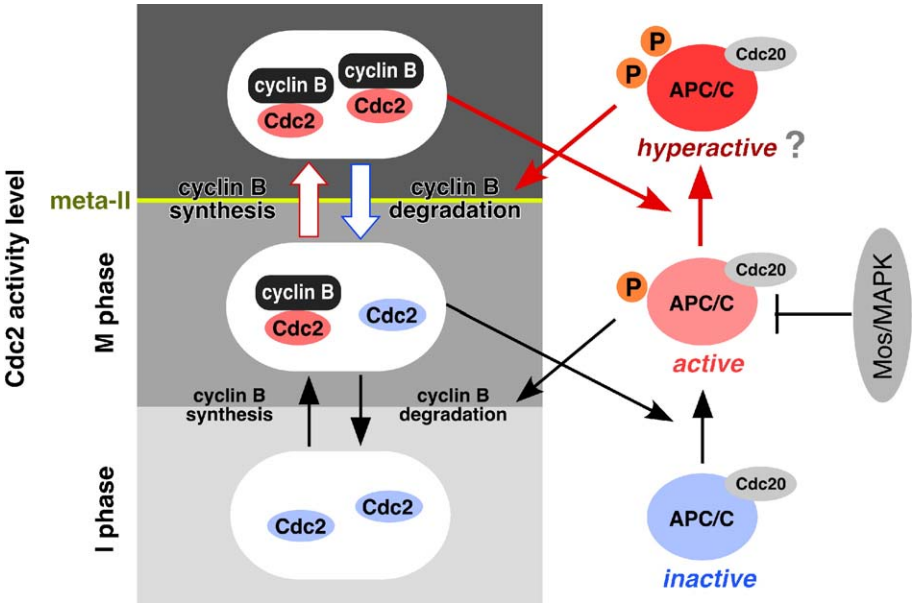

Fig. 7. Model for the regulation of cyclin B degradation under CSF arrest. Cyclin B synthesis continues under CSF arrest and Cdc2 activity is elevated (upward open arrow). APC/C–Cdc20-mediated cyclin B degradation is suppressed by a Mos/MAPK-dependent activity when Cdc2 activity is lower than the meta-II level, but activated through hyperphosphorylation of APC/C components when Cdc2 activity is elevated beyond the meta-II level, resulting in a decline in Cdc2 activity to meta-II levels (downward open arrow). Cdc2 activity is thus maintained at the meta-II level through the dynamic regulation for APC/C–Cdc20-mediated cyclin B degradation (red arrows). See text for details.

revealed that the APC/C isolated from CSF-arrested extracts possesses activity to catalyze polyubiquitination of cyclin B (Vorlaufer and Peters, 1998) and to bind its destruction box (Yamano et al., 2004). Thus, it appears that under CSF arrest, the APC/C is in the active state but is prevented from catalyzing cyclin B ubiquitination by Mos/MAPK activity when Cdc2 activity is lower than the meta-II level. This putative effect of Mos/MAPK is thought to be ineffective for the APC/C whose components are hyperphosphorylated since, in the presence of Cdc2 activity higher than the meta-II level, the APC/C effectively mediated cyclin B degradation, by a Cdc20-dependent mechanism (Figs. 3 and 4). Our data do not exclude the possibility that high Cdc2 activity antagonizes the suppression of the APC/C by Mos/MAPK activity.

It has been reported that Cdc20 is inhibited by Emi1 in MII, and that this inhibition is responsible, at least in part, for CSF arrest (Reimann and Jackson, 2002). However, according to our analysis, Emi1 is not present in CSF-arrested eggs, and arrest caused by Emi1 is distinct from CSF arrest in both dependency on MAPK activity and sensitivity to Ca^{2+}-induced activation (Ohsumi et al., 2004). Thus, the regulation of Cdc20 under CSF arrest is not clear, although our results clearly show that its activity is essential for cyclin B degradation under CSF arrest.

Taken together, our results lead us to the following model. We propose that under CSF arrest, cyclin B degradation is suppressed through inhibition of the APC/C by a mechanism that depends on Mos/MAPK activity, whenever Cdc2 activity decreases below the meta-II level. However, this suppression is ineffective for the APC/C whose components are hyperphosphorylated in the presence of Cdc2 activity higher than the meta-II level (Fig. 7).

The role of the Mos/MAPK pathway in CSF arrest

Although there is a consensus that Mos/MAPK activity is essential for the establishment of CSF arrest, there are contradictory arguments regarding whether or not Mos/MAPK activity is required for the maintenance of CSF arrest (Tunquist and Maller, 2003). Our results favor the positive role of Mos/MAPK activity in the maintenance of CSF arrest, and are consistent with previous studies showing that inhibition of Mos or MAPK with a neutralizing antibody and addition of Mkp1, a MAPK-inactivating phosphatase, causes release from CSF arrest in unfertilized eggs or CSF-arrested extracts (Furuno et al., 1997; Minshull et al., 1994). On the contrary, others have reported that inhibition of MAPK with U0126 does not cause release from CSF arrest (Reimann and Jackson, 2002; Tunquist et al., 2002). In our unpublished experiments, however, U0126-treatment of CSF extracts failed to completely inactivate MAPK, even at the highest concentration used (500 μM) (Yamamoto, unpublished). The small fraction of MAPK remaining active, which was likely to be missed in detection by immunoblotting with anti-phospho-MAPK antibodies, is significant since even partial activation of MAPK in meiotic extracts was sufficient to suppress precocious cyclin B degradation in MII (Fig. 5). The requirement of Mos/MAPK activity for the maintenance of CSF arrest has also been challenged by studies showing that CSF arrest is maintained when extracts are immunodepleted of Rsk or Bub1, downstream components of the MAPK pathway, which are known to be involved in the establishment of CSF arrest (Bhatt and Ferrell, 1999; Tunquist et al., 2002). However, the downstream component of Mos/MAPK that is depleted should be taken into account, since it has been suggested that there is an alternative pathway other than the one leading to activation of Rsk that possibly contributes to the maintenance of M-phase arrest (Horne and Guadagno, 2003; Lefebvre et al., 2002). Although the reasons for the discrepancies discussed above are not clear, we emphasize that when Mos activity is ablated from CSF-arrested eggs and extracts, CSF arrest is not maintained (Furuno et al., 1997).

In good agreement with a precocious decline of Cdc2 activity in MII in Mos-ablated maturing oocytes (Dupre et al., 2002), our data showed that in the absence of Mos/MAPK activity APC/C–Cdc20-mediated cyclin B degradation was activated at a Cdc2 activity level much lower than the meta-II level (Fig. 6). Therefore, the failure of CSF arrest in both Mos-ablated oocytes and MAPK-inhibited meiotic extracts is ascribable to the precocious activation of APC/C–Cdc20-mediated cyclin B degradation by the low level of Cdc2 activity. According to our results, the Mos/MAPK pathway plays a consistent role in both establishment and maintenance of CSF arrest; namely, it prevents APC/C–Cdc20-mediated cyclin B degradation when Cdc2 activity is lower than the meta-II level. More importantly, the dynamics of Cdc2 activity during MII is well explained by this regulation of cyclin B degradation in the presence of continuous cyclin B synthesis. We, therefore, propose that the Mos/MAPK pathway plays an essential role in both establishment and maintenance of CSF-arrest through prevention of polyubiquitination of cyclin B by APC/C–Cdc20 when Cdc2 activity is lower than the meta-II level. The mechanism involved in the prevention of APC/C–Cdc20 remains to be elucidated.

In summary, we have demonstrated that under CSF arrest, Cdc2 activity is kept constant at the meta-II level through the precise turning on and off of APC/C–Cdc20-mediated cyclin B degradation in response to the Cdc2 activity level. The present finding that APC/C–Cdc20 does mediate cyclin B degradation under CSF arrest provides a novel view of the regulatory mechanism for CSF arrest in unfertilized eggs of vertebrates.

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