Developmental Biology **231,** 279–288 (2001)
doi:10.1006/dbio.2000.0142, available online at http://www.idealibrary.com on IDEAL®

Interplay between Cdc2 Kinase and the c-Mos/MAPK Pathway between Metaphase I and Metaphase II in *Xenopus* Oocytes

Marie Frank-Vaillant, Olivier Haccard, René Ozon, and Catherine Jessus[1]

Laboratoire de Physiologie de la Reproduction, INRA/ESA-CNRS 7080, Université Pierre et Marie Curie, boîte 13, 4 place Jussieu, 75252 Paris Cedex 05, France

***Xenopus* oocytes arrested in prophase I resume meiotic division in response to progesterone and arrest at metaphase II. Entry into meiosis I depends on the activation of Cdc2 kinase [M-phase promoting factor (MPF)]. To better understand the role of Cdc2, MPF activity was specifically inhibited by injection of the CDK inhibitor, Cip1. When Cip1 is injected at germinal vesicle breakdown (GVBD) time, Cdc25 and Plx1 are both dephosphorylated and Cdc2 is rephosphorylated on tyrosine. The autoamplification loop characterizing MPF is therefore not only required for MPF generation before GVBD, but also for its stability during the GVBD period. The ubiquitine ligase anaphase-promoting complex/cyclosome (APC/C), responsible for cyclin degradation, is also under the control of Cdc2; therefore, Cdc2 activity itself induces its own inactivation through cyclin degradation, allowing the exit from the first meiotic division. In contrast, cyclin accumulation, responsible for Cdc2 activity increase allowing entry into metaphase II, is independent of Cdc2. The c-Mos/mitogen-activated protein kinase (MAPK) pathway remains active when Cdc2 activity is inhibited at GVBD time. This pathway could be responsible for the sustained cyclin neosynthesis. In contrast, during the metaphase II block, the c-Mos/MAPK pathway depends on Cdc2. Therefore, the metaphase II block depends on a dynamic interplay between MPF and CSF, the c-Mos/MAPK pathway stabilizing cyclin B, whereas in turn, MPF prevents c-Mos degradation.**

***Key Words: Xenopus* oocyte; c-Mos; Cdc2; cyclins; meiotic maturation.**

INTRODUCTION

Two protein kinases play a pivotal role during the oocyte meiotic maturation in all studied animal species: the M-phase promoting factor (MPF),[2] a complex of Cdc2 kinase and cyclin B, and mitogen-activated protein kinase (MAPK) (Gebauer and Richter, 1997; Jessus and Ozon, 1993). Depending on the species, both kinases, that are activated with various time courses, are connected to each other by different molecular cross-talks. Surprisingly, the MAPK pathway appears to be differentially involved at different steps of the meiotic maturation process. It is therefore of crucial interest to clarify the interplays between Cdc2 kinase and the MAPK pathway during this biological process.

In *Xenopus*, oocyte maturation is triggered by progesterone. This cellular process allows resting oocytes, arrested at the first meiotic prophase, to resume the first meiotic division and to arrest at metaphase II. The meiotic maturation period can be divided into three phases (for review, see Ferrell, 1999): (1) A pre-GVBD period, starting after hormonal addition and lasting between 3 and 5 h until GVBD; (2) The metaphase I/metaphase II transition, or interkinesis. Nuclear envelopes do not reform, chromosomes remain condensed, and DNA replication does not occur; (3) The metaphase II arrest.

During the pre-GVBD period, Cdc2 and MAPK turn on at about the same time, just before GVBD (Ferrell *et al.*, 1991; Gotoh *et al.*, 1995; Jessus *et al.*, 1991; Nebreda and Hunt, 1993). MPF is activated by the Cdc25 phosphatase, which removes the inhibitory phosphates on Thr14 and Tyr15 of Cdc2. MAPK activation depends on the c-Mos protein

[1] To whom correspondence should be addressed. Fax: (33) 1 44 27 34 72. E-mail: jessus@ccr.jussieu.fr.

[2] Abbreviations: GVBD, germinal vesicle breakdown; MAPK, mitogen-activated protein kinase; MPF, M-phase promoting factor; APC, anaphase-promoting complex; APC/C, APC/cyclosome.

accumulation. c-Mos protein kinase is an efficient activator of MAPK kinase, that in turn activates MAPK (Nebreda and Hunt, 1993; Posada *et al.*, 1993). How are the Cdc2 pathway and the MAPK pathway connected? c-Mos synthesis is required for Cdc2 activation (Sagata *et al.*, 1988), leading to the conclusion that MPF activation depends on the MAPK pathway (Gotoh *et al.*, 1991, 1995; Posada *et al.*, 1993). Conversely, inhibition of Cdc2 kinase activation by different specific proteins prevents c-Mos accumulation and MAPK activation, leading to the opposite hypothesis (Frank-Vaillant *et al.*, 1999; Nebreda *et al.*, 1995). Moreover, the inhibition of MAPK activation does not prevent Cdc2 activation induced by progesterone, although the extent of Cdc2 kinase activity is lowered, GVBD induction is delayed, and oocytes do not complete meiosis normally in the absence of MAPK activity (Fisher *et al.*, 1999; Gross *et al.*, 2000). It is therefore probable that progesterone initiates the process of Cdc2 activation independently of MAPK. Cdc2 kinase would stabilize c-Mos, leading to MAPK activation, which in turn participates to the full activation of Cdc2.

Soon after GVBD, MPF activity falls during the metaphase I/anaphase I transition, due to partial cyclin degradation, and then rises again leading to entry into meiosis II. What does control cyclin degradation and cyclin neosynthesis during interkinesis? It is established that inactivating Cdc2 kinase during this period leads to the rephosphorylation of Cdc2 on Tyr15 and reentry into an interphase-DNA-replicating state (Furuno *et al.*, 1994; Jessus *et al.*, 1991). The reincrease in MPF activity is therefore crucial to enter the second meiotic division and to avoid an interphase state between both meiotic divisions. This increase is attributed to a sustained cyclin neosynthesis (Thibier *et al.*, 1997). c-Mos is also subjected to an active turnover at GVBD time, although the level of the protein is stable during the metaphase I–metaphase II transition (Nishizawa *et al.*, 1992). Indeed, addition of protein synthesis inhibitors at GVBD time leads to the disappearance of c-Mos and cyclins, and consequently to the inactivation of Cdc2 and MAPK (Nishizawa *et al.*, 1992; Thibier *et al.*, 1997). How are the Cdc2 pathway and the MAPK pathway connected during interkinesis? It has been proposed that Cdc2 reactivation just before metaphase II requires c-Mos function (Furuno *et al.*, 1994). However, it is not known whether MAPK requires Cdc2 function during the metaphase I–metaphase II transition. It is also not known to what extent Cdc2 does control cyclin degradation and cyclin accumulation during this period.

During the metaphase II arrest, c-Mos protein is stabilized. Inhibition of protein synthesis neither affects the level of c-Mos protein nor MAPK activity (Nishizawa *et al.*, 1992; Thibier *et al.*, 1997). In the presence of protein synthesis inhibitors, MPF activity is stable, although the cyclin B level is slightly decreased (Thibier *et al.*, 1997). This shows that the majority of cyclin proteins are stabilized, as explained by the presence of the CSF activity, initially described by Masui and Markert (1971). The cytostatic function of the c-Mos/MAPK pathway during the metaphase II arrest is well established (Sagata, 1997). However, the molecular connection between MAPK and the ubiquitine ligase, APC/C, responsible for cyclin degradation (Hershko *et al.*, 1994; King *et al.*, 1995; Sudakin *et al.*, 1995), is not yet known. It is also not known whether the MAPK pathway requires Cdc2 function during the metaphase II arrest.

Fertilization induces the release of the metaphase II arrest. The egg–sperm fusion leads to an intracellular increase of calcium concentration, assumed to activate the Ca^{2+}-calmodulin kinase II, that subsequently directs cyclin degradation via the ubiquitination and proteasome pathway (Glotzer *et al.*, 1991; Lorca *et al.*, 1993; Murray *et al.*, 1989). While cyclin degradation is achieved within 10 min after the Ca^{2+} wave, c-Mos is degraded later, between 30 and 60 min after oocyte activation, and MAPK is subsequently inactivated (Lorca *et al.*, 1991; Watanabe *et al.*, 1991). It is therefore clear that MPF inactivation is not induced by the disappearance of c-Mos. The CSF activity, attributed to the MAPK pathway, and more recently to the MAPK substrate $p90^{rsk}$ (Bhatt and Ferrell, 1999; Gross *et al.*, 1999), is bypassed by the Ca^{2+} surge that promotes cyclin degradation despite the presence of c-Mos and active MAPK. It can be hypothesized that c-Mos degradation is driven by MPF inactivation.

Therefore, during these different phases of the meiotic divisions, a dynamic cross-talk exists between Cdc2 and the MAPK pathway. In order to better understand how the activities of both kinases are linked, we investigated the consequences of the specific *in ovo* Cdc2 inhibition. We have recently reported that, when injected into prophase oocytes, the CDK inhibitor Cip1 binds endogenous Cdc2-cyclin B complexes and prevents their activation normally induced by progesterone (Frank-Vaillant *et al.*, 1999, 2000). We demonstrate here that Cip1 is also able to inhibit the active form of Cdc2 present at both GVBD time and metaphase II. This allowed us to study the role of this kinase from GVBD to metaphase II.

MATERIALS AND METHODS

Materials

Xenopus laevis adult females (CNRS, Rennes, France) were bred and maintained under laboratory conditions. $\gamma[^{32}P]$ATP was purchased from Dupont NEN (Boston, MA). Reagents, unless otherwise specified, were from Sigma (Saint Quentin Fallavier, France). Recombinant GST-$p21^{cip1}$ was expressed and purified as described by Frank-Vaillant *et al.* (1999).

Xenopus Oocyte Treatment

Fully grown *Xenopus* oocytes were isolated and prepared as described (Jessus *et al.*, 1987). For injections at GVBD time, 500 oocytes were incubated in the presence of 1 μM progesterone. Oocytes were referred as to germinal vesicle breakdown stage when the first pigment rearrangement appeared; at this stage, they were collected by groups of 10, injected or not with GST-$p21^{cip1}$ at 1 μM

intracellular concentration, and immediately incubated or not in the presence of 100 μg/ml cycloheximide. Extracts were then prepared at different times following the injection. For injections at metaphase II, oocytes were incubated in the presence of 1 μM progesterone until metaphase II, i.e., 3 h after GVBD. Then metaphase II-arrested oocytes were incubated in the presence of 50 mM EGTA for 1 h, before GST-p21^{cip1} microinjection. Oocytes were collected 1 or 2 h after the injection. In some cases, control oocytes and GST-p21^{cip1}-microinjected oocytes were extensively washed and then activated or not by electric shock, as previously described (Karsenti *et al.*, 1984). Oocytes were collected 1 h later.

Xenopus Oocyte Extracts

Oocytes were lysed at 4°C in 4 volumes of extraction buffer (EB: 80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors: 25 μg/ml leupeptin and aprotinin, 10 μg/ml pepstatin, 1 mM benzamidine, 1 μM AEBSF from Pentapharm AG, Basel, Switzerland). To visualize the electrophoretic shift of Cdc25, 1 mM orthovanadate was included in EB. Lysates were centrifuged at 15,000*g* at 4°C for 15 min (Sigma 302K centrifuge). The supernatant was collected and analyzed.

Histone H1 Kinase Assays

Extracts (15 μl; i.e., equivalent of three oocytes) were collected for p13^{suc1} binding followed by histone H1 kinase assay in the presence of γ[^{32}P]ATP (Dupont NEN) according to Jessus *et al.* (1991).

Immunoblotting

Extracts in Laemmli buffer (Laemmli, 1970) equivalent to three oocytes were loaded on each lane, electrophoresed on 12.5% SDS–PAGE Anderson gels (Anderson *et al.*, 1973), and transferred to nitrocellulose filters (Schleicher and Schull, Ecquevilly, France) using a semi-dry blotting system (Millipore, Saint Quentin, France) as described in Jessus *et al.* (1991). The proteins of interest were visualized by use of the appropriate primary antibody; the following antibodies were used: sheep polyclonal anti-cyclin B2 antibody (Gautier *et al.*, 1990), sheep polyclonal anti-cyclin B1 antibody (Gautier *et al.*, 1990), mouse anti-Cdc27 antibody (Transduction Laboratories, Montluçon, France), rabbit polyclonal anti-Cdc2 phosphorylated on tyrosine antibody (New England Biolabs, Saint Quentin, France), rabbit polyclonal anti-Cdc25 antibody (Izumi *et al.*, 1992), rabbit polyclonal anti-Plx1 antibody (Abrieu *et al.*, 1998), rabbit polyclonal anti-c-Mos antibody (Santa Cruz Biotechnologies), and mouse anti-phosphorylated MAPK antibody (New England Biolabs). The secondary antibodies were HRP-conjugated (Jackson Immunoresearch, West Grove, PA). The chemoluminescence reagent was from NEN.

RESULTS

Microinjection of Cip1 at GVBD Inhibits MPF Activity

Cip1 protein is a broad-spectrum inhibitor of cdk/cyclin complexes (Xiong *et al.*, 1993). When injected into prophase-arrested oocytes, recombinant human GST-p21^{Cip1} prevents MPF activation and GVBD normally induced by progesterone by direct binding to Cdc2/cyclin B complexes (Frank-Vaillant *et al.*, 1999). To better understand the role of Cdc2 during the metaphase I–metaphase II transition, we attempted to inhibit MPF activity at GVBD in order to analyze the molecular consequences of MPF inhibition at this period.

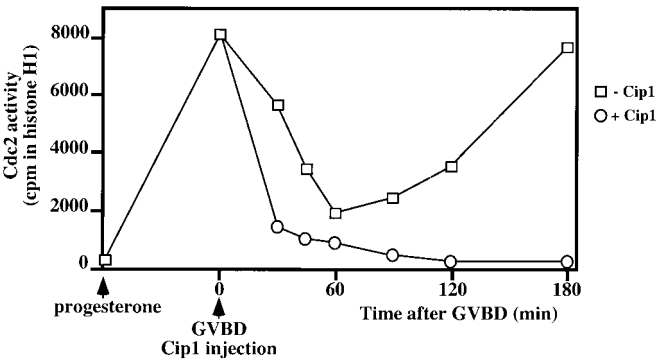

FIG. 1. Effect of Cip1 on Cdc2 activity at GVBD time. Oocytes were collected at GVBD time and injected (○) or not (□) with Cip1. Histone H1 kinase activity of Cdc2 was measured in prophase oocytes, at GVBD, and at 30, 45, 60, 90, 120, and 180 min after GVBD.

We first examined whether Cip1 was able to inhibit the active form of Cdc2 present in GVBD oocytes. Oocytes were incubated in the presence of 1 μM progesterone and GVBD was monitored by the appearance of a white spot at the animal pole. At GVBD time, oocytes were injected or not with Cip1 at 1 μM intracellular concentration and collected at different times following the injection. The activity of Cdc2 was assayed by measuring histone H1 kinase activity (Fig. 1). Cdc2 was inactive in prophase oocytes and was activated at GVBD time. As previously reported in control oocytes, MPF activity decreased after GVBD when cyclins are degraded and increased again at entry into metaphase II, when cyclins accumulate. In Cip1-injected oocytes, MPF activity sharply decreased after injection; 30 min after GVBD, Cdc2 activity level was reduced by 80% in the presence of Cip1, whereas it was decreased by 30% in control oocytes at the same time. Cdc2 activity reached the prophase level within 90 min after Cip1 injection and did not reincrease during the following 2 h. This experiment shows that injection of Cip1 at GVBD rapidly leads to the inhibition of MPF activity and prevents its subsequent reactivation at metaphase II, which normally requires cyclin neosynthesis. The inhibition of Cdc2 activity by Cip1 injection during the metaphase I–metaphase II transition allowed us to study the role of this kinase during this period.

Cdc2 Controls Cdc25 Phosphatase and Plx1 Kinase in Metaphase I

Cdc2 is activated at GVBD by dephosphorylation on Tyr15 and Thr14 by the Cdc25 phosphatase (Dunphy and

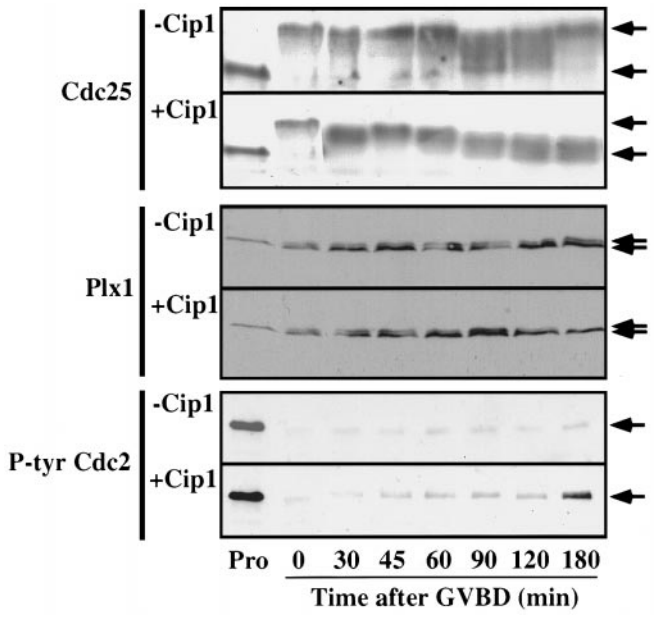

FIG. 2. Cdc2 inhibition at GVBD time leads to Cdc25 and Plx1 inactivation. Oocytes were collected at GVBD time and injected (+Cip1) or not (−Cip1) with Cip1. Western blots were performed with extracts prepared from prophase oocytes (Pro), or from oocytes taken at various times after GVBD, with an anti-Cdc25 antibody, an anti-Plx1 antibody, and an anti-phospho-Tyr-Cdc2 (P-tyr Cdc2) antibody. Arrows indicate the various migration levels of the indicated proteins.

Kumagai, 1991; Gautier *et al.*, 1991). Cdc25 activity is itself positively regulated by Cdc2 activity, contributing to the positive feedback loop that allows the abrupt activation of MPF just before GVBD. We addressed the question of whether Cdc25 activity is still under the control of Cdc2 after the amplification loop has been completed at GVBD, leading to a full MPF activation. For this purpose, we examined whether Cdc25 electrophoretic mobility was altered following the inactivation of Cdc2 by Cip1 at GVBD. Oocytes were microinjected with Cip1 at GVBD time and collected at different times following the injection. Oocyte extracts were subjected to Western blot analysis with an anti-*Xenopus* Cdc25 antibody. At GVBD, Cdc25 underwent an electrophoretic mobility shift (Fig. 2), corresponding to the phosphorylation and the activation of the protein (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). In control oocytes, the Cdc25 phosphorylation level was stable during interkinesis. A slight decrease in Cdc25 phosphorylation was observed between 90 and 120 min after GVBD (Fig. 2), corresponding to the decreased level of Cdc2 activity at this period (Fig. 1). When Cdc2 was inactivated at GVBD time by Cip1 injection, Cdc25 was progressively dephosphorylated, as shown by the appearance of multiple mobility bands (Fig. 2). It almost reached the prophase level 3 h after injection, not completely returning to its faster migrating unphosphorylated form (Fig. 2). This suggests that its phosphatase activity was almost turned off, as supported by the tyrosine phosphorylation level of Cdc2 at this period (see later, Fig. 2).

Plx1 kinase activity is also required to phosphorylate and activate Cdc25 in *Xenopus* oocyte (Karaiskou *et al.*, 1999; Kumagai and Dunphy, 1996). As previously reported (Qian *et al.*, 1998), Plx1 was activated at GVBD by phosphorylation, that is visualized by the electrophoretic migration of the protein as a doublet (Fig. 2). In control oocytes, Plx1 phosphorylation was stable from GVBD to metaphase II (Fig. 2). In contrast, the inhibition of Cdc2 activity by Cip1 microinjection at GVBD time led to Plx1 dephosphorylation, completed within 2 h after the injection (Fig. 2). These results indicate that the phosphorylation and therefore activation of both Plx1 and Cdc25 require Cdc2 activity during the metaphase I–metaphase II transition.

Cdc25 and Plx1 inactivation prompted us to analyze the tyrosine phosphorylation level of Cdc2 following Cip1 injection at GVBD time. Tyrosine phosphorylation of Cdc2 was monitored at different times following the injection by Western blotting using an antibody directed against the tyrosine-phosphorylated form of Cdc2. Cdc2, that was phosphorylated on tyrosine in prophase oocytes, was then dephosphorylated at GVBD and remained under this dephosphorylated form until metaphase II in control oocytes (Fig. 2). Three hours after Cip1 microinjection at GVBD, Cdc2 was rephosphorylated on tyrosine, in parallel with Cdc25 and Plx1 inactivation (Fig. 2).

Cyclin B1 and Cyclin B2 Accumulation Is Independent of MPF Activity after GVBD

We next addressed the question of whether cyclin B degradation and accumulation that normally take place after GVBD are under the control of Cdc2. Cip1 was injected into GVBD oocytes and Western blots were performed using anti-*Xenopus* cyclin B1 and anti-*Xenopus* cyclin B2 antibodies (Fig. 3). In control oocytes, cyclin B1 was almost undetectable in prophase oocytes and then accumulated from GVBD to metaphase II (Fig. 3). The accumulation pattern of cyclin B1 was identical in oocytes where Cdc2 was inactivated at GVBD time by Cip1 injection (Fig. 3).

As already reported (for review, Taieb and Jessus, 1997), cyclin B2 is associated with inactive tyrosine-phosphorylated Cdc2 in prophase oocytes and this inactive form migrates as a doublet (Fig. 3). The protein was electrophoretically shifted at GVBD, at time of Cdc2 activation. It remained under this form until metaphase II in control oocytes (Fig. 3). As expected, a slight decrease in cyclin B2 level occurred between metaphase I and metaphase II. The level of cyclin B2 protein appeared to be stable for 3 h in oocytes that have been injected with Cip1 at GVBD (Fig. 3). Under these conditions, the electrophoretic mobility of the protein was progressively accelerated following Cip1 injection. Three hours after Cip1 injection, cyclin B2 migrated as

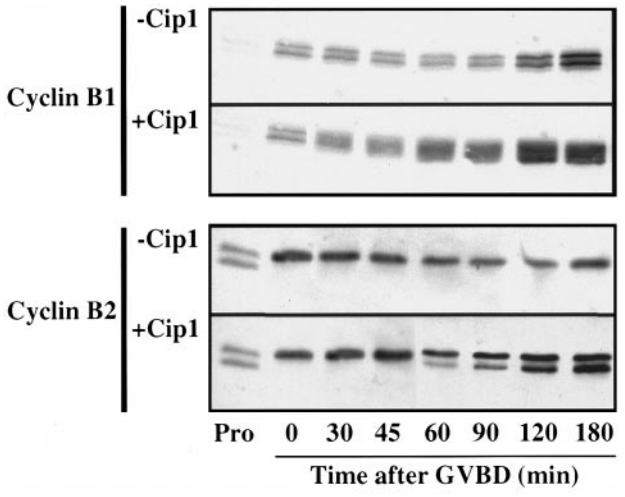

FIG. 3. Effect of Cdc2 inhibition at GVBD time on cyclins accumulation. GVBD oocytes were injected (+Cip1) or not (−Cip1) with Cip1. Oocytes were then collected at various times after GVBD and subjected to Western blot analysis with an anti-cyclin B1 antibody and an anti-cyclin B2 antibody. Extracts from prophase oocytes (Pro) were also probed.

a doublet, identical to the inactive prophase form of the protein (Fig. 3). This observation is correlated with the rephosphorylation of Cdc2 on Tyr15 following Cip1 injection (Fig. 2). Altogether, these results show that B-type cyclins accumulation does not depend on Cdc2 activity during the metaphase I–metaphase II transition.

APC Phosphorylation and Cyclin Degradation Are under the Control of Cdc2 Activity in Metaphase I Oocytes

To estimate whether the degradation of B-type cyclins is controlled by Cdc2, the same experiments as above were performed in the presence of cycloheximide. Preventing cyclin neosynthesis allows the visualization of B-type cyclins degradation. Oocytes were incubated in the presence of cycloheximide at GVBD time and simultaneously injected or not with Cip1. Cyclin B1 level rapidly decreased in the presence or in the absence of Cip1, being totally degraded within 1 h following GVBD (Fig. 4). The degradation time course was delayed in the presence of Cip1 (Fig. 4). In the absence of Cip1, cyclin B2 was degraded between 90 min and 2 h following GVBD, therefore slower than cyclin B1 (Fig. 4). As for cyclin B1, cyclin B2 decrease was delayed in response to Cip1 injection (Fig. 4). The delay observed for cyclin B2 degradation was longer than for cyclin B1, since significant amounts of cyclin B2 were still detected 3 h after GVBD (Fig. 4). Under these conditions, i.e., in the absence of protein synthesis, cyclin B2 migrated as the up-shifted form and not as a doublet. The cyclin B2 doublet observed following Cip1 injection (Fig. 3) therefore depends on protein synthesis.

Since B-type cyclin degradation was affected by Cdc2 inhibition, we analyzed the phosphorylation level of Cdc27, an APC/C component whose phosphorylation level is highly correlated with APC/C activity (King *et al.*, 1995). Western blot analysis using an anti-Cdc27 antibody revealed that, in control oocytes, Cdc27 underwent a slight electrophoretic mobility shift at GVBD and a subsequent larger shift at time of metaphase II (Fig. 4). In Cip1-microinjected oocytes, the slightly shifted form of Cdc27 observed at GVBD was converted back into a nonphosphorylated form within 90 min to 2 h following Cip1 injection (Fig. 4). Moreover, the large shift observed at metaphase II was totally prevented by Cip1 injection (Fig. 4). These electrophoretic mobility changes in Cdc27 strongly suggest that APC/C is inactivated in response to Cdc2 inhibition at GVBD. It has been previously reported that APC/C activation is dependent on Cdc2 (Lahav-Baratz *et al.*, 1995). Our data demonstrate, according to the observed dephosphorylation of Cdc27 (Fig. 4), that continuation of APC/C activity might also depend on Cdc2 during the metaphase

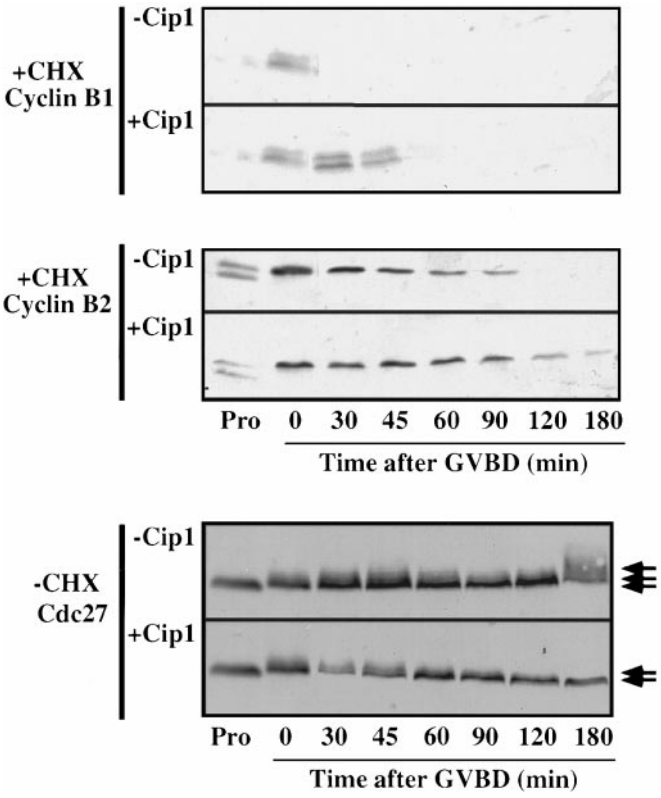

FIG. 4. Effect of Cdc2 inhibition at GVBD time on cyclins degradation. GVBD oocytes were injected (+Cip1) or not (−Cip1) with Cip1 in the presence (+CHX) or in the absence (−CHX) of cycloheximide. Oocytes were then collected at various times after GVBD and subjected to Western blot analysis with an anti-cyclin B1 antibody, an anti-cyclin B2 antibody, and an anti-Cdc27 antibody. Extracts from prophase oocytes (Pro) were also probed.

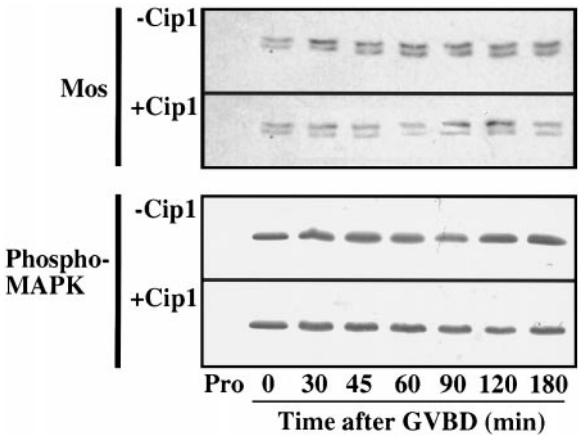

FIG. 5. Effect of Cdc2 inhibition at GVBD time on the c-Mos/MAPK pathway. Oocytes were collected at GVBD time and injected (+Cip1) or not (−Cip1) with Cip1. Western blots were performed with extracts prepared from prophase oocytes (Pro), or from oocytes at various times after GVBD, with an anti-c-Mos antibody and an anti-phospho-MAPK antibody.

I–metaphase II transition. The inactivation of APC/C explains why cyclin B degradation is delayed in the presence of Cip1 (Fig. 4). The different time course of degradation of both cyclins, B1 and B2, suggests that APC/C exhibits a stronger affinity for cyclin B1 than for cyclin B2. Consequently, cyclin B1 is quickly degraded once APC/C is phosphorylated and therefore activated at GVBD time, and its proteolysis is almost completed when APC/C is dephosphorylated and presumably inactivated in response to Cdc2 downregulation (Figs. 3 and 4). In contrast, when APC/C is turned off around 60 min after Cdc2 inactivation, cyclin B2 degradation is not yet achieved, and consequently, the protein is better stabilized than cyclin B1 (Figs. 3 and 4).

Maintenance of the c-Mos/MAPK Pathway Is Independent of MPF Activity during the Metaphase I–Metaphase II Transition

We next examined whether the c-Mos/MAPK pathway is affected by Cip1 microinjection. Oocytes were microinjected with Cip1 at GVBD and extracts were subjected to Western blot analysis using an anti-c-Mos antibody. As expected, c-Mos was undetectable in prophase oocytes and was accumulated at GVBD, migrating as a doublet (Fig. 5). In control oocytes, the protein was detected at a constant level from GVBD to metaphase II (Fig. 5). Cip1 injection did not modify the c-Mos protein pattern for at least 3 h, the protein being detected as in control oocytes. This result shows that MPF activity does not control c-Mos turnover during interkinesis. The same oocyte extracts were subjected to Western blot analysis with an antibody raised against the phosphorylated active form of MAPK (Fig. 5). As expected, active MAPK was not detectable in prophase oocytes, and the kinase was activated at GVBD time (Fig. 5). The phosphorylation level of MAPK was then stable during the metaphase I–metaphase II transition in control oocytes (Fig. 5). In Cip1-injected oocytes, MAPK remained under its active form as in control oocytes. This result shows that Cdc2 activity is not required for maintaining the c-Mos/MAPK pathway active during interkinesis.

c-Mos Stability Is under the Control of Cdc2 in Metaphase II Oocytes

We next investigated the consequences of inhibiting Cdc2 during the metaphase II arrest. We first tested whether Cip1 is able to inhibit the active form of Cdc2 that is stabilized by CSF in metaphase II-arrested oocytes. Oocytes were incubated in the presence of progesterone. The metaphase II stage was reached 2 h after GVBD, according the cytological criteria described by Huchon *et al.* (1993) and Thibier *et al.* (1997). At that stage, oocytes were injected with Cip1. Injections were performed in the presence of 50 mM EGTA in order to prevent oocyte activation. One hour later, oocytes were collected and subjected to histone H1 kinase assay. Injection of Cip1 led to the inactivation of Cdc2 kinase, which returned to the prophase level within 1 h (Fig. 6, lane 4).

Metaphase II-arrested oocytes were electrically activated. One hour after, Cdc2 kinase activity level was similarly decreased to the prophase level (Fig. 6, lane 3). When metaphase II oocytes were first injected with Cip1 and then subjected 1 h later to an electric shock, the basal level of Cdc2 activity was not further reduced (Fig. 6, lane 6).

To ascertain that Cip1 injection did not promote egg activation, the level of cyclin B2 was analyzed by Western blot. Figure 6 shows that cyclin B2 is not degraded within the 2 h following Cip1 injection and is progressively converted into a doublet, characteristic of the prophase stage (Fig. 6, lanes 4–5). In contrast, electric activation of metaphase II oocytes led to a sharp decrease of cyclin B2 level (Fig. 6, lane 3). These results demonstrate that MPF inhibition triggered by Cip1 injection in metaphase II oocytes is not a result of oocyte activation, but is rather due to the direct inhibition of Cdc2 by Cip1. When the electric shock was applied 1 h after Cip1 injection, cyclin B2 was no further degraded (Fig. 6, lane 6), indicating that APC/C was turned off in parallel with Cip1-induced Cdc2 inactivation.

c-Mos protein level was monitored in oocytes where MPF activity had been inactivated by Cip1 injection during the metaphase II arrest. Western blot using an anti-c-Mos antibody shows that c-Mos was degraded within 1 h after Cip1 microinjection, as it is in response to an electric activation (Fig. 6). Therefore, c-Mos stability is dependent on Cdc2 activity in metaphase II-arrested oocytes, and c-Mos degradation can occur in the absence of any Ca^{2+} influx, a hypothesis recently suggested by Bodart *et al.* (1999), by using a nonspecific kinase inhibitor, the purine analog 6-DMAP. To estimate MAPK activity, a Western blot was performed with the antibody recognizing the

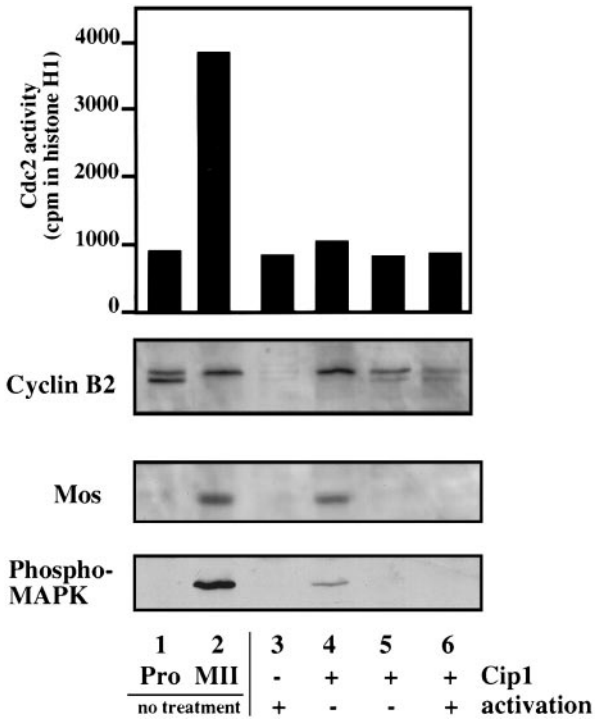

FIG. 6. Effects of Cip1 on Cdc2 activity and the c-Mos/MAPK pathway in metaphase II oocytes. Histone H1 kinase activity assay and Western blot analysis using either an anti-cyclin B2 antibody or an anti-c-Mos antibody or an anti-phospho-MAPK antibody were performed on oocytes after the following treatments: (1) prophase oocytes; (2) metaphase II oocytes; (3) metaphase II treated by an electric shock and collected 1 h later; (4) metaphase II oocytes microinjected with Cip1 and collected 1 h after the injection; (5) metaphase II oocytes microinjected with Cip1 and collected 2 h after the injection; and (6) metaphase II oocytes microinjected with Cip1, treated by an electric shock 1 h after the injection and collected 1 h later.

phosphorylated active form of MAPK. As expected, MAPK was inactivated in parallel with c-Mos degradation after Cip1 injection (Fig. 6).

DISCUSSION

We have previously reported a new experimental approach using the CDK inhibitor Cip1 to prevent the activation of pre-MPF normally induced by progesterone in *Xenopus* oocytes (Frank-Vaillant *et al.*, 1999, 2000). In this study, Cip1 was injected into *Xenopus* oocytes either at GVBD (entry into metaphase I) or at metaphase II arrest, in an attempt to inhibit active MPF. Our results demonstrate that Cip1 injection leads to a rapid inhibition of the fully active Cdc2 kinase, correlated with cyclin stabilization and rephosphorylation of Cdc2 on Tyr15. It can be concluded that injection of Cip1 induces direct MPF inactivation, which leads to the reformation of inactive cyclin B complexes, comparable to the complexes found in prophase. Using the specific Cip1 inhibitor, we were therefore able to analyze the consequences of Cdc2 inactivation from GVBD to metaphase II.

GVBD period. It is well known that the abrupt activation of MPF that occurs just before GVBD is the result of a complex autoamplification process (Masui and Markert, 1971), depending on the positive feedback loop between Cdc2 and Cdc25. Recent reports have established that Plx1 is involved in this process (Abrieu *et al.*, 1998; Karaiskou *et al.*, 1998, 1999; Kumagai and Dunphy, 1996). Our results demonstrate that in the whole oocyte after GVBD, Cdc25 and Plx1 are still under the control of Cdc2. Therefore, once MPF is fully activated at GVBD, the positive feedback loop remains dynamic and actively sustained by Cdc2 (Fig. 7). The amplification loop is therefore not only required for the rapid generation of MPF before GVBD, but also for the stability of its activity during the short GVBD period.

Metaphase I–metaphase II transition or interkinesis. During interkinesis, two sets of events are differentially regulated by Cdc2.

Cdc25, Plx1, APC/C, and cyclin degradation are strictly dependent upon Cdc2 activity. Therefore, once MPF is activated at GVBD, it first maintains its own activity through the positive regulation of Cdc25 and Plx1. The drop of Cdc2 activity that is then initiated soon after GVBD could be the consequence of Cdc2 activity itself: by activating APC/C, it would indirectly promote cyclin degradation (Fig. 7). This leads to the exit from the first meiotic division.

In contrast, the elevation of the cyclin level and the c-Mos/MAPK pathway, once switched on at GVBD, becomes independent of Cdc2 activity during the metaphase I/metaphase II transition. As already suggested by previous reports (Furuno *et al.*, 1994; Gross *et al.*, 2000), the c-Mos/MAPK pathway could be necessary for cyclin neosynthesis, inducing a precocious reincrease in Cdc2 activity that results in the entry into the second meiotic division in the absence of interphase (Fig. 7).

Metaphase II block. Strikingly, once the metaphase II block is reached, the c-Mos/MAPK pathway becomes strictly dependent on Cdc2 activity. It is therefore probable that c-Mos proteolysis that follows cyclin degradation after fertilization (Sagata *et al.*, 1989) does not directly depend on the Ca^{2+} surge and the Ca^{2+}-calmoduline kinase II activation induced by sperm fusion (Lorca *et al.*, 1993), but rather results from Cdc2 inactivation, as already proposed by Bodart *et al.* (1999). It is also highly probable that c-Mos degradation, although depending on ubiquitination and proteasome (Ishida *et al.*, 1993; Nishizawa *et al.*, 1993), is not mediated through APC/C that is rapidly turned off once Cdc2 is inactivated. It is well established that the c-Mos/MAPK pathway is one of the essential components of the CSF activity, implying that MPF is stabilized by this pathway in metaphase II-arrested oocytes (Gross *et al.*, 1999; Haccard *et al.*, 1993; Sagata *et al.*, 1989). Interestingly, we show that *in vivo*, the c-Mos/MAPK pathway is recip-

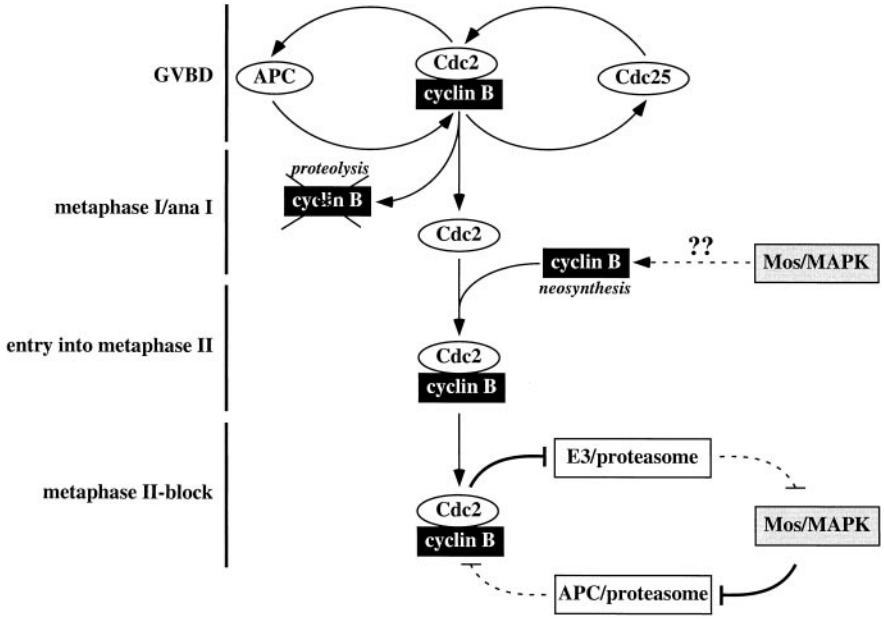

FIG. 7. Model. MPF is fully activated at GVBD, and the positive feedback loop between Cdc25, Plx1, and Cdc2 remains active. APC/C is then activated by Cdc2 and leads to cyclin degradation. In contrast, cyclin accumulation is initiated independently of Cdc2. The c-Mos/MAPK pathway functions independently of Cdc2 and could regulate cyclin neosynthesis, promoting Cdc2 reactivation and entry into metaphase II. The metaphase II block is characterized by a reciprocal control between Cdc2 and the c-Mos/MAPK pathway, each of them stabilizing the other one.

rocally stabilized by MPF during the metaphase II arrest. In other words, the c-Mos/MAPK pathway prevents cyclin B degradation by APC/proteasome, whereas MPF prevents c-Mos from being degraded by a specific ubiquitination/proteasome system (Fig. 7). The metaphase II block is therefore based not only on CSF activity, but also on an MPF-CSF feedback loop. The inactivation of any of them leads to the degradation of the other one. Although the metaphase II block looks very static at the level of the protein turnover, it depends on a dynamic interplay between MPF and CSF, whose molecular basis remains to be elucidated.

Establishment of the metaphase II arrest. There is a crucial switch between the entry into metaphase II and the block into metaphase II (Fig. 7). In both cases, Cdc2 and the c-Mos/MAPK pathway are active. However, in the case of metaphase II entry, the c-Mos/MAPK pathway is independent of Cdc2 (our results) and CSF activity is not yet established (Masui and Markert, 1971; Thibier *et al.*, 1997). In the case of the metaphase II block, the c-Mos/MAPK pathway requires Cdc2 activity (our results) and CSF is activated (Masui and Markert, 1971). What does ensure the transition between both states? One can postulate that during this essential transition characterizing the vertebrate oocyte meiotic maturation, the proteolytic machineries specific of c-Mos and cyclin B are modified, rendering c-Mos degradation sensitive to Cdc2 and cyclin B degradation sensitive to the c-Mos/MAPK pathway.

ACKNOWLEDGMENTS

We thank all members of the laboratory for helpful discussions. We are grateful to Dr. D. Beach for providing the p13 overexpressing *E. coli* strain, Dr. M. Dorée for the anti-Plx1 antibody, Dr. T. Hunt for $p21^{cip1}$ cDNA, and Dr. J. Maller for the anti-cyclin B2, anti-cyclin B1, and anti-Cdc25 antibodies. This research was supported by grants from INRA, CNRS, University Pierre and Marie Curie, and ARC (9473).

REFERENCES

Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J. C., and Doree, M. (1998). The Polo-like kinase Plx1 is a component of the MPF amplification loop at the G2/M-phase transition of the cell cycle in *Xenopus* eggs. *J. Cell Sci.* **111,** 1751–1757.

Anderson, C. W., Baum, P. R., and Gesteland, R. F. (1973). Processing of adenovirus 2-induced proteins. *J. Virol.* **12,** 241–252.

Bhatt, R. R., and Ferrell, J. E., Jr. (1999). The protein kinase p90rsk as an essential mediator of cytostatic factor activity. *Science* **286,** 1362–1365.

Bodart, J. F., Bechard, D., Bertout, M., Gannon, J., Rousseau, A., Vilain, J. P., and Flament, S. (1999). Activation of *Xenopus* eggs by the kinase inhibitor 6-DMAP suggests a differential regulation of cyclin B and p39(mos) proteolysis. *Exp. Cell Res.* **253,** 413–421.

Dunphy, W. G., and Kumagai, A. (1991). The Cdc25 protein contains an intrinsic phosphatase activity. *Cell* **67,** 189–196.

Ferrell, J. E., Wu, M., Gerhart, J. C., and Martin, G. S. (1991). Cell cycle tyrosine phosphorylation of p34cdc2 and a microtubule-associated protein kinase homolog in *Xenopus* oocytes and eggs. *Mol. Cell. Biol.* **11,** 1965–1971.

Ferrell, J. E. (1999). *Xenopus* oocyte maturation: New lessons from a good egg. *Bioessays* **21,** 833–842.

Fisher, D. L., Brassac, T., Galas, S., and Doree, M. (1999). Dissociation of MAP kinase activation and MPF activation in hormone-stimulated maturation of *Xenopus* oocytes. *Development* **126,** 4537–4546.

Frank-Vaillant, M., Haccard, O., Thibier, C., Ozon, R., Arlot-Bonnemains, Y., Prigent, C., and Jessus, C. (2000). Progesterone regulates the accumulation and the activation of Eg2 kinase in *Xenopus* oocytes. *J. Cell Sci.* **113,** 1127–1138.

Frank-Vaillant, M., Jessus, C., Ozon, R., Maller, J. L., and Haccard, O. (1999). Two distinct mechanisms control the accumulation of cyclin B1 and Mos in *Xenopus* oocytes in response to progesterone. *Mol. Biol. Cell* **10,** 3279–3288.

Furuno, N., Nishizawa, M., Okazaki, K., Tanaka, H., Iwashita, J., Nakajo, N., Ogawa, Y., and Sagata, N. (1994). Suppression of DNA replication via Mos function during meiotic divisions in *Xenopus* oocytes. *EMBO J.* **13,** 2399–2410.

Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J. L. (1990). Cyclin is a component of maturation-promoting factor from *Xenopus. Cell* **60,** 487–494.

Gautier, J., Solomon, M. J., Booher, R. N., Bazan, J. F., and Kirschner, M. W. (1991). Cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. *Cell* **67,** 197–211.

Gebauer, F., and Richter, J. D. (1997). Synthesis and function of Mos: The control switch of vertebrate oocyte meiosis. *Bioessays* **19,** 23–28.

Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* **349,** 132–138.

Gotoh, Y., Moriyama, K., Matsuda, S., Okumura, E., Kishimoto, T., Kawasaki, H., Suzuki, K., Yahara, I., Sakai, H., and Nishida, E. (1991). *Xenopus* M-phase MAP kinase: Isolation of its cDNA and activation by MPF. *EMBO J.* **10,** 2661–2668.

Gotoh, Y., Masuyama, N., Dell, K., Shirakabe, K., and Nishida, E. (1995). Initiation of *Xenopus* oocyte maturation by activation of the mitogen-activated protein kinase cascade. *J. Biol. Chem.* **270,** 25898–25904.

Gross, S. D., Schwab, M. S., Lewellyn, A. L., and Maller, J. L. (1999). Induction of metaphase arrest in cleaving *Xenopus* embryos by the protein kinase p90(Rsk). *Science* **286,** 1365–1367.

Gross, S. D., Schwab, M. S., Taieb, F. E., Lewellyn, A. L., Qian, Y. W., and Maller, J. L. (2000). The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by p90(Rsk). *Curr. Biol.* **10,** 430–438.

Haccard, O., Sarcevic, B., Lewellyn, A., Hartley, R., Roy, L., Izumi, T., Erikson, E., and Maller, J. L. (1993). Induction of metaphase arrest in cleaving *Xenopus* embryos by MAP kinase. *Science* **262,** 1262–1265.

Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L. H., Luca, F. C., Ruderman, J. V., and Eytan, E. (1994). Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase Cdc2. *J. Biol. Chem.* **269,** 4940–4946.

Huchon, D., Rime, H., Jessus, C., and Ozon, R. (1993). Control of metaphase I-formation in *Xenopus* oocyte—Effects of an indestructible cyclin B and of protein synthesis. *Biol. Cell* **77,** 133–141.

Ishida, N., Tanaka, K., Tamura, T., Nishizawa, M., Okazaki, K., Sagata, N., and Ichihara, A. (1993). Mos is degraded by the 26S proteasome in a ubiquitin-dependent fashion. *FEBS Lett.* **324,** 345–348.

Izumi, T., Walker, D. H., and Maller, J. L. (1992). Periodic changes in phosphorylation of the *Xenopus* Cdc25 phosphatase regulate its activity. *Mol. Biol. Cell* **3,** 927–939.

Jessus, C., and Ozon, R. (1993). Regulation of cell divisions during oogenesis of vertebrates: The *Xenopus* oocyte paradigm. *Comp. Biochem. Physiol. A* **106,** 431–448.

Jessus, C., Rime, H., Haccard, O., Van, L. J., Goris, J., Merlevede, W., and Ozon, R. (1991). Tyrosine phosphorylation of $p34^{cdc2}$ and p42 during meiotic maturation of *Xenopus* oocyte. Antagonistic action of okadaic acid and 6-DMAP. *Development* **111,** 813–820.

Jessus, C., Thibier, C., and Ozon, R. (1987). Levels of microtubules during the meiotic maturation of *Xenopus* oocytes. *J. Cell Sci.* **87,** 705–712.

Karaiskou, A., Cayla, X., Haccard, O., Jessus, C., and Ozon, R. (1998). MPF amplification in *Xenopus* oocyte extracts depends on a two-step activation of cdc25 phosphatase. *Exp. Cell Res.* **244,** 491–500.

Karaiskou, A., Jessus, C., Brassac, T., and Ozon, R. (1999). Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. *J. Cell Sci.* **112,** 3747–3756.

Karsenti, E., Newport, J., Hubble, R., and Kirschner, M. (1984). Interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into *Xenopus* eggs. *J. Cell Biol.* **98,** 1730–1745.

King, R. W., Peters, J.-M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M. W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* **81,** 279–288.

Kumagai, A., and Dunphy, W. G. (1992). Regulation of the Cdc25 protein during the cell cycle in *Xenopus* extracts. *Cell* **70,** 139–151.

Kumagai, A., and Dunphy, W. G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science* **273,** 1377–1380.

Laemmli, U. K. (1970). Cleavage of structural proteins during theassembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lahav-Baratz, S., Sudakin, V., Ruderman, J. V., and Hershko, A. (1995). Reversible phosphorylation controls the activity of cyclosome-associated cyclin-ubiquitin ligase. *Proc. Natl. Acad. Sci. USA* **92,** 9303–9307.

Lorca, T., Cruzalegui, F. H., Fesquet, D., Cavadore, J. C., Mery, J., Means, A., and Doree, M. (1993). Calmodulin-dependent protein kinase-II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366,** 270–273.

Lorca, T., Galas, S., Fresquet, D., Devault, A., Cavadore, J. C., and Doree, M. (1991). Degradation of the proto-oncogene product $p39^{mos}$ is not necessary for cyclin proteolysis and exit from meiotic metaphase: Requirement for a Ca^{2+}-calmodulin dependent event. *EMBO J.* **10,** 2087–2093.

Masui, Y., and Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177,** 129–146.

Murray, A. W., Solomon, M. J., and Kirschner, M. W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339,** 280–286.

Nebreda, A., Gannon, J., and Hunt, T. (1995). Newly synthesized protein(s) must associate with p34cdc2 to activate MAP kinase

and MPF during progesterone-induced maturation of *Xenopus* oocytes. *EMBO J.* **14,** 5597–5607.

Nebreda, A. R., and Hunt, T. (1993). The c-mos proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of *Xenopus* oocytes and eggs. *EMBO J.* **12,** 1979–1986.

Nishizawa, M., Furuno, N., Okazaki, K., Tanaka, H., Ogawa, Y., and Sagata, N. (1993). Degradation of Mos by the N-terminal proline (Pro2)-dependent ubiquitin pathway on fertilization of *Xenopus* eggs: Possible significance of natural selection for Pro2 in Mos. *EMBO J.* **12,** 4021–4027.

Nishizawa, M., Okazaki, K., Furuno, N., Watanabe, N., and Sagata, N. (1992). The "second-codon rule" and autophosphorylation govern the stability and activity of Mos during the meiotic cell cycle in *Xenopus* oocytes. *EMBO J.* **11,** 2433–2446.

Posada, J., Yew, N., Ahn, N. G., Vande Woude, G. F., and Cooper, J. A. (1993). Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase kinase in vitro. *Mol. Cell Biol.* **13,** 2546–2553.

Qian, Y. W., Erikson, E., Li, C., and Maller, J. L. (1998). Activated polo-like kinase Plx1 is required at multiple points during mitosis in *Xenopus laevis*. *Mol. Cell. Biol.* **18,** 4262–4271.

Sagata, N. (1997). What does Mos do in oocytes and somatic cells? *Bioessays* **19,** 13–21.

Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J., and Vande Woude, G. F. (1988). Function of c-mos proto-oncogene product in meiotic maturation in *Xenopus* oocytes. *Nature* **335,** 519–525.

Sagata, N., Watanabe, N., Vande Woude, G. F., and Ikawa, Y. (1989). The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342,** 512–518.

Sudakin, V., Ganoth, D., Dahan, A., Heller, H., Hershko, J., Luca, F. C., Ruderman, J. V., and Hershko, A. (1995). The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclin for destruction at the end of mitosis. *Mol. Biol. Cell* **6,** 185–198.

Taieb, F., and Jessus, C. (1997). On cyclins, oocytes and eggs. *Mol. Reprod. Dev.* **48,** 397–411.

Thibier, C., De Smedt, V., Poulhe, R., Huchon, D., Jessus, C., and Ozon, R. (1997). In vivo regulation of cytostatic activity in *Xenopus* metaphase II-arrested oocytes. *Dev. Biol.* **185,** 55–66.

Watanabe, N., Hunt, T., Ikawa, Y., and Sagata, N. (1991). Independent inactivation of MPF and cytostatic factor (Mos) upon fertilization of *Xenopus* eggs. *Nature* **352,** 247–248.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* **366,** 701–704.

Received for publication July 28, 2000
Accepted December 13, 2000
Published online January 30, 2001