Two Distinct Mechanisms Control the Accumulation of Cyclin B1 and Mos in *Xenopus* Oocytes in Response to Progesterone

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Progesterone-induced meiotic maturation of *Xenopus* oocytes requires the synthesis of new proteins, such as Mos and cyclin B. Synthesis of Mos is thought to be necessary and sufficient for meiotic maturation; however, it has recently been proposed that newly synthesized proteins binding to p34^{cdc2} could be involved in a signaling pathway that triggers the activation of maturation-promoting factor. We focused our attention on cyclin B proteins because they are synthesized in response to progesterone, they bind to p34^{cdc2}, and their microinjection into resting oocytes induces meiotic maturation. We investigated cyclin B accumulation in response to progesterone in the absence of maturation-promoting factor-induced feedback. We report here that the cdk inhibitor p21^{cip1}, when microinjected into immature *Xenopus* oocytes, blocks germinal vesicle breakdown induced by progesterone, by maturation-promoting factor transfer, or by injection of okadaic acid. After microinjection of p21^{cip1}, progesterone fails to induce the activation of MAPK or p34^{cdc2}, and Mos does not accumulate. In contrast, the level of cyclin B1 increases normally in a manner dependent on down-regulation of cAMP-dependent protein kinase but independent of cap-ribose methylation of mRNA.

INTRODUCTION

Xenopus fully grown oocytes are naturally arrested in prophase of the first meiotic division (prophase I). In response to progesterone, they undergo meiotic maturation and then arrest a second time in metaphase of the second meiotic division (metaphase II) until fertilization. The steroid hormone progesterone induces with a lag of 3–5 h activation of maturation-promoting factor (MPF), breakdown of the germinal vesicle (GVBD), and entry into M-phase of the first meiotic division (see Masui and Clarke, 1979, for review). MPF is a protein kinase composed of a catalytic subunit, p34^{cdc2} (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Draetta *et al.*, 1989; Meijer *et al.*, 1989), and a regulatory subunit, cyclin B (Gautier *et al.*, 1990), that accumulates during terminal oocyte growth to form a complex called

preMPF (Wasserman and Masui, 1975; Gerhart *et al.*, 1984; Gautier and Maller, 1991). PreMPF is kept inactive by inhibitory phosphorylations on threonine 14 and tyrosine 15 of the p34^{cdc2} subunit. The conversion of preMPF into active MPF occurs several hours after progesterone stimulation, just before GVBD; it depends on activation of the protein phosphatase Cdc25, which catalyzes the dephosphorylation of threonine 14 and tyrosine 15 of p34^{cdc2}. At about the same time, i.e., around GVBD, MAPK becomes fully activated (Gotoh *et al.*, 1991; Matsuda *et al.*, 1992; Roy *et al.*, 1996). Little is known about the transduction pathway that connects the initial effects of progesterone to activation of preMPF.

It is well established that progesterone-induced maturation depends on the synthesis of new proteins and is inhibited by the cAMP pathway (Maller and Krebs, 1977). The protein kinase Mos is the only newly synthesized protein that has been shown to be necessary for progesterone-induced maturation (Sagata *et al.*, 1988). Mos is a MAP kinase kinase that leads to the activation of MAPK through the activation of MAP kinase kinase (also called MEK) (Nebreda *et al.*, 1993; Posada *et al.*, 1993; Shibuya and Ruderman, 1993); therefore, it is generally proposed that in ovo the translation of stored *mos* mRNA induces MAPK activation

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Abbreviations used: GVBD, germinal vesicle breakdown; MPF, maturation-promoting factor; OA, okadaic acid; PKA, cAMP-dependent protein kinase; PKAc, catalytic subunit of cAMP-dependent protein kinase; PKI, thermostable inhibitor of the catalytic subunit of cAMP-dependent protein kinase; SIBA, S-isobutylthioadenosine.

in maturing oocytes. In the absence of p34^{cdc2} activity, Mos is synthesized but does not attain normal levels (Nebreda *et al.*, 1995). This raises the question of whether the accumulation of Mos is stimulated by p34^{cdc2} activity. In theory, the accumulation of a protein absolutely required for preMPF activation must depend on progesterone stimulation and be independent of p34^{cdc2} activity. It is important, therefore, to distinguish the newly made proteins that accumulate during the lag period before p34^{cdc2} activation from those whose accumulation is stimulated downstream of p34^{cdc2} activation.

We report here a direct approach for identifying proteins that accumulate independently of p34^{cdc2} activity after progesterone stimulation. For this purpose, we microinjected into prophase oocytes recombinant p21^{cip1}, a well-known inhibitor of cdk/cyclin complexes (Xiong *et al.*, 1993). We show that microinjected p21^{cip1} binds endogenous p34^{cdc2}/cyclin complexes and prevents their activation in ovo. Therefore, we used p21^{cip1} as a tool to study the accumulation of proteins such as cyclin B1, cyclin B2, and Mos under conditions in which progesterone-induced MPF activation and GVBD were abolished.

MATERIALS AND METHODS

Materials

Xenopus laevis adult females (Centre National de la Recherche Scientifique, Rennes, France) were bred and maintained under laboratory conditions. Reagents, unless otherwise specified, were from Sigma (Saint Quentin Fallavier, France).

Purification of Recombinant Proteins

Cip1 cloned into the *NcoI* site of pGEX-KG (a kind gift of Dr. Tim Hunt) was transfected into TG1 cells. Expression of the GST-p21^{cip1} fusion protein was induced by 0.1 mM isopropyl- β -D-thiogalactopyranoside for 3 h at room temperature. After centrifugation at 10,000 $\times$ g for 15 min at 4°C, the pellets were frozen. Bacteria were lysed in 50 mM Tris-HCl, pH 7.3, 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM benzamidine, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pentapharm, Basel, Switzerland), 1 mg/ml lysozyme (Boehringer Mannheim, Indianapolis, IN) by sonication in the presence of detergents (0.5% Triton, 0.5% NP40). After ultracentrifugation (100,000 $\times$ g, 4°C, 1 h), the supernatant containing recombinant p21^{cip1} was loaded onto an equilibrated glutathione-agarose column, washed, and eluted with 20 mM Tris-HCl, pH 9, 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM glutathione. The purified proteins were concentrated with a Microcon-50 (Millipore, Saint Quentin en Yvelines, France) to a final concentration of 0.75 mg/ml. MBP-Mos was purified as described by Roy *et al.* (1996).

Synthesis of [³⁵S]Methionine-labeled Cyclin B1

Twenty micrograms of circular DNA containing the sequence coding for *Xenopus* cyclin B1 were incubated for 3 h at 30°C in 200 μ l of TNT-coupled reticulocyte lysate system containing T7 polymerase (Promega, Charbonnières, France) in the presence of 400 μ Ci of [³⁵S]methionine (New England Nuclear, Boston, MA). The newly synthesized protein was concentrated and washed on Microcon-10 (Millipore) before injection into oocytes.

Oocyte Treatment

Isolated oocytes were prepared and maintained as described (Jessus *et al.*, 1987). Fully grown oocytes, referred to as "prophase oocytes," were injected or not with p21^{cip1} to an internal concentration of 1

μ M. One hour after microinjection, oocytes were induced to mature either by the addition of 1 μ M progesterone in the medium, by MPF transfer, by microinjection of 50 nl of 10⁻⁵ M okadaic acid (OA) (ICN, Orsay, France), by microinjection of the recombinant thermostable inhibitor of the catalytic subunit of cAMP-dependent protein kinase (PKI) from rabbit muscle (8 pmol/oocyte), or by microinjection of recombinant MBP-Mos (50 μ g/oocyte).

In other experiments, maturation was inhibited by microinjection of 10 ng of the catalytic subunit of PKA (PKAc) per oocyte (Promega) 1 h before the addition of progesterone, by preincubation in the presence of 0.75 mM 5-isobutylthioadenosine (SIBA) for 2 h, or by preincubation in the presence of 100 μ g/ml cycloheximide for 1 h.

Maturation was monitored by the appearance of a white spot at the animal pole of the oocyte. Oocytes were collected when 100% GVBD was reached in control oocytes, or at metaphase II (2 h after 100% GVBD in progesterone controls), or at the indicated times. Oocytes matured in vitro (metaphase II) were injected or not with p21^{cip1} to an internal concentration of 1 μ M in the presence of 100 mM EGTA. Oocytes were homogenized at 4°C in 5 volumes of extraction buffer (80 mM β -glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 1 mM DTT, pH 7.3, 25 μ g/ml leupeptin and aprotinin, 10 μ g/ml pepstatin, 1 mM benzamidine, 1 μ M 4-(2-aminoethyl)-benzenesulfonyl fluoride [Pentapharm]) and centrifuged at 7000 rpm for 15 min at 4°C. Clear supernatant was used for Western blot analysis or for recovery of proteins on glutathione-agarose beads.

Histone H1 Kinase Assays

The supernatant (15 μ l, i.e., three oocytes) was collected for p13^{suc1} binding followed by histone H1 kinase assays in the presence of [γ -³²P]ATP (New England Nuclear) according to Jessus *et al.* (1991).

Immunoblotting

Western blotting was performed with anti-Mos, anti-MAPK (Santa Cruz Biotech, Santa Cruz, CA), anti-p34^{cdc2} (a kind gift from Dr. Tim Hunt, Imperial Research Fond, London, United Kingdom), anti-cyclin B1, anti-cyclin B2, or anti-cyclin A antibodies. The antisera raised in sheep against *Xenopus* cyclins A, B1, and B2 have been described by Gautier *et al.* (1990); antisera to cyclins B1 and B2 were blot purified using recombinant cyclin B1 and B2 as described (Olmsted, 1981; Rempel *et al.*, 1995). Proteins were subjected to electrophoresis in Laemmli buffer (Laemmli, 1970) on a 12.5% SDS-PAGE Anderson gel (Anderson *et al.*, 1973) or on a 15% SDS-PAGE Laemmli gel (Laemmli, 1970) and then transferred to nitrocellulose filters (Schleicher & Schull, Ecqueville, France). The proteins of interest were visualized by use of the appropriate primary antibody, HRP-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA), and renaissance chemoluminescence reagent (New England Nuclear). We observed that the amount of cyclin B1 detected in prophase-arrested oocytes varied from female to female. Cyclin B1 was resolved as two bands on 12.5% Anderson gels and as one band on 15% Laemmli gels (see Figure 6, A and B).

Binding of GST-p21^{cip1} to Glutathione-Agarose Beads

Fifteen milligrams of glutathione-agarose beads saturated with extraction buffer containing 10% BSA was added to a supernatant prepared from 30 oocytes. After incubation for 2 h, the beads were washed three times with 20 mM Tris, 5 mM EDTA, 1% Triton X-100 containing 100 mM NaCl, then 1 M NaCl, and finally 100 mM NaCl. Proteins bound to the beads were eluted with SDS-sample buffer, subjected to electrophoresis, and analyzed by Western blotting.

Autoradiography of [³⁵S]Methionine-labeled Cyclin B1

Oocytes injected with [³⁵S]methionine-labeled cyclin B1 were collected at different times, homogenized, and analyzed by autoradiog-

Figure 1. Effect of p21^{cip1} microinjection on meiotic maturation and preMPF activation in *Xenopus* oocytes. Thirty prophase oocytes were microinjected or not with 50 nl of p21^{cip1} at various concentrations (50 nl of the 0.75 mg/ml solution results in an internal concentration of p21^{cip1} of $\sim 1 \mu\text{M}$). One hour after microinjection, oocytes were treated with 10^{-6} M progesterone (Pg), cultured, and scored for GVBD (A). Histone H1 kinase activity was measured 2 h after GVBD in control and progesterone-treated oocytes injected or not with GST-Cip1, as indicated (B).

raphy on a 12.5% SDS-PAGE Anderson gel (Anderson *et al.*, 1973). An amount equivalent to four oocytes was loaded on each lane.

RESULTS

p21^{cip1} Inhibits Progesterone-induced Maturation

Human p21^{cip1} is an inhibitor of all cdk/cyclin complexes (Xiong *et al.*, 1993). To determine whether p21^{cip1} could inhibit oocyte maturation, recombinant p21^{cip1} prepared as a GST fusion protein (GST-p21^{cip1}) was microinjected into fully grown prophase oocytes. At a final intracellular concentration of $1 \mu\text{M}$ (0.75 mg/ml in the pipette), p21^{cip1} totally inhibited progesterone-induced GVBD (Figure 1A). This effect was obtained with oocytes isolated from more than 30 different females and was dose dependent, with a 50% inhibitory concentration of $\sim 0.4 \mu\text{M}$. The activity of p34^{cdc2} was assayed by measuring histone H1 kinase activity in progesterone-treated control and p21^{cip1}-injected oocytes (Figure 1B). Histone H1 kinase activity was high 2 h after GVBD in control oocytes. In contrast, in p21^{cip1}-injected oocytes, preMPF activation did not occur and activity remained at the basal level, comparable to that in prophase control oocytes. Histone H1 kinase activation was blocked in p21^{cip1}-injected oocytes for as long as 24 h in the continuous presence of progesterone.

p21^{cip1} Interacts with p34^{cdc2}/Cyclin Complexes and Inhibits MPF Autoamplification but Not the Mos-induced MAPK Pathway

We next examined whether injected p21^{cip1} inhibits p34^{cdc2} activation and GVBD by directly binding to endogenous p34^{cdc2}/cyclin complexes. p21^{cip1} was microinjected into

prophase oocytes; 1 h later, oocytes were collected, homogenized, and centrifuged, and the supernatant was incubated in the presence of glutathione-agarose beads. Proteins bound to the beads were then analyzed by immunoblotting with various antibodies. Microinjected p21^{cip1} was recovered in association with the glutathione beads. p34^{cdc2}, cyclin B1, and cyclin B2 were also bound to the beads (Figure 2, A–C). A similar experiment was also performed with oocytes matured in vitro and arrested in metaphase II. As in prophase oocytes, p34^{cdc2}/cyclin B2 and p34^{cdc2}/cyclin B1 were recovered on the beads (Figure 2, A–C); as expected, cyclin A, which is absent in prophase oocytes and is synthesized de novo during maturation, was also bound to the beads (Figure 2D). These results indicate that p21^{cip1} acts in ovo by direct binding to active or inactive p34^{cdc2}/cyclin complexes.

We next verified whether p21^{cip1} could inhibit the MAPK pathway. When recombinant MBP-Mos was injected into p21^{cip1}-treated oocytes, activation of MPF and GVBD were blocked. However, the electrophoretic mobility shift of MAPK was still observed (Figure 2E). Moreover, it has been shown that the activity of oocyte MAPK measured by an in-gel assay was not affected by the presence of p21^{cip1} (Karaiskou *et al.*, 1998). These results show that p21^{cip1} does not inhibit components of the MAPK pathway.

If p21^{cip1} acts by direct binding to MPF, then it should also inhibit meiotic maturation induced by MPF transfer because it also requires activation of preMPF. When a small amount of cytoplasm taken from matured oocytes is microinjected into prophase oocytes, p34^{cdc2} activation and GVBD occur less than 3 h later (Masui and Markert, 1971). When oocytes were first microinjected with p21^{cip1} and then 1 h later with 50 nl of cytoplasm taken from a matured oocyte, GVBD did

Figure 2. p21^{cip1} interacts with p34^{cdc2}/cyclin complexes and does not inhibit the MAPK pathway. (A–D) Recovery of complexes bound to p21^{cip1}. Thirty prophase (P) or metaphase II (MII) oocytes were microinjected or not with p21^{cip1} to an internal concentration of 1 μ M and cultured for 1 h. After homogenization of oocytes, glutathione-agarose beads were added to the clear supernatant. Beads were washed, and bound proteins were electrophoresed and subjected to Western blot analysis for p34^{cdc2} (A), cyclin B1 (B), cyclin B2 (C), and cyclin A (D). (E) Effect of p21^{cip1} on the MAPK electrophoretic mobility shift induced by injection of MBP-Mos. Twenty prophase oocytes were microinjected or not with p21^{cip1} (1 μ M). One hour later, they were microinjected with 50 μ g of recombinant MBP-Mos or treated with progesterone (Pg). Oocytes were collected at the time of 100% GVBD in MBP-Mos-injected oocytes. An amount equivalent to three oocytes was subjected to Western blot analysis for MAPK.

not occur, H1 kinase activity was not increased, and the electrophoretic mobility shift of cyclin B2 was blocked (Figure 3, A and B).

OA is an inhibitor of types 1 and 2A Ser/Thr protein phosphatases (Bialojan and Takai, 1988). Microinjection of 50 nl of 10^{-5} M OA into prophase oocytes induces rapid p34^{cdc2} activation (Goris *et al.*, 1989) and a subsequent cytologically abnormal GVBD (Rime *et al.*, 1990). It has been proposed that microinjected OA, by promoting phosphorylation/activation of Cdc25, initiates the positive feedback loop that controls activation of p34^{cdc2} in ova (Izumi and Maller, 1993). When OA was microinjected into p21^{cip1}-injected oocytes, p34^{cdc2} activation and the electrophoretic mobility shift of cyclin B2 were totally blocked for at least 8 h (Figure 3, C and D).

Figure 3. Effect of p21^{cip1} on MPF autoamplification. Twenty prophase oocytes were microinjected or not with p21^{cip1} (1 μ M). One hour later, they were microinjected with either 50 nl of cytoplasm taken from metaphase II oocytes containing active MPF (A and B) or 50 nl of 10^{-5} M OA (C and D). All control oocytes underwent GVBD, whereas none of the p21^{cip1}-injected oocytes underwent GVBD. Oocytes were collected at the time when 100% GVBD was reached in the control oocytes (3.5 h after MPF transfer or 6 h after OA injection). MPF activity was measured by assaying histone H1 kinase activity (A and C), and an amount equivalent to three oocytes was subjected to Western blot analysis for cyclin B2 (B and D). Pg, progesterone.

Progesterone Induces Accumulation of Cyclin B1 in p21^{cip1}-microinjected Oocytes

As shown above, microinjection of recombinant p21^{cip1} inhibited preMPF activation induced by progesterone. The electrophoretic mobility shift of cyclin B2, which normally correlates with p34^{cdc2} activation (Gautier *et al.*, 1990; Roy *et al.*, 1996), was also totally blocked (Figure 4A). Although the synthesis of Mos can be detected before the conversion of preMPF to active MPF (Sagata *et al.*, 1989; Nebreda *et al.*, 1995), accumulation of Mos does not become detectable before GVBD (Roy *et al.*, 1996). Surprisingly, in p21^{cip1}-injected oocytes, the level of Mos did not increase after progesterone stimulation (Figure 4B); as expected from the absence of Mos under these conditions, the electrophoretic mobility of MAPK was not retarded (Figure 4C). Therefore, the progesterone-dependent accumulation of Mos does not occur in the absence of p34^{cdc2} activity. Unexpectedly, however, in p21^{cip1}-inhibited oocytes, cyclin B1 accumulated normally after progesterone treatment (Figure 4D). The time course of the accumulation of cyclin B1 in control progesterone-treated oocytes and in p21^{cip1}-injected progesterone-treated oocytes was similar (Figure 5). Interestingly, in both control (Kobayashi *et al.*, 1991) and p21^{cip1}-inhibited oocytes, the accumulation of cyclin B1 after progesterone stimulation takes place after a lag period of several hours and is thus not

Figure 4. Effect of p21^{cip1} microinjection on the electrophoretic mobility of cyclin B2 (A), accumulation of Mos (B), electrophoretic mobility of MAPK (C), and accumulation of cyclin B1 (D). Twenty prophase oocytes were microinjected or not with p21^{cip1} to an internal concentration of 1 μ M, and progesterone (Pg) was added 1 h later. Oocytes were collected 2 h after 100% GVBD was reached in progesterone control oocytes and homogenized for Western blot analysis with the indicated antibodies. An amount equivalent to three oocytes was loaded on each lane.

an early effect of the hormone. These results show that accumulation of cyclin B1 is regulated differently from that of Mos in response to progesterone. In particular, the amount of cyclin B1 is up-regulated by progesterone independently of p34^{cdc2} and MAPK activities.

It has been reported that SIBA, a methyltransferase inhibitor, prevents mRNA cap-ribose methylation, Mos synthesis, and oocyte maturation (Kuge *et al.*, 1998). Therefore, we examined the effect of SIBA on the accumulation of cyclin B1. Oocytes were preincubated or not in the presence of 750 μ M SIBA for 2 h, p21^{cip1} was then microinjected, and 1 h later progesterone was added or not in the continuous presence of SIBA. As expected, meiotic maturation induced by progesterone was totally prevented by SIBA. As reported by

Figure 6. Effect of SIBA on the accumulation of cyclin B1. Thirty prophase oocytes, microinjected or not with p21^{cip1}, were cultured with or without of 0.75 mM SIBA for 2 h. Then, progesterone (Pg) was added or not and oocytes were cultured overnight. Oocytes were collected, homogenized, and analyzed by immunoblotting for Mos (A) or cyclin B1 (B). An amount equivalent to three oocytes was loaded on each lane.

Kuge *et al.* (1998), accumulation of Mos was blocked (Figure 6A); however, accumulation of cyclin B1 was still observed (Figure 6B). This result shows that the increased level of cyclin B1 does not depend on new cap-ribose methylation of mRNAs, not even that of *mos* mRNA.

Progesterone Does Not Change the Stability of Microinjected Cyclin B1

We then addressed the question of whether cyclin B1 accumulation was the consequence of either the increased rate of synthesis and/or a decrease in the rate of degradation. Oocytes were first incubated in the presence of cycloheximide, a strong inhibitor of protein synthesis. They were then injected or not with p21^{cip1}, and 1 h later they were incubated in the presence of progesterone. As expected, cyclin B1 did not accumulate (Figure 7A). This finding shows that cyclin B1 accumulation is a consequence of a new synthesis and does not result from decreased degradation.

To estimate the in ovo stability of cyclin B1, [³⁵S]methionine-labeled cyclin B1 was injected into prophase oocytes in the presence or in the absence of p21^{cip1}, stimulated or not with progesterone. We injected trace amounts of radioactive cyclin B1 that were not able to induce meiotic maturation. In prophase oocytes, ectopic cyclin B1 remained stable from more than 20 h (Figure 7B, upper left panel). When oocytes were first microinjected with radioactive cyclin B1 and then

Figure 5. Time course of the accumulation of cyclin B1. Two hundred prophase oocytes were microinjected (bottom) or not (top) with p21^{cip1} to an internal concentration of 1 μ M. One hour later, they were incubated in the presence of progesterone. Groups of 10 oocytes were collected at the indicated times after progesterone addition and homogenized for Western blot analysis with the anti-cyclin B1 antibody. An amount equivalent to three oocytes was loaded on each lane.

Figure 7. Synthesis and degradation of cyclin B1. (A) Twenty prophase oocytes were microinjected or not with p21^{cip1} (1 μ M) and incubated or not in the presence of cycloheximide (CHX) at 100 μ g/ml for 1 h before progesterone (Pg) addition. Oocytes were cultured overnight, collected, homogenized, and analyzed by Western blotting for cyclin B1. An amount equivalent to two oocytes was loaded on each lane. (B) Stability of microinjected cyclin B1. Seventy oocytes were microinjected or not with p21^{cip1} (1 μ M), microinjected with [³⁵S]methionine-labeled cyclin B1, and incubated in the presence or absence of progesterone. Ten oocytes of each group were collected at different times, homogenized, and analyzed by autoradiography. An amount equivalent to four oocytes was loaded on each lane. (C) Extracts from B (lower left panel) were analyzed by Western blotting for cyclin B1. An amount equivalent to two oocytes was loaded on each lane.

treated with progesterone, cyclin B1 was stable until GVBD, after which it was abruptly degraded (Figure 7B, upper right panel). This result demonstrates that the ectopic protein is the target of the anaphase-promoting complex (APC), which is activated at the metaphase/anaphase transition in meiosis I and promotes the degradation of cyclins (Furuno *et al.*, 1994; Roy *et al.*, 1996; Thibier *et al.*, 1997). Radioactive microinjected cyclin B1 was as stable in p21^{cip1}-injected oocytes whether progesterone was added or not (Figure 7B, lower panels). Although exogenous radioactive cyclin B1 levels remained stable in oocytes treated with progesterone in the presence of p21^{cip1}, it was ascertained by Western blotting that under these conditions endogenous cyclin B1 accumulated after progesterone stimulation (Figure 7C). These results suggest that cyclin B1 accumulation induced by progesterone does not involve a change in the stability of cyclin B1.

PKA Inhibits the Accumulation of Cyclin B1

The decrease in cAMP concentration and the subsequent inactivation of PKA are the first early events known to be induced by progesterone. To evaluate whether cyclin B1 accumulation is regulated by PKA activity, we microinjected the thermostable inhibitor of the catalytic subunit of PKA (PKI) into oocytes. Under these conditions, PKI induces

100% of the oocytes to undergo maturation (Maller and Krebs, 1977; Huchon *et al.*, 1981). However, coinjection of p21^{cip1} blocked the ability of PKI to induce maturation. It also blocked the accumulation of Mos induced by PKI, but the increase in cyclin B1 was not affected (Figure 8A). The inhibition of PKA by progesterone treatment, therefore, is sufficient to promote the accumulation of cyclin B1. To determine whether this inhibition is also necessary, 10 ng of PKAc was microinjected per oocyte. Under these conditions, PKAc inhibited progesterone-induced maturation, and the accumulation of both Mos and cyclin B1 was prevented (Figure 8B). These results clearly show that the accumulation of cyclin B1 requires a decrease in the activity of PKA.

MPF Transfer or OA Induces Accumulation of Cyclin B1

Because MPF transfer is known to induce all the events of meiotic maturation (Masui and Markert, 1971), it was interesting to investigate the effects of MPF transfer on cyclin B1 accumulation. As shown in Figure 9A, when cytoplasm taken from matured oocytes was microinjected into prophase oocytes, cyclin B1 accumulated in the presence or in the absence of p21^{cip1}. In contrast, in the presence of p21^{cip1} Mos did not accumulate and MAPK remained in its inactive, unshifted form (Figure 9, B and C). Similar results were obtained when OA was microinjected into p21^{cip1}-

Figure 8. Accumulation of cyclin B1 is down-regulated by PKA. (A) PKI microinjection. Thirty prophase oocytes were microinjected or not with p21^{cip1} to an internal concentration of 1 μ M. One hour later, they were microinjected with 8 pmol of PKI per oocyte. Control oocytes were treated with progesterone (Pg). Oocytes were cultured overnight, collected, homogenized, and analyzed by Western blotting for Mos (top) or cyclin B1 (bottom). An amount equivalent to two oocytes was loaded on each lane. (In this experiment, cyclin B1 was resolved as a single band on a 15% Laemmli gel.) (B) PKAc microinjection. Thirty prophase oocytes were microinjected or not with p21^{cip1} to an internal concentration of 1 μ M and/or with 10 ng of PKAc per oocyte. One hour later, progesterone was added or not and oocytes were cultured for 5.5 h (50% GVBD occurred at 4 h). Oocytes were collected, homogenized, and analyzed by Western blotting for Mos (top) or cyclin B1 (bottom). An amount equivalent to two oocytes was loaded on each lane. (In this experiment, cyclin B1 was resolved as a single band on a 15% Laemmli gel.)

treated oocytes (Figure 9, D–F). This argues that a phosphorylation step might be involved in the pathway leading to the accumulation of cyclin B1.

DISCUSSION

In this study, we developed a new experimental approach to the study of proteins whose abundance is regulated by progesterone during the lag period preceding p34^{cdc2} activation in *Xenopus* oocytes. This approach used the cdk inhibitor p21^{cip1} to inhibit the activation of preMPF and facilitated the study of the early effects of progesterone. The results show that the level of cyclin B1 is directly regulated by initial progesterone stimulation and does not depend on increased p34^{cdc2} activity. In contrast, the progesterone-dependent increase of Mos protein was totally inhibited in p21^{cip1}-treated oocytes, indicating that in vivo the activity of p34^{cdc2} is absolutely required to promote Mos accumulation. Together, these results indicate that regulation of the levels of Mos and cyclin B1 is mediated by two different biochemical pathways.

Figure 9. Cyclin B1 and Mos accumulation after MPF transfer or OA microinjection in the presence of p21^{cip1}. Twenty prophase oocytes were microinjected or not with p21^{cip1} (1 μ M). One hour later, they were microinjected with either 50 nl of cytoplasm taken from metaphase II oocytes (A–C) or 50 nl of 10⁻⁵ M OA (D–F). Oocytes were collected at the time when 100% GVBD was reached in the control oocytes. An amount equivalent to three oocytes was subjected to Western blot analysis for cyclin B1 (A and D), Mos (B and E), and MAPK (C and F). Pg, progesterone.

p21^{cip1} binds to and inhibits in vitro and in vivo a number of cdk/cyclin complexes (Gu *et al.*, 1993; Xiong *et al.*, 1993; Su *et al.*, 1995). Recently, it was reported that microinjection of p21^{cip1} into *Xenopus* oocytes at a final intracellular concentration of 0.1 μ M does not inhibit progesterone-induced maturation but totally inhibits the activation of cdk2 that normally occurs after GVBD (Furuno *et al.*, 1997). Because in vitro the affinity of p21^{cip1} for cdk2/cyclin complexes is higher than its affinity for p34^{cdc2}/cyclin complexes (Su *et al.*, 1995), we microinjected a higher concentration of p21^{cip1} (1 μ M) to inhibit in ovo the activation of p34^{cdc2}/cyclin B complexes. We found that at this concentration p21^{cip1} binds to endogenous p34^{cdc2}/cyclin B complexes and totally inhibits GVBD and activation of histone H1 kinase. Therefore, under our Westerns, p21^{cip1} acts by binding and inhibiting

Figure 10. Model. Cyclin B1 accumulates in response to the decrease of PKA activity induced by progesterone and independently of MPF. Although Mos synthesis has been reported to occur upstream of MPF activation, progesterone does not lead to Mos accumulation unless MPF has been activated.

p34^{cdc2}. This conclusion is strengthened by the observation that p21^{cip1} also inhibits GVBD after MPF transfer or OA microinjection, i.e., experimental conditions that bypass the early effects of progesterone.

It has been shown that the activity of MAPK depends on a critical threshold of Mos protein (Chen and Cooper, 1997; Ferrell and Bhatt, 1997). The accumulation of Mos leading to this threshold level could result from regulation of the synthesis and/or turnover of the protein. Nebreda *et al.* (1995) have shown that overexpression of a kinase-inactive p34^{cdc2} (K33R) or microinjection of the A17 anti-p34^{cdc2} antibody inhibits progesterone-induced GVBD, activation of p34^{cdc2}, accumulation of Mos, and the MAPK cascade. Interestingly, in the presence of the A17 anti-p34^{cdc2} antibody, progesterone stimulates the synthesis of ³⁵S-labeled Mos, as detected by immunoprecipitation, but its accumulation is not detectable by immunoblotting (Nebreda *et al.*, 1995). A main finding presented here is that Mos accumulation also does not occur when activation of p34^{cdc2} is blocked by p21^{cip1}, arguing that the Mos/MAP kinase kinase/MAPK pathway is under the control of p34^{cdc2} activity (Figure 10). Although not studied directly in this paper, the accumulation of Mos may depend on a change in stability. Indeed, Nishizawa *et al.* (1993) reported that phosphorylation at a proline-directed site near the N terminus might regulate stability.

In contrast to the p34^{cdc2}-dependent accumulation of Mos and activation of the MAPK pathway, we show in this report that the accumulation of cyclin B1 induced by progesterone is independent of p34^{cdc2} activity. It is also independent of the activation of the MAPK pathway because both Mos accumulation and MAPK activation were blocked by p21^{cip1}. This was confirmed by the injection of *mos*-specific antisense oligonucleotides into prophase oocytes in the presence of progesterone. Under these conditions and as previously shown (Sagata *et al.*, 1988; Roy *et al.*, 1996), p34^{cdc2} activation, GVBD, and MAPK activation normally induced by progesterone were inhibited, but the accumulation of cyclin B1 was still observed (our unpublished results). Taken together, these results show that cyclin B1 accumulation occurs in response to progesterone independently of p34^{cdc2} activity (Figure 10).

The discrepancy between Mos and cyclin B1 protein accumulation is also reflected at the mRNA level. Barkoff *et al.* (1998) reported that polyadenylation of *mos* mRNA is not sufficient for accumulation of the protein in the absence of

progesterone. Moreover, when meiotic maturation is inhibited by the presence of kinase-inactive p34^{cdc2} (K33R), *mos* mRNA is still polyadenylated in response to progesterone (Ballantyne *et al.*, 1997) but the protein does not accumulate (Nebreda *et al.*, 1995). Conversely, under the same conditions, cyclin B1 mRNA does not undergo additional polyadenylation (Ballantyne *et al.*, 1997) but the protein does accumulate (Nebreda *et al.*, 1995). Furthermore, it has been shown that inhibition of cap-ribose methylation by SIBA prevents *mos* mRNA translation (Kuge *et al.*, 1998); in contrast, our results demonstrate that, unlike Mos, cyclin B1 accumulates normally in the presence of SIBA. Thus, polyadenylation and cap-ribose methylation appear to be necessary but not sufficient for the accumulation of Mos, whereas the level of cyclin B1 can increase in the absence of polyadenylation or cap-ribose methylation of mRNA. Accumulation of cyclin B1 occurs over several hours. Our results with injected cyclin B1 suggest that the accumulation of cyclin B1 induced by progesterone in the presence of p21^{cip1} does not involve a decreased rate of degradation. However, we cannot rigorously exclude the possibility that a slight change in the balance between synthesis and degradation over several hours could contribute to the accumulation of cyclin B1 induced by progesterone.

One biochemical change known to control *Xenopus* oocyte maturation is a decrease in the activity of PKA. Maller and Krebs (1977) showed that PKAc inhibits progesterone-induced maturation, whereas PKI and the regulatory subunit of PKA induce meiotic maturation directly in the absence of progesterone. A decrease in cAMP concentration after progesterone addition correlates with the inhibition of PKA and is the first early event known to occur upstream of Mos synthesis and p34^{cdc2} activation (Speaker and Butcher, 1977; Maller *et al.*, 1979). The results in this paper provide evidence that the accumulation of cyclin B1 is another response of the oocyte to progesterone that is independent of Mos synthesis and p34^{cdc2} activation. We also analyzed directly the effects of an increase or decrease in PKA activity on cyclin B1 accumulation. Our results demonstrate that inhibition of PKA is sufficient to allow cyclin B1 accumulation when p34^{cdc2} activation is inhibited by p21^{cip1}. The reciprocal experiment shows that injection of PKAc prevents the accumulation of cyclin B1 induced by progesterone. The hormone, therefore, triggers cyclin B1 accumulation by acting through a decrease in PKA activity (Figure 10). This

again differentiates cyclin B1 from Mos in terms of regulation, because it was recently shown that PKA does not exert any inhibitory effect on Mos translation induced by exogenous Mos injection (Faure *et al.*, 1998). However, GVBD does not occur when exogenous Mos is injected in the presence of PKAc (Daar *et al.*, 1993). These results favor the view that accumulation of cyclin B1 may be required for Mos to trigger meiotic maturation.

What could be the functional role of cyclin B1 accumulation? It is well established that microinjection of cyclin B protein induces GVBD in the absence of protein synthesis (Roy *et al.*, 1991). Our findings demonstrate that cyclin B1 accumulates in response to progesterone in the absence of MPF activation. Furthermore, a recent report by de Moor and Richter (1999) shows that the stimulation of endogenous cyclin B1 translation is sufficient to induce meiotic maturation.

Together, these experimental data suggest that cyclin B1 accumulation could be a physiological trigger of preMPF activation in oocytes. This pathway is indeed the shortest link between progesterone and preMPF activation (Figure 10).

Recently, a new cdk2-interacting protein encoded by the *spy1* gene was cloned in *Xenopus* (Lenormand *et al.*, 1999). The *spy1* mRNA is capable of inducing MAPK and MPF activation as well as triggering meiotic maturation when injected into prophase oocytes, with a kinetic close to the one observed after cyclin B mRNA microinjection. Although there is no evidence at this time that *spy1* protein is present and/or translated in oocytes, this protein represents a new potential player in the transduction pathway leading to MPF activation.

In summary, the accumulation of cyclin B1 represents an early step in the transduction pathway induced by progesterone in the oocyte. It clearly lies downstream of the decrease in PKA activity induced by the hormone. It is significant that this accumulation is independent of the mechanisms that lead to Mos accumulation, including stimulation of cap-ribose methylation. Additional studies on the mechanism of cyclin B1 accumulation should advance our understanding of the signal transduction pathways regulated by progesterone during oocyte maturation.

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