Cdc2-Cyclin B Triggers H3 Kinase Activation of Aurora-A in Xenopus Oocytes

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Xenopus oocytes are arrested in meiotic prophase I and resume meiotic divisions in response to progesterone. Progesterone triggers activation of M-phase promoting factor (MPF) or Cdc2-cyclin B complex and neo-synthesis of Mos kinase, responsible for MAPK activation. Both Cdc2 and MAPK activities are required for the success of meiotic maturation. However, the signaling pathway induced by progesterone and leading to MPF activation is poorly understood, and most of the targets of both Cdc2 and MAPK in the oocyte remain to be determined. Aurora-A is a Ser/Thr kinase involved in separation of centrosomes and in spindle assembly during mitosis. It has been proposed that in Xenopus oocytes Aurora-A could be an early component of the progesterone-transduction pathway, acting through the regulation of Mos synthesis upstream Cdc2 activation. We addressed here the question of Aurora-A regulation during meiotic maturation by using new in vitro and in vivo experimental approaches. We demonstrate that Cdc2 kinase activity is necessary and sufficient to trigger both Aurora-A phosphorylation and kinase activation in Xenopus oocyte. In contrast, these events are independent of the Mos/MAPK pathway. Aurora-A is phosphorylated in vivo at least on three residues that regulate differentially its kinase activity. Therefore, Aurora-A is under the control of Cdc2 kinase in the Xenopus oocyte and could be involved in meiotic spindle establishment.

Amphibian oocyte meiotic maturation is a pioneer model system to study an unconventional post-transcriptional pathway induced by a steroid hormone, progesterone, and the regulation of M-phase promoting factor (MPF) or Cdc2-cyclin B complex, the universal inducer of mitotic and meiotic divisions.

(1). Xenopus oocytes are arrested in prophase of the first meiotic division, assimilated to the G2 arrest of the cell cycle. When stimulated by progesterone, they resume meiotic division through the activation of MPF. The signaling pathway induced by progesterone and leading to MPF activation is not well understood (1). It involves a drop in cAMP, induced within minutes after progesterone addition, leading to the inhibition of the cAMP-dependent protein kinase, protein kinase A (2). Protein kinase A down-regulation could stimulate the synthesis of new proteins, a process required to activate Cdc2-cyclin B complex (3). The identification of the one or more proteins whose synthesis is needed for Cdc2 activation represents a major challenge. Mos kinase is synthesized and activated simultaneously with that of MPF activation and indirectly induces MAPK activation (4). It has been recently proposed that Mos synthesis and MAPK activation are not required to induce Cdc2-cyclin B activation in Xenopus oocyte; however, they most probably facilitate this process (5–7). Thus, one or more other proteins, not identified yet, must be synthesized in response to progesterone to allow Cdc2-cyclin B activation.

Recently, the Aurora kinases emerged as a new family of mitotic Ser/Thr kinases conserved from yeast to humans (8–13). The vertebrate Aurora family is composed of three members, termed Aurora-A, -B, and -C (14). Aurora-A localizes to the centrosomes and the spindle poles during mitosis (11). Ablation or inactivation of Aurora-A in Caenorhabditis elegans embryos and mammalian culture cells prevents centrosome separation and spindle assembly, showing a function of Aurora-A in centrosome separation and bipolar establishment of the spindle (9, 15, 16). It has also been demonstrated that in Xenopus, one substrate of Aurora-A could be the kinesin-related motor Eg5, involved in centrosome separation (17).

Given the regulation of spindle function by Aurora-A (11, 18), it could be proposed that this kinase plays a role downstream MPF activation in the oocyte, as described in human mitotic cell lines (19). Xenopus Aurora-A has been cloned and originally termed “Eg2” in a screen designed to identify mRNAs that are polyadenylated during meiotic maturation and deadenylated at fertilization (11, 20, 21). Aurora-A protein accumulates during meiotic maturation, in response to progesterone independently of MPF activation, but its kinase activity would be under the control of MPF (22). The inhibition of Aurora-A by microinjection of a specific antibody in Xenopus oocytes neither affects nuclear envelope breakdown (GVBD, for germinal vesicle breakdown) nor Cdc2 activation. However, oocytes arrest in metaphase I without extruding the first polar body (23). This suggests again that Aurora-A functions downstream of MPF activation and controls the meiotic spindle. However,
Andrésson and Ruderman (24) cloned Aurora-A cDNA using a screen designed to identify oocyte proteins whose electrophoretic migration is retarded within 30 min after progesterone stimulation. Moreover, they reported that Aurora-A overexpression accelerates GVBD in response to progesterone (24). It was then shown in Xenopus oocyte that Aurora-A phosphorylates and regulates CPRB, a protein that binds the CPR sequence present in the 3'-non-coding region of several mRNAs, including Mos mRNA, and allows their transduction (25). Therefore, Aurora-A could be an early component of the progesterone pathway stimulating Mos synthesis upstream of Cdc2 activation. Because the Mos/MAPK pathway appears to be dispensable for Cdc2 activation (5–7), it is difficult to assume that the physiological function of Aurora-A would be restricted to Cdc2 activation.

Whether Aurora-A functions upstream or downstream of Cdc2 is important to further understand the regulation of the meiotic process. In this study, we addressed two main questions. First, is Aurora-A kinase activation physiologically controlled by MPF, or by the Mos/MAPK pathway, or by an upstream regulator of MPF? Second, what is the molecular basis of this activation? Several reports have shown that Aurora-A activation correlates with the phosphorylation of several of its residues (26). However, the exact role of these phosphorylations remains unclear. Some of them could target the protein to the degradation pathway, others could play a docking function, while a third category could function as activators (19, 26, 27).

The role of Aurora-A in meiotic prophase is still under investigation. First, is Aurora-A kinase activation physiologically controlled by MPF? Second, what is the molecular basis of this activation? Several reports have shown that Aurora-A activation correlates with the phosphorylation of several of its residues (26). However, the exact role of these phosphorylations remains unclear. Some of them could target the protein to the degradation pathway, others could play a docking function, while a third category could function as activators (19, 26, 27).

To answer these questions, we took advantage of a new in vitro substrate, histone H3, that allows us to assay Aurora-A kinase activity in a reliable manner. We demonstrate that, in vivo during oocyte maturation as well as in vitro using prophase cytosolic extracts, active MPF is necessary and sufficient to induce Aurora-A phosphorylation and activation, independently of the Mos/MAPK pathway.

EXPERIMENTAL PROCEDURES

Material—Xenopus laevis adult females (Horst Kähler, Germany) were bred and maintained under laboratory conditions. Reagents, unless otherwise specified, were from Sigma.

Purification of Recombinant Proteins—The 21 N-terminal amino acids of either wild type histone H3 (WT H3) or histone H3 mutant where Ser10 was replaced by Ala (S10A H3) fused with GST were cloned into pGEX vector (28). WT H3 and S10A H3 fusion proteins were expressed in bacteria and purified using glutathione-agarose beads (Amersham Biosciences). Recombinant GST-p21cip1, sea urchin cyclin B, and GST-Cdc25A were expressed in bacteria and purified as described, respectively, in Refs. 29–31.

Xenopus Oocyte Treatments and Homogenization—Fully grown Xenopus oocytes were isolated and prepared as described before (32). Oocytes were injected with various proteins: recombinant GST-p21cil-1at 40 ng per oocyte, recombinant Cdc25A at 25 ng per oocyte, sea urchin cyclin B at 5 ng per oocyte, okadaic acid at 5 × 10−6 M intracellular concentration, and morpholino antisense oligonucleotides (Gene Tools LLC) raised against c-Mos mRNA at 100 ng per oocyte (5). Oocytes were also incubated in the presence of various reagents: 1 μM progesterone, 100 μg/ml cycloheximide (CHX), 1 μM 3-isobutyl-1-methylxanthine (IBMX), or 50 μM U0126 (Promega). GVBD was monitored by the appearance of a white spot at the animal pole. Oocytes were referred to as “metaphase II-arrested oocytes” when they were collected at least 2 h after GVBD. Activation of metaphase II-arrested oocytes was induced by electric shock (33).

Oocytes were collected by groups of 10 or 20, and lysed at 4 °C in 4 volumes of EB (80 mM β-glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM MgCl2, 1 mM dithiothreitol), supplemented with protease inhibitor mixture (Sigma, P8340) and 1 μM okadaic acid (ICN). Lysates were centrifuged at 15,000 × g at 4 °C for 15 min and frozen at −80 °C. Western blot analysis and kinase activity assays were then performed by using the same lysate.

In Vitro Extracts—Xenopus prophase oocyte lysates prepared in the absence of okadaic acid were incubated at 30 °C in the presence of an ATP-regenerating system (10 mM creatine phosphate, 80 μg/ml creatine phosphokinase, 1 mM ATP, 1 mM MgCl2) and either 1 μM okadaic acid

FIG. 1. Kinetic parameters of the Xenopus Aurora-A kinase for ATP and histone H3-peptide. Aurora-A was immunoprecipitated from metaphase II-arrested extracts with the anti-Aurora-A antibody and assayed for Aurora-A kinase activity, using histone WT H3-peptide as substrate. Kinase assays were carried out as described under “Experimental Procedures,” except that various ATP concentrations (10 μM to 1 mM, A) or various histone H3-peptide concentrations (2.5–500 μg/ml, B) were used.

(ICN) or 4 μg/ml recombinant Cdc25A. Samples were collected at indicated times for Western blot analysis and kinase assays.

Western Blotting—Samples equivalent to two oocytes were electrophoresed on 12.5% SDS-PAGE Anderson system (34) and transferred to nitrocellulose filters (Schleicher and Schuell) using a semi-dry blotting system (Millipore). The rabbit anti-Xenopus Aurora-A polyclonal antibody and the polyclonal antibody raised against Xenopus cyclin B2 were described previously in Refs. 35 and 36, respectively. The mouse monoclonal antibody directed against the active phosphorylated form of MAPK, P-MAPK, was purchased from New England BioLabs. The rabbit polyclonal antibodies raised against ERK1 and Xenopus Mos protein were purchased from Santa Cruz Biotechnologies. The primary antibodies were detected with appropriated horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and the Western blot Chemiluminescence Renaissance kit from PerkinElmer Life Sciences.

Kinase Assays and Phosphatase Treatment—To measure Cdc2 kinase activity, histone H1 assays were performed on p13agar pull-down extracts (three oocytes equivalent) in the presence of 1 μCi of [γ-32P]ATP (ICN), 100 μM ATP and 0.2 mg/ml histone H1 (Roche Applied Science) in kinase buffer (50 mM Tris-HCl, pH 7.2, 15 mM MgCl2, 5 mM EGTA, 1 mM dithiothreitol).

Histone H3 kinase activity of Aurora-A was assayed in immunoprecipitates, incubated for 30 min at 30 °C in the presence of kinase buffer containing 3 μCi of [γ-32P]ATP (ICN), 50 μM ATP, and 0.5 mg/ml of either WT H3 or S10A H3. Kinase reactions were stopped by adding Laemmli buffer (37) and boiling. After electrophoresis and autoradiography, the bands corresponding to histone H1 and histone H3 were excised, and the associated radioactivity was measured in a Wallac

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Biochemical Characterization of Aurora-A Kinase Activity—
To study the regulation of Aurora-A kinase activity, a reproducible kinase assay was developed. MBP and casein are often used to assay Aurora-A kinase activity in vitro (12, 22, 24, 26, 27, 38, 39). However, the activity of Xenopus oocyte Aurora-A kinase toward both substrates is low, rendering the quantification of the kinase activity difficult. It is established that Aurora-B phosphorylates in vivo histone H3 on a single residue, Ser\(^{10}\) (40). Given the high homology between the kinase domains of Aurora-A and Aurora-B, we checked whether histone H3 could be an in vitro substrate of Xenopus Aurora-A. Aurora-A was immunoprecipitated from metaphase II-arrested oocytes that have been reported to contain active Aurora-A kinase (22, 24). The kinase activity was measured in immunoprecipitates using the 21 N-terminal amino acids of histone H3 (ARTKQTARK\(^{10}\)TGGKAPRKQLC) fused with a GST tag, as substrate. As a control, we used a mutated histone H3, where Ser\(^{10}\), the unique residue in H3 phosphorylated by Aurora-B, was replaced by Ala (S10A H3). This S10A H3-peptide was never phosphorylated under our conditions. To determine the optimal conditions for the kinase assay, H3-peptide phosphorylation was measured in the presence of various ATP concentrations (from 10 \(\mu\)M to 1 mM) or various histone H3-peptide concentrations (from 2.5 to 500 \(\mu\)g/ml) at 30 °C for 30 min (Fig. 1). The \(K_m\) for ATP and histone H3-peptide were, respectively, 80 \(\mu\)M and 35 \(\mu\)g/ml (Fig. 1). These values are comparable to those reported for the Cdc2-cyclin B kinase (75 \(\mu\)M for ATP and 40 \(\mu\)g/ml for histone H1 (41)).
Aurora-A Is Activated at GVBD Time during Meiotic Maturation—To investigate how Aurora-A is regulated during meiotic maturation, the activity of the protein was estimated, in parallel with its electrophoretic migration. Oocytes were collected at different times following progesterone stimulation (Fig. 2). As expected, MPF activity, measured by histone H1 kinase assay, increased just before GVBD, in correlation with cyclin B2 electrophoretic mobility retardation. Its activity decreased at the metaphase I-metaphase II transition and increased again, inducing entry into metaphase II. MAPK activity was estimated by Western blot, using an antibody that specifically recognizes the phosphorylated active form of the protein. As already reported, MAPK was stably activated at time of GVBD, due to Mos synthesis, until the end of the maturation process (Fig. 2A). Aurora-A kinase activity was similarly measured during meiotic maturation using histone H3-peptide as substrate (Fig. 2B). In prophase-arrested oocytes and until GVBD, Aurora-A was inactive and migrated as a single band (Fig. 2). At GVBD time, Aurora-A accumulated, as previously reported (22) and underwent an electrophoretic shift, leading to a doublet, in correlation with its kinase activation (Fig. 2). In this experiment, the level of Aurora-A activity increased continuously from GVBD until the arrest in metaphase II. In some cases, Aurora-A kinase activity is maximal at GVBD time and is maintained at a high level until metaphase II (see Figs. 4, 8, and 10 below).

Metaphase II oocytes were parthenogenetically activated by electric shock. This treatment inactivated MPF within 10 min, due to cyclin B2 degradation (Fig. 2). Mos was degraded 30 min after activation, and consequently, MAPK was inactivated at the same time (Fig. 2A). When oocytes re-entered interphase in response to activation, the upper band of Aurora-A partially disappeared, in correlation with a drop in its kinase activity (Fig. 2).

The H3 Kinase Activity of Aurora-A Depends on Its Phosphorylation—We next addressed the question whether Aurora-A activation results from its phosphorylation, as suggested by the electrophoretic retardation that correlates with the active form of the H3 kinase activity. Active Aurora-A kinase was immunoprecipitated from metaphase II-arrested oocytes and incubated in the presence of phosphatase λ (PPλ), that exhibits a broad range of specificity. PPλ treatment abolished Aurora-A activity, whereas heat-inactivated PPλ had no effect (Fig. 3A). The electrophoretic migration of Aurora-A was further analyzed in immunoprecipitates that allow a better resolution than crude extracts. Active Aurora-A migrated as a doublet when metaphase II oocyte extracts were loaded on a gel (see Fig. 2A). The active kinase isolated by immunoprecipitation migrated under three bands (Fig. 3B). The lower band migrated at the same level as the inactive prophase form (Fig. 3B). PPA treatment induced the disappearance of the upper band, a decrease in the middle band, and the enrichment of the lower band (Fig. 3B). These observations indicate that Aurora-A is activated by phosphorylation and that at least two residues of Aurora-A are phosphorylated in metaphase II-arrested oocytes, resulting in the electrophoretic retardation of the protein.

Aurora-A Phosphorylation and Activation Are under the Control of cAMP and Protein Synthesis—Aurora-A phosphorylation and H3 kinase activation take place in response to progesterone at GVBD time. It is well established that progesterone induces an early drop in cAMP and the synthesis of new proteins, both events being required for Cdc2 activation and GVBD (42, 43). To ascertain whether both events are necessary for Aurora-A activation, prophase-arrested oocytes were incubated in the presence of either cycloheximide (CHX), a protein synthesis inhibitor, or IBMX, a phosphodiesterase inhibitor that maintains a high level of cAMP in the cell. The oocytes were then stimulated by progesterone. In control oocytes, Cdc2 activation and GVBD occurred 4 h after progesterone addition (Fig. 4). As expected, CHX totally prevented Cdc2 activation, as ascertained by H1 kinase assay and cyclin B2 electrophoretic migration (Fig. 4). MAPK was not activated (Fig. 4), due to the inhibition of Mos synthesis. Under these conditions, Aurora-A mobility was not shifted and no kinase activity could be detected (Fig. 4). Identical results were obtained when oocytes were first incubated in the presence of IBMX (Fig. 4). Taken together, these results show that Aurora-A phosphorylation and activation are under the control of the drop of cAMP and protein synthesis induced by progesterone.

Aurora-A Phosphorylation and Activation Are Independent of the Mos/MAPK Cascade—As shown in Fig. 2, H3 kinase activity of Aurora-A switched on at the same time as Cdc2 kinase and MAPK, the major kinases activated during meiotic maturation. We then asked whether Aurora-A activation could be dependent on one of these kinases. We first investigated the potential implication of the Mos/MAPK pathway in Aurora-A activation. Oocytes were incubated in the presence of U0126, a pharmacological MEK inhibitor (44), and progesterone was then added. GVBD occurred with 1-h delay in comparison to
control progesterone-treated oocytes. Mos was synthesized, but MAPK was not activated (Fig. 5). In the absence of active MAPK, Cdc2 was activated at GVBD as shown by the mobility shift of cyclin B2 and H1 kinase assay. Under these conditions, Aurora-A was phosphorylated and its H3 kinase activity was increased (Fig. 5). This result shows that Aurora-A activation and phosphorylation do not depend on MAPK activation. However, because Mos is synthesized in response to progesterone in the presence of U0126, Mos could account for Aurora-A kinase activation independently of MAPK activity. To investigate this possibility, we adopted an antisense strategy to ablate Mos in the oocytes. Morpholino antisense oligonucleotides directed against Mos mRNA have been shown to prevent Mos synthesis, and consequently MAPK activation, induced by progesterone (5). In control oocytes, GVBD started 4 h after progesterone addition, whereas oocytes microinjected with morpholino Mos antisense underwent GVBD with a 1-h delay. As shown by Western blot, Mos was not synthesized under these conditions and consequently MAPK was not activated (Fig. 5). Cdc2 kinase was activated at GVBD (Fig. 5), as previously described (5). In the absence of Mos and MAPK activation, Aurora-A was still phosphorylated and activated at GVBD (Fig. 5). Taken together, these results show that neither Mos synthesis nor MAPK activation are required for Aurora-A phosphorylation and activation.

Activation of Cdc2 by Injection of Cdc25, OA, or Cyclin B Triggers Aurora-A Kinase Activation—We then addressed the question whether Aurora-A activation could be under the control of Cdc2 activity. To bypass the upstream signaling pathway induced by progesterone, Cdc2 was directly activated by injection into the oocytes of either Cdc25 or cyclin B, in the absence of progesterone. The injection of Cdc25, the phosphatase directly responsible for the dephosphorylation of Cdc2 (45), induced GVBD and Cdc2 activation in the absence of progesterone (Fig. 6A). As expected, MAPK was also activated, as judged by electrophoretic retardation of the protein visualized on Western blot with an antibody recognizing all the forms of MAPK (Fig. 6A). Aurora-A was phosphorylated and activated in response to the direct activation of Cdc2 by injected cyclin (Fig. 6B). Okadaic acid, an inhibitor of types 1 and 2A Ser/Thr phosphatases, is a potent Cdc2 activator in the oocyte (46), although its precise targets are not well defined yet. Okadaic acid injection induced GVBD within 90 min in the absence of progesterone, leading to Cdc2 kinase activation and partial MAPK activation (Fig. 6B), as previously reported (47). Okadaic acid injection induced the activation of Aurora-A and a supershift of the doublet usually observed in progesterone-treated oocytes (Fig. 6B). Therefore, the in vivo inhibition of okadaic acid-sensitive phosphatases allows the phosphorylation of Aurora-A.
on residues that are not phosphorylated under physiological conditions. These additional phosphorylations did not significantly increase the kinase activity (Fig. 6B). These results show that Cdc2 activation by three different agents, Cdc25, cyclin B, and okadaic acid, is sufficient to induce Aurora-A phosphorylation and activation in the absence of progesterone.

To determine whether Cdc2 induces Aurora-A activation in a protein synthesis-dependent manner, oocytes were injected with okadaic acid or cyclin B in the presence of cycloheximide. As expected, Cdc2 was still activated, whereas MAPK was not (Fig. 6B). Interestingly, Aurora-A phosphorylation and activation were still observed under these conditions, demonstrating that Aurora-A activation is induced in vivo by Cdc2 activation but requires neither the synthesis of new proteins nor the Mos/MAPK pathway. We have earlier shown that Aurora-A phosphorylation and activation induced by progesterone depend on protein synthesis (Fig. 4). This is obviously because of the protein synthesis dependence of the progesterone pathway leading to Cdc2 activation. Once Cdc2 is activated, then Aurora-A activation does not require any new proteins.

To better analyze the regulation of Aurora-A kinase activation, an in vitro approach was adopted based on prophase cytosolic extracts that do not support protein synthesis (31, 48). Prophase cytosolic extracts were incubated in the presence of either okadaic acid or Cdc25, in the presence of an ATP-regenerating system (Fig. 7). In the presence of okadaic acid, Cdc2 was rapidly activated within 30 min (Fig. 7A and B). As previously reported (31), MAPK was activated 60 min after okadaic acid addition, despite the absence of Mos (Fig. 7A), suggesting that MAPK activity results in vitro from a balance between kinase activities and okadaic acid-sensitive phosphatases. Aurora-A was activated between 30 and 60 min after okadaic acid addition (Fig. 7C). Kinase assays of Aurora-A performed with S10A H3-peptide as substrate remained at a basal level throughout the time course of the experiment (data not shown). Okadaic acid induced in vitro (Fig. 7A) as well as in vivo (Fig. 6B) a supershift of Aurora-A mobility, suggesting that, under both conditions, inhibition of okadaic acid-sensitive phosphatases leads to additional phosphorylations of the protein.

When extracts were supplemented with Cdc25, Cdc2 was activated after a lag period of 60 min, and MAPK was not activated (Fig. 7A and B), as previously reported (31), arguing that in vitro activation of MAPK cannot occur in the absence of Mos, unless okadaic acid-sensitive phosphatases are inhibited. Aurora-A phosphorylation and activation occurred shortly after Cdc2 activation in response to Cdc25 addition (Fig. 7A). The electrophoretic shift of Aurora-A induced by Cdc25 addition in the extracts was similar to its migration observed in vivo in maturing oocytes. Taken together, these results show that Cdc2 activation is sufficient to trigger Aurora-A kinase activation and phosphorylation, in the absence of progesterone, independently of protein synthesis and of the Mos-MAPK pathway.

Inhibition of Cdc2 Kinase Activation by p21^{cip1} Prevents Aurora-A Kinase Activation by Progesterone—The next question was to determine whether Cdc2 activity is not only sufficient but also necessary for physiological Aurora-A activation during meiotic maturation induced by progesterone. We used p21^{cip1}, a protein known to specifically inhibit the cyclin-dependent kinase-cyclin complexes (49) and to prevent MPF activation normally induced by progesterone by direct binding to Cdc2-cyclin B complexes (29). As expected, oocytes injected with p21^{cip1} and incubated with progesterone did not achieve GVBD, and neither Cdc2 nor MAPK was activated (Fig. 8). Interestingly, Aurora-A was neither phosphorylated nor activated (Fig. 8). These results were confirmed in vitro (Fig. 9). Prophase oocytes extracts were activated by okadaic acid, in the presence or not of p21^{cip1}. In the presence of p21^{cip1}, Cdc2 activation was strongly delayed (Fig. 9A and B). MAPK was activated 60 min after OA addition. As previously reported (31), MAPK activation in these extracts is independent of Cdc2 activity. In contrast, Aurora-A phosphorylation and its kinase activation were inhibited (Fig. 9A and C). In conclusion, Cdc2 activity is necessary and sufficient to trigger the phosphorylation and the consequent activation of Aurora-A both in vivo during oocyte meiotic maturation and in vitro in extracts reproducing the Cdc2 auto-amplification feedback loop.

Because Aurora-A activation occurs downstream of Cdc2 activation, our next goal was to determine whether MPF directly
activates Aurora-A. To test this possibility, an in vitro kinase assay was performed using Cdc2-cyclin B kinase purified from metaphase II-arrested oocytes, either by p13-binding affinity or by chromatography (36), as an enzyme source, and the bacterially expressed Aurora-A or inactive immunoprecipitated Aurora-A from prophase oocytes as a substrate. Aurora-A was not phosphorylated and not activated by purified Cdc2-cyclin B (data not shown). These results demonstrate that Aurora-A is not a direct substrate of MPF and that Cdc2-cyclin B indirectly regulates the phosphorylation and activation of Aurora-A kinase during meiotic maturation.

Cdc2 Kinase Inhibition at GVBD Does Not Inhibit Aurora-A Kinase Activity—Although Aurora-A activation is clearly Cdc2-dependent, it was important to know whether the kinase further requires Cdc2 activity to remain active. To answer this question, Cdc2 kinase activity was inhibited at GVBD stage by either inhibiting protein synthesis (incubation in the presence of CHX) or by direct Cdc2 inhibition (p21cip1 injection). Both treatments are known to induce inactivation of Cdc2 within 2 h (50–52) (Fig. 10A). When oocytes are incubated in the presence of CHX at GVBD, cyclin B2 completely disappears, as a consequence of its degradation occurring normally at this period and of the prevention of its synthesis (52–54). After p21cip1 injection, cyclin B2 was not degraded and pre-MPF was reformed as previously reported (50); cyclin B2 migrated as a doublet, as in prophase oocytes (Fig. 10A), in parallel to tyrosine phosphorylation of Cdc2 (data not shown). Under both conditions, MAPK was totally inactivated. Interestingly, Aurora-A remained active during the 2-h period following Cdc2 kinase inhibition (Fig. 10A). However, analysis of Aurora-A electrophoretic migration showed that the upper band disappeared under these conditions (Fig. 10A) suggesting that the protein was at least partially dephosphorylated. To determine whether the activity of this non-shifted form of the kinase was dependent on phosphorylation, it was treated by PP1/PP2A. PP1/PP2A totally abolished the kinase activity of Aurora-A (Fig. 10B). Therefore, when Cdc2 is inactivated after GVBD, the migration of Aurora-A is accelerated, but the protein is still phosphorylated on sites required for its activity. It can be concluded that the two phosphorylated sites inducing an electrophoretic shift require Cdc2 activity to be stabilized but are not necessary for the maintenance of the kinase activity. This experiment also reveals the existence of an initially phosphorylated site in response to Cdc2 activation that does not modify the migration of the protein in SDS-PAGE, does not require Cdc2 to be maintained under a phosphorylated state, and is crucial for the H3 kinase activity of Aurora-A. Cdc2 kinase activity is therefore no longer necessary to maintain Aurora-A kinase activity after GVBD.

DISCUSSION

The major aim of this study was to determine how Aurora-A activation is controlled during Xenopus oocyte meiotic maturation. To answer this question, we undertook a dual in vitro and in vivo approach, using a polyclonal anti-Aurora-A antibody and histone H3 as an Aurora-A substrate, used in the in vitro kinase assays. We demonstrate that Aurora-A is phosphorylated and activated at GVBD time and that this activity is regulated by phosphorylation. Because Aurora-A is activated at
the same period as Cdc2 and MAPK, the implication of both kinases in Aurora-A activation was investigated. Preventing Mos synthesis by morpholino antisense oligonucleotides, or MAPK activation by U0126, did not inhibit GVBD and Cdc2 activation as previously reported (5–7), nor did it inhibit Aurora-A phosphorylation or its activation. In contrast, our results show that 1) Aurora-A phosphorylation and activation can be induced by Cdc2 kinase activation, and 2) inhibition of Cdc2 kinase activation by injection of p21cip1 prevents Aurora-A phosphorylation and activation normally induced by progesterone. This means that the Mos/MAPK pathway is not involved in Aurora-A regulation and that Cdc2 activation is necessary and sufficient for Aurora-A activation. Therefore, Aurora-A is a physiological target of Cdc2 in Xenopus oocyte. However, Cdc2 is unable to directly phosphorylate and activate Aurora-A. These results suggest that other kinases under the control of Cdc2 kinase phosphorylate Aurora-A. It is also not excluded that active Cdc2 activates an adaptor of Aurora-A or inhibits a specific phosphatase rendering the kinase able to auto-phosphorylate and to auto-activate. Recently, it has been shown that human Aurora-A is activated during mitosis by phosphorylation, under the indirect control of Cdc2-cyclin B (19). Taken together, these results show that Aurora-A is phosphorylated and activated downstream of Cdc2-cyclin B kinase, either in mitosis or in meiosis, in different species.

In apparent contradiction with the present study and others (22, 23), it has been shown that Aurora-A is phosphorylated soon after progesterone addition, using as a criterion its electrophoretic mobility in SDS-PAGE, and that injection of the protein facilitates GVBD in response to progesterone (24). However, inactivation of Aurora-A by the microinjection of a blocking antibody does not affect the timing of GVBD (23). Because CPEB was further shown to be an Aurora-A substrate, it was proposed that Aurora-A could participate to the polyadenylation of Mos mRNA leading to Mos synthesis (25). However, Mos synthesis and MAPK activation do not appear to be required for Cdc2 activation (5–7); it is therefore not probable that the putative function of Aurora-A in Cdc2 activation could be exerted through the recruitment of the Mos/MAPK pathway. One possible way to reconcile our results and those of others (24, 25) is to assume that an active phosphorylated form of Aurora-A is generated soon after progesterone addition but that its phosphorylation cannot be detected by SDS-PAGE under our conditions and that this active form does not use histone H3 as an in vitro substrate. Then later, a hyperphosphorylated form could be generated under Cdc2 control at GVBD, leading to an active H3 kinase activity. The biological significance of both active forms, the first one controlling Mos synthesis and the latest one the spindle assembly, remain to be explored. Using a broad range of in vitro substrates, we were not able to assay the kinase activity of the putative first form of Aurora-A. We therefore studied the regulation of the active H3 kinase activity of Aurora-A, generated by phosphorylation during GVBD in response to Cdc2 activation.

The second task of our study was to approach the molecular basis of Aurora-A activation. It has been shown that Xenopus Aurora-A activation correlates with the phosphorylation of three residues of the protein (26). However, the exact role of these phosphorylations is still unclear. Some of them could target the protein to the degradation pathway (as Ser349, Ser351, Ser353) (27, 35), others could play a docking or structural function (as Ser249), whereas a third category could function as activators (as Thr295) (19, 27). We have demonstrated by PP1 treatment that Aurora-A kinase activation during meiotic maturation depends on its phosphorylation. Our data establish that the phosphorylation of at least two residues of Aurora-A induce a retardation of the electrophoretic mobility of the protein. The phosphorylation as well as the maintenance of the phosphorylated state of both residues depends on Cdc2 activity. This result suggests that the phosphates on both residues are subject to an active turnover, involving the activity of one or more Cdc2-dependent kinases and opposing phosphatase(s). Because the presence of okadaic acid in the lysis buffer is required to preserve the electrophoretic shift of Aurora-A (22), it can be concluded that the phosphatases regulating both phosphorylations are sensitive to okadaic acid. They most probably correspond to a type 1 phosphatase, which is assumed to regulate the Aurora-A function in various species (55, 56).
Interestingly, the phosphorylations of both residues, responsible for the appearance of the two retarded bands at GVBD, are not required for the stabilization of Aurora-A activity. Indeed, when Cdc2 kinase activity is inhibited at GVBD time, Aurora-A kinase activity remains high, showing that Cdc2 is not necessary to stabilize Aurora-A kinase activity. Under these conditions, Aurora-A electrophoretic migration is not retarded anymore, showing that it was dephosphorylated on the two "shifting" residues, although the kinase remains active. This finding led us to propose that Aurora-A could be subjected to three phosphorylations, in agreement with a previous report (26). At GVBD time, active Cdc2 could indirectly induce Aurora-A phosphorylation on both residues that promote an electrophoretic retardation of the protein. Both phosphorylations are not sufficient to activate Aurora-A kinase. They could correspond to a permissive event that allows the phosphorylation of a third residue responsible for Aurora-A kinase activation. This third phosphorylation does not induce any change in the electrophoretic migration of Aurora-A. When Cdc2 kinase activity is inhibited at GVBD, i.e. just after Aurora-A activation, the protein returns to its faster migration form but remains active, because the phosphorylation of the third residue is preserved. Because stabilization of Aurora-A kinase activity requires the presence of okadaic acid in the lysis buffer (22), it can be concluded that the phosphorylation of this third activation site is also dependent on an okadaic acid-sensitive phosphatase, as PP1.

Interestingly, Aurora-A kinase activity is down-regulated when oocytes re-enter interphase after activation, presumably by dephosphorylation. A previous study has reported that the protein was degraded at this period (22). Indeed, we observed that Aurora-A is proteolyzed in an anaphase promoting complex-independent manner after extended periods after electric...
Cdc2 kinase activities are expressed in cpm incorporated in the sub-
strate. Cdc2 kinase activity using histone H1 as substrate. Aurora-A and
anti-Aurora-A antibody and assayed for Aurora-A kinase activity using
(P-P MAPK) antibody, as indicated, or immunoprecipitated with
MAPK (P-MAPK) with anti-cyclin B2 antibody, anti-Aurora-A antibody, or anti-phospho-
/h11001/h11002 antibody and incubated (H) or not (H). Lysates were immunoprecipitated with anti-Aurora-A
CHX (H). 2h
incubated for 2h in the presence (H) or in the absence (H) of cyclohexi-
mide (CHX). These observations demonstrate that Aurora-A could be a cru-
ential element of spindle organization in mitosis and in meiosis,
under the control of Cdc2 kinase. It is now of high interest to
undergo a structural analysis of Aurora-A localization in meiotic spindles and to study the consequences of its functional
ation on the microtubular organization of such spindles.

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Aurora-A Regulation in Xenopus Oocyte

Aurora-A kinase activity is stabilized independently of Cdc2-cyclin B and protein synthesis after GVBD. Prophase
uclei were incubated in the presence of progesterone, and oocytes were collected either in prophase (Pro), at GVBD, or 2 h after GVBD (GVBD + 2h). A. GVBD nuclei were either immediately homogenized, incubated in the presence of cycloheximide (CHX), or injected with p21(H11001) and collected 2 h later. Lysates were subjected to Western blotting with anti-cyclin B2 antibody, anti-Aurora-A antibody, or anti-phospho-
/P-P MAPK (P-P MAPK) antibody, as indicated, or immunoprecipitated with
anti-Aurora-A antibody and assayed for Aurora-A kinase activity using histone H3-peptide as substrate. A pull-down on p13 beads was assayed for Cdc2 kinase activity using histone H1 as substrate. Aurora-A and
Cdc2 kinase activities are expressed in cpm incorporated in the sub-
strate. B. GVBD nuclei were either immediately homogenized or
incubated for 2 h in the presence (+) or in the absence (–) of cyclohexi-
mide (CHX). Lysates were immunoprecipitated with anti-Aurora-A
antibody and incubated (+) or not (–) in the presence of λ phosphatase (PPi) for 1 h at 30 °C. Immunoprecipitates were then assayed for Aurora-A kinase activity using histone H3-peptide as substrate.

Aurora-A is hyperphosphorylated when okadaic acid is either injected into oocytes or added in cytosolic extracts that reproduce in vitro Cdc2 and Aurora-A activation. However, the
additional phosphorylation level generated by inhibition of okadaic acid-sensitive phosphatases does not increase the ki-
rase activity level of Aurora-A. Therefore, because the phos-
phylation on these additional sites is never observed under physiological conditions and does not control Aurora-A kinase activity, okadaic acid addition could have simply unmasked a kinase activity that does not control Aurora-A under normal conditions.

In somatic cells, Aurora-A plays a role in the mitotic spindle organization (18, 57). This function could also exist in meiosis, although no regular centrosomes are present in vertebrate acentriolar oocytes. During GVBD in Xenopus oocyte, a giant monopolar spindle is organized at the basal part of the germi-
nal vesicle, associated to chromosomes. It migrates toward the animal pole, where it is reorganized in the two successive bipolar metaphase spindles (58–60). These two metaphase
spindles could be regulated by Aurora-A. Supporting this idea, inhibition of Aurora-A association by antibody injection in prophase oocytes induces an arrest in metaphase I, because oocytes are unable to extrude the first polar body (23). It has been published that Eg5, a kinesin-related motor protein is a substrate of Xenopus Aurora-A (17). Moreover, it has recently been shown that Aurora-A binds to and phosphorylates TPX2, a prominent component of the spindle, inducing the recruit-
ment of Aurora-A to the spindle microtubules (61). All together, these observations demonstrate that Aurora-A could be a cru-
ial element of spindle organization in mitosis and in meiosis,
under the control of Cdc2 kinase. It is now of high interest to
undergo a structural analysis of Aurora-A localization in meiotic spindles and to study the consequences of its functional
ation on the microtubular organization of such spindles.
Cdc2-Cyclin B Triggers H3 Kinase Activation of Aurora-A in *Xenopus* Oocytes
Gilliane Maton, Catherine Thibier, Anna Castro, Thierry Lorca, Claude Prigent and Catherine Jessus

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