Aurora-A kinase Ser349 phosphorylation is required during *Xenopus laevis* oocyte maturation

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**A B S T R A C T**

*Xenopus laevis* Aurora-A is phosphorylated in vivo onto three amino acids: Ser53, Thr295 and Ser349. The activation of the kinase depends on its autophosphorylation on Thr295 within the T-loop. The phosphorylation of Ser53 by still unknown kinase(s) prevents its degradation. The present work focused on the regulation of Aurora-A function via Ser349 phosphorylation. Mutagenesis of Ser349 to alanine (S349A) had few impact in vitro on the capability of the kinase to autophosphorylate as well as on its activity. These data in addition to in gel kinase assays and site-specific proteolytic digestion experiments prove that Ser349 is clearly neither a primary autophosphorylation site, nor an autophosphorylation site depending on the priming phosphorylation of Thr295. Using specific antibodies, we also show that the phosphorylation of Aurora-A Ser349 is a physiological event during Xenopus oocyte maturation triggered by progesterone. A peak of phosphorylation paralleled the decrease of Aurora activity observed between meiosis I and II. In response to progesterone, *X. laevis* stage VI oocytes microinjected with the Aurora-A S349A mutant proceeded normally to germinal vesicle breakdown (GVBD), but degenerated rapidly soon after. Since phosphorylation of Ser349 is responsible for a decrease in kinase activity, our results suggest that a down-regulation of Aurora-A activity involving Ser349 phosphorylation is required in the process of maturation.

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**Introduction**

Aurora-A is a member of the serine/threonine kinase family involved in centrosome maturation, spindle formation and stability (Giet and Prigent, 2000; Roghi et al., 1998). In somatic cells, Aurora-A defects can lead to abnormal chromosome segregation and cell cycle arrest (Katayama et al., 2001). Its overexpression is sufficient to transform cells, identifying Aurora-A as an oncogene (Bischoff et al., 1998; Zhou et al., 1998). The protein is synthesized throughout the cell cycle to reach a maximum level at the M phase. Upon exit from mitosis, Aurora-A is degraded through a proteasome-dependent pathway mediated by APC/cdh1 (Arlot-Bonnemains et al., 2001; Castro et al., 2002a; Walter et al., 2000). Aurora-A contains two sequences necessary for its degradation: a Destruction box (D-box) in its C-terminal end and a Destruction box Activating Domain (DAD or A-box) in its N-terminal end (Castro et al., 2002b; Littlepage and Ruderman, 2002).

In *Xenopus laevis*, Aurora-A is also required for oocyte maturation (Andresson and Ruderman, 1998; Ma et al., 2003; Mendez et al., 2000). The kinase is activated after progesterone stimulation, around the time when MPF (maturation promoting factor) activation occurs. But, the precise timing and function of Aurora-A activation in the regulation of oocyte maturation remain controversial (Andresson and Ruderman, 1998; Frank-Vaillant et al., 2000). Nevertheless, Aurora-A has been shown to phosphorylate and activate CPEB, a cytoplasmic polyadenylation factor important for controlling the translation of various mRNAs during maturation (Mendez et al., 2000), as well as maskin (Pascreau et al., 2005).

*X. laevis* Aurora-A incubated in the presence of M phase extracts has been found phosphorylated on three sites: Ser53 in the DAD box, Thr295 in the T-loop and Ser349 in the C-terminal domain (Fig. 1A) (Littlepage et al., 2002). Fourteen residues were also found phosphorylated in recombinant Aurora-A (purified from bacteria), four of them were present in the additional N-terminal 49 amino acid long tail containing the His 6 tag. The authors suggested that the sites were likely to be autophosphorylation sites (Haydon et al., 2003).

In many kinases, the phosphorylation of a Thr residue located in the activation-loop of the kinase domain is required for constitutive kinase autophosphorylation (Huse and Kuriyan, 2002). Autophosphorylation within the T loop induces large conformational changes which allow the kinase to switch from inactive to active state (Johnson et al., 1996). The activity of *Xenopus* Aurora-A depends on the autophosphorylation of the T-loop Thr295 residue. If the autophosphorylation of Thr295 is not required for the kinase activity of Aurora-A, it has to be noted that some physiological substrates, such as TPX2, does enhance the autophos-
Fig. 1. Analysis of the autophosphorylation and activity of wild type and mutant Aurora-A(His)6 proteins. (A) Organisation of Aurora-A and mutagenesis sites. The central domain (grey) contains the catalytic domain. The underlined amino acids correspond to the mutated amino acids. (B) Recombinant Aurora-A proteins (2 μg) were incubated in the presence of 5 μCi [32P]ATP without substrate (group a, autophosphorylation) or in presence of 10 μg of GST-p17 (group b). The mixture was electrophoresed on a 12.5% polyacrylamide gel. The gel was stained with Coomassie Blue (lower panel for each group) and kinase activity was monitored by autoradiography (upper panel for each group). The autoradiography exposure times of gels containing kinase assays were five times shorter for the GST-p17 substrate than for the autophosphorylation. (C) In-gel kinase assays performed in absence or presence of protein substrates embedded in the gel: no protein (lane 1), K169R (lane 2), T294A–T295A–S349A (lane 3), T294A–T295A (lane 4) or T295A (lane 5) as substrates. Recombinant wild type Aurora-A (3 μg) was electrophoresed on the gels. The phosphorylation assay was then performed on the gels. The radioactivity incorporated in the substrates was visualized by autoradiography. (D) 10 μg of recombinant wild type (lane 1) or S349A mutant (lane 3) Aurora-A were autophosphorylated in the presence of 5 μCi [32P]ATP. The proteins were then treated with Factor Xa (lanes 2 and 4). All the samples were analyzed on a 10–20% Tris–Tricine gel. The gel was stained with Coomassie Blue (upper panel) and autoradiographed (lower panel). (E) Quantification of the different bands was performed using the Image Quant software.
phorylation process (Eyers and Maller, 2003; Kufer et al., 2002; Tsai et al., 2003).

The sequence (...GPS*NVP...) around Ser53 differs from (K/R)XX (S/T), the putative consensus site for phosphorylation by Aurora-A, suggesting that the site is probably not an autophosphorylation site. Mutations of Ser53 have revealed that the phosphorylation of this amino acid is not involved in the regulation of the kinase activity but rather controls the degradation of the protein (Littlepage et al., 2002). The Ser53 residue is indeed located within an A box, a short N terminal region required for Aurora A destruction. Its phosphorylation stabilizes Aurora-A, and its mutation into an aspartic residue which mimics the effect of phosphorylation, prevents the degradation of Aurora-A through APC/Cdh1 (Littlepage and Ruderman, 2002).

The Ser349 residue is included in a characteristic Aurora-A consensus sequence (...RRIRIS*K...) (Sarkissian et al., 2004). Ser349 phosphorylation has not been investigated in vivo, and the few data suggests a role of this modification in the regulation of the kinase activity. It has been proposed that Ser349 autophosphorylation is an event occurring after a primary phosphorylation on Ser290/291 by the Glycogen synthase kinase 3 (GSK3) (Sarkissian et al., 2004). The phosphorylation of Ser349 reduces the activity of the kinase, and its mutation into an Asp residue completely abolishes the kinase activity (Littlepage et al., 2002; Sarkissian et al., 2004). In contrast, the kinase purified from bacteria was found entirely phosphorylated on Ser349 but paradoxically still fully active (Haydon et al., 2003).

These discrepancies lead us to reinvestigate the phosphorylation of Ser349 both in vitro and in vivo. Our results clearly demonstrate that Ser349 is neither a primary autophosphorylation site nor a site requiring the primary Thr295 autophosphorylation. We also show that, in vitro, XI-Aurora-A can be specifically phosphorylated on Ser349 by the X. laevis p21 activated kinase (XpAK1), a member of the Xenopus Ste20/PAK protein kinases involved in the arrest of G2/prophase oocytes. We confirm that Ser349 phosphorylation reduces the kinase activity. In vivo, using a specific anti-phospho-Ser49 antibody, we show that Aurora-A is phosphorylated on Ser349 in X. laevis stage VI oocytes and that the level of this phosphorylation fluctuates during their maturation after progesterone stimulation. Microinjection studies of various recombinant Aurora-A mutants led us to conclude that the phosphorylation of Ser349 is essential to allow proper progression of oocyte maturation.

Materials and methods

Antibodies

The monoclonal antibodies against XI-Aurora-A (1C1) and against XI-Aurora-A (His6, 6E3) were produced in the laboratory (Roghi et al., 1998). The polyclonal antibodies against anti-XI-Aurora-A and cdck were kind gifts from T. Lorca (CRBM, Montpellier, France) and M. Mechali (IGH, Montpellier, France) respectively. The polyclonal antibody directed against the phospho-Ser349 of the XI-Aurora-A protein was produced by Eurogentec (Searing, Belgium) using the pentadecapeptide GETYRRISS PVVEFQYP as antigen.

Site directed mutagenesis

The mutant forms of Aurora-A (His6) were obtained by PCR amplification, using the pET21a-wt-Aurora-A(His)6 construction as a DNA template. The mutations were performed by the first round of PCR in the presence of the sense (S) (CTTCGGCCGCTG-AAATGCTGCTG) or the antisense (AS) (CCCCCTACCTTTGAAGGC) primers associated with the primer corresponding to the expected mutations described:

Ser349A: Sense: TCCACCCCTTGACTATGCTCTGA

Antisense: TACAGCAGTTGATCCGAGTGAA

T295A: Sense: GCTCTCTTCCAGGACGGCTCTGTCGAGGACTCTG

Antisense: CCAACAGGGCTGCTTGGATGTGACAGTCTT

T294AT295A: Sense: ATCTTCACCTTTGAAGGC

Antisense: ACAGACGGCCCTCGTGGAGAT

The second PCR was performed with both the sense primer and the antisense primer, using the two products from the first round of PCR as the DNA template. PCR products were directly cloned in a pET21a vector (Novagen). The T294A–T295A–S349A mutant was obtained by insertion of the NotI/NcoI fragment of S349A mutant (containing the S349A mutation) into the T294A–T295A construction cut with the same enzymes.

Phosphorylation of recombinant proteins

All histidine-tagged recombinant Aurora-A proteins were produced from BL21 (DE3)-plysS and purified by a Ni-NTA-agarose affinity chromatography following the manufacturer's instructions (Qiagen).

The recombinant GST-p17 and GST-GSK-3 proteins were purified as previously described from BL21(DE3)-plyssS transformed with constructions in pGEX-4T (Fisher et al., 1999; Pasceau et al., 2005). All constructs were sequenced in full (Genome Express). Cloning and purification of rMal-xpAK1 and its K279R mutant have been reported previously (Faure et al., 1999).

In vitro kinase assay

The assays were performed in 20 μL of kinase buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 10 μM MgCl2, 10 μM ATP) in the presence of 5 μCi (γ-32P) ATP at 3000 Ci/mmol and different recombinant kinase proteins (2 μg) or immunoprecipitates, with or without 10 μg of the appropriate protein substrate. Incubations were performed at 30 °C for 10 min. Ten microliters of the incubate was mixed with 10 μL of 2×-Laemmli sample buffer and the proteins were separated on a 12.5% SDS-polyacrylamide gel. Gel was stained with Coomassie Blue and autoradiographed.

In gel kinase assay

Recombinant wild type Aurora-A protein was electrophoresed onto a 12.5% SDS-polyacrylamide gel, containing (or not containing) 500 μg/mL of one of the following Aurora proteins: K169, T295A, T294A–T295A, and T294A–T295A–S349A. The phosphorylation reactions were performed in the presence of 100 μCi (γ-32P) ATP at 3000 Ci/mmol. The gels were processed as previously described (Wooten, 2002).

Clavage of the autophosphorylated Aurora-A by Factor Xa

WT and S349A Aurora-A recombinant proteins autophosphorylated in presence of [32P]ATP were treated with 0.6 units of Factor Xa (Qagen) in the digestion buffer (50 mM Tris–HCl pH 8, 100 mM NaCl, 5 mM CaCl2) during 1 h at 37 °C. The reaction was stopped by addition of Laemmli buffer and the proteins were separated on a 10–20% Tris–Tricine gel (Biogel). Gel was stained with Coomassie blue and autoradiographed.

Oocyte experimentations

Xenopus oocytes were enzymatically isolated from fragments of ovaries which were previously treated with 40 mg of dispase in 100 mL of OR-2 medium (10 mM Hepes pH 7.6, 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2) for 3 h, then in 10,000 units of collagenase in 100 mL of OR-2 medium for 45 min. Isolated oocytes were then directly recovered and stored for 12 h at 16 °C in Merriam buffer (Merriam, 1971). Groups of 20 oocytes were injected with 368 ng of wild type or mutant proteins, or with the dilution buffer (Merriam buffer) as a control. After injections, the oocytes were incubated with 3× 10–5 M of progesterone at 21 °C. GBVD was monitored by the appearance of the white spot. Immunoprecipitation experiments and kinase activity assays were performed using extracts prepared from oocytes at different stage of maturation as previously described (Chen and Murray, 1997).

Immunoprecipitations

10 μL of dried Affigrep protein-A (BioRad) were washed with 500 μL of Immunopure (A) IgG binding buffer (IIB-Pierce) and were incubated for 2 h at 4 °C with 500 μL of 1C1 antibody (dil: 1/20) in IIB (dil: 1/2), and then washed twice with 500 μL TBS (50 mM Tris–HCl pH 7.5; 150 mM NaCl). Beads were then incubated with a 10 equivalent oocytes extract for 2 h at 4 °C on a wheel. The beads were washed once in 500 μL of 0.5 mM NaCl and 5 times with 500 μL TBS (TBS–0.05% Tween 20). Round proteins were eluted in 10 μL of 2×-Laemmli sample buffer.

Western blotting

Proteins were loaded on a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane using the BioRad system. The membranes were washed with TBS (50 mM Tris–HCl pH 7.5; 150 mM NaCl) and saturated with 5% low fat milk in TBS for 2 h at room temperature. The membranes were incubated overnight at 4 °C in 2.5% low fat milk in TBS with the proper antibody: anti-XI-Aurora-A 1C1 monoclonal antibody (dil: 1/2000), polyclonal antibodies raised against phospho-Ser349 XI-Aurora-A (dil: 1/5000), or cdck (dil: 1/2000). After incubation with appropriate secondary antibodies conjugated with horseradish peroxidase (dil: 1/ 50,000; Jackson Immuno-Research Laboratories), the western blot chemiluminescence Super Signal kit (Pierce) was used for revelation.
Results

Aurora-A autophosphorylation sites

In order to validate our assay used to analyse the autophosphorylation sites of Aurora-A, wild type and mutant forms of Aurora-(His)6 were produced in bacteria. Mutant forms bear single or combined point mutation of Thr295 and Ser349 (Fig. 1A).

Autophosphorylation of the various forms of Aurora-A was analyzed after incubation of the enzymes in presence of [32P]ATP in an adequate buffer and electrophoretic separation (Fig. 1B, group a). The autoradiography revealed incorporation of [32P] in the wild-type kinase and the S349A mutant (lanes 1 and 3). This was accompanied by a decrease in the electrophoretic mobility of both proteins. In contrast, none of the other mutants had incorporated radioactivity.

The kinase activity of the various forms of recombinant Aurora-(His)6 was determined using GST-p17, a protein previously described as a physiological substrate of Aurora-A (Pascreau et al., 2005), in presence of [32P] ATP in an adequate buffer (Fig. 1B, group b). The K169R and the T295A mutations had a dramatic effect on the activity of the kinase. The K169R mutation completely abolished the activity of the kinase (lane 2). The activity of the T295A mutant was also dramatically reduced but a residual kinase activity was observed (lane 4). This residual activity was completely lost in the double T294A–T295A mutant in which the adjacent Thr294 was also mutated into an alanine (lane 5). In contrast, the kinase with the Ser349 replaced by alanine was fully active (lane 3). We also tested the activity of all mutants with two other substrates MPB and H3, and observed similar results than with GST-p17 (data not shown). We finally carried out all kinase activity in the presence of the GST-p17 substrate.

Trans-phosphorylation analysis by in-gel kinase assay

To determine whether Aurora-A Thr295 and Ser349 residues can be trans-phosphorylated by Aurora-A, we performed an "in gel kinase assay", a method currently used to identify kinase substrates. The assay consisted in electrophoresing an active Aurora kinase in a polyacrylamide gel cast with another form of Aurora kinase which acts as the substrate for the kinase reaction. As the kinase assay is performed with GST-p17 (data not shown). We finally carried out all kinase activity in the presence of the GST-p17 substrate.

Site-specific proteolytic digestion of autophosphorylated Aurora-A kinase

It is now clear that Thr295 is an autophosphorylation site. It is also obvious from the above results that Ser349 is not a primary autophosphorylation site. Nevertheless, it is still conceivable that the primary autophosphorylation of Thr295 leads to structural remodelling that renders the Ser349 susceptible to auto- or trans-phosphorylation. To check this hypothesis, the recombinant wild type Aurora-A and the S349A mutant were autophosphorylated in vitro in the presence of [32P] ATP. Then, the proteins were subjected to a proteolytic cleavage using Factor Xa: this protease generates a 34 kDa fragment containing the Thr295 (Aurora-A–34 kDa), and a 11 kDa (Aurora-A–11 kDa) fragment enclosing the Ser 349. As shown in Fig. 1D, in our experimental conditions, the cleavage of Aurora-A was not complete. In addition to the 34 and 11 kDa fragments, some native 45 kDa protein was still observed. The autoradiography shows that in absence of digestion both the wild type and the S349A mutant proteins had incorporated radioactivity (Fig. 1D lanes 1 and 3). After digestion, radioactivity was found associated to the 34 kDa fragment of Factor Xa-cleaved Aurora-A as well as to the 45 kDa undigested protein. But no radioactivity was found associated to the 11 kDa fragment (Fig. 1D lanes 2 and 4, panel b — see quantification in Fig. 1E). This clearly demonstrates that, when an active Aurora-A kinase is incubated in presence of ATP, Ser349 is not phosphorylated while Thr295 is.

Altogether our results demonstrate that the Aurora-A Ser349 is neither a primary autophosphorylation site, nor an autophosphorylation site depending on the priming phosphorylation of Thr295.

Impact of Ser 349 phosphorylation on Aurora-A kinase activity

A previous report has shown that Ser349 is phosphorylated in the presence of mitotic extract (Littlepage et al., 2002). It has been proposed that the phosphorylation of this residue might be an autophosphorylation event requiring an initial phosphorylation of Ser290/291 by the glycogen synthase kinase 3 (Sarkissian et al., 2004). However, these results did not rule out the possibility that Ser349 is a XI-GSK3 phosphorylation site requiring the primary phosphorylation of Ser290/291. Nevertheless, it was observed in this last study that the phosphorylation of Aurora-A by XI-GSK3 reduced by 50% the activity of the kinase. However, it is not clear whether the XI-GSK3-induced drop of activity is due to the sole phosphorylation of Ser349 or if it is the consequence of the double phosphorylation on Ser290/291 and Ser349. The S349D mutant where the Ser349 is replaced by an Asp residue (to mimick phosphorylation; mutation S/D) was found fully active in our hand (data not shown) while it has been reported to be fully inactive in other laboratories (Littlepage et al., 2002; Sarkissian et al., 2004). However, these last results were in apparent contradiction with another work reporting that the wild-type kinase purified from bacteria is entirely phosphorylated on Ser349 but still active (Haydon et al., 2003). Altogether, these studies do not allow to draw a clear figure of the impact of the phosphorylation of Ser349 on the activity of the enzyme.

As we demonstrated that the Ser349 is not an autophosphorylation site, we searched for a kinase able to phosphorylate Aurora-A on Ser349. This residue is included in a sequence RISK similar to a consensus domain xxRXSxx… found in substrates of the Xenopus PAK1 kinase, a kinase known to regulate the dynamics of the microtubule network and to be involved in the regulation of the oocyte maturation process (Fauze et al., 1997). Recently, Zhao and collaborators reported the phosphorylation of both Thr288 and Ser 342 of human Aurora-A (which are equivalent to Thr295 and Ser349, respectively, in XI-Aurora-A) by hs-PAK1 (Zhao et al., 2005).

We studied the phosphorylation of Aurora-A by xPAK1 by incubating various forms of Aurora-A with [32P]ATP in the presence of either active xPAK1 (Fig. 2A lanes 1, 3 and 5) or inactive K729R xPAK1 (Fig. 2A lanes 2 and 4). Inactive recombinant mutant Aurora-A proteins
Is Aurora-A Ser349 phosphorylated during Xenopus oocyte maturation

A previous report has shown that Aurora-A Ser349 is phosphorylated in the presence of extracts of Xenopus oocytes (Littlepage et al., 2002; Sarkissian et al., 2004). But there is no evidence for this phosphorylation event in vivo.

The anti-phospho-Ser349 antiserum was then used to investigate the phosphorylation of Aurora-A Ser349 during oocyte maturation. Aurora-A was immunoprecipitated from extracts prepared from immature oocytes (Prophase-arrested oocytes), from progesterone-stimulated maturing oocytes (at the time of GVBD, 30 min post-GVBD and 1 h post-GVBD) and from mature oocytes (metaphase II-arrested oocytes). Immunoprecipitates were analyzed for Ser349 phosphorylation (Fig. 3A, upper panel) and Aurora-A content (Fig. 3A, upper middle panel) by western blot and for Aurora-A activity using GST-p17 as substrate (Fig. 3A, lower middle panel). In parallel, to confirm the specificity of the signal, a western blot was performed using the anti-phosphoSer349 antibody preabsorbed with the recom-

**Fig. 2.** In vitro phosphorylation of Aurora-A Ser349. (A) Mutant K/R, T294A–T295A, and T294A–T295A–S349A Aurora-A(His)6 were incubated with purified active xPK1 protein in presence of [γ-32P]ATP (lanes 1, 3 and 5). The same proteins were subjected to the same reaction in the presence of K279R xPK1 inactive (lanes 2 and 4). Samples were separated by SDS-PAGE and the gel was stained with Coomassie blue (lower panel). The Aurora-A(His)6 phosphorylation for each protein was analyzed by autoradiography (upper panel). A western blot was performed with the antibody against the phospho-Ser 349 Aurora-A (middle panel). (B) Wild type Aurora-A(His)6 was first phosphorylated in presence (lane 1) of purified xPK1 (lane 2) or with XI-GSK3 (lane 3). Then the activity of Aurora-A is measured in presence of γ-32P[ATP and the substrate GST-p17, Histone H3 and MBP. The assay was analyzed on a 12.5% SDS-PAGE. The gel was stained with Coomassie Blue and autoradiographed.

Since xPK1 specifically phosphorylates Aurora-A on Ser349, it is a good way to study the consequence of this phosphorylation on the activity of Aurora-A. We examined the kinase activity of wild type Aurora after its phosphorylation by xPK1. As shown in Fig. 2B (lane 2), the phosphorylation of Aurora-A by xPK1 reduced by 50% its kinase activity. Similar result was observed when Aurora-A was phosphorylated by XI-GSK3 (Fig. 2B lane 3), in agreement with a previous report (Sarkissian et al., 2004). A similar pattern was observed when histone H3 or MBP were used as substrates for the kinase, indicating that the reduction of the kinase activity of Aurora-A phosphorylated on Ser349 was not substrate dependent (Fig. 2B middle and lower panel). Altogether, these observations indicated that the phosphorylation of the Ser349 is responsible for a reduction of Aurora-A activity.

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**Fig. 3.** In vivo phosphorylation of Aurora-A Ser349. (A) Cytolysic extracts were prepared from oocytes collected at different stage of maturation. Aurora-A was immunoprecipitated using the specific IC1 antibody directed against the native Aurora-A protein and analysed by western blot to evaluate the amount of protein (middle panel). The phosphorylation of the Ser349 was analyzed with the antibody anti phospho-Ser349-Aurora-A (upper panel). The activity of the kinase was tested in the immunoprecipitate using the GST-p17 as a substrate (lower middle panel). The immunoprecipitated Aurora-A protein was also analysed using a preadsorbed antibody with the Ser349 phosphorylated Aurora-A(His)6 (lower panel). (B) Quantification of the activity of the kinase, the phosphorylation of Ser349 Aurora-A and the amount of Aurora A was performed with the ImageQuant software. Results are expressed as means ± standard deviation of three different experiments.
binant phosphorylated Aurora-A (Fig. 3A, lower panel). The amount of Aurora-A protein increased as the oocytes entered GVBD and progressed until meiosis II arrest; in parallel the kinase activity showed a biphasic profile: it increased up to 30 min after GVBD, dropped 1 h post GVBD and reincreased in meiosis II arrested oocytes (Fig. 3A, upper middle panel). As shown in Fig. 3A (upper panel), a signal corresponding to the phosphorylation of Ser349 was detected as the oocytes progressed in meiosis. A peak of phosphorylation on Ser349 was observed 1 h post GVBD and the level dropped in MII arrested mature oocytes. This peak of phosphorylation paralleled the decrease of Aurora activity (Fig. 3B).

**Is the Ser349 phosphorylation required during Xenopus oocyte maturation?**

To enlighten the function of the Ser349 and its phosphorylation during oocyte maturation, oocytes were injected with the different recombinant Aurora-A mutant proteins. The injected oocytes were then stimulated with progesterone and checked at regular intervals for appearance of the white spot in the animal hemisphere which indicates the GVBD.

Each oocyte received by microinjection a quantity of recombinant protein equivalent to the quantity of the endogenous protein, and similar for all recombinant proteins (Fig. 4A). After stimulation with progesterone, maturation was scored by the appearance of a white spot, an indicator of germinal vesicle breakdown (Fig. 4B). For all recombinant proteins, a (GVBD)-50 (time when 50% of the oocytes show a white spot) was determined (Fig. 4C).

K169R or T294A–T295A or T294A–T295A–S349A mutants-injected oocytes reached (GVBD)-50 ~1 h faster than the oocytes injected with the dilution buffer alone. In contrast, oocytes injected either with the wild-type or the S349A mutant had a kinetic similar to the control oocytes (Figs. 4B and C).

Biochemical analysis were conducted to better understand the effect of the microinjected recombinant mutant protein on the oocyte maturation. The MPF activity was determined by measuring the Histone H1 kinase activity. As shown in Fig. 4D, the MPF was activated in all microinjected oocytes (Fig. 4D middle panel). The MPF being a key activity that catalyses entry into M-phase of meiosis I and meiosis II, this indicates that the microinjected recombinant proteins did not impair oocyte maturation. In addition, all microinjected oocytes expressed Cdc6, a factor undetectable in fully-grown oocytes but present in mature oocytes. Then the microinjected recombinant proteins did not prevent the meiosis to progress into metaphase II (Fig. 4D lower panel).

The microscopic observation revealed that the configuration of the white spot was normal in oocytes injected with the T295A, the T294A–T295A or the T294A–T295A–S349A mutants (Fig. 4E, panels e, f and g) but was unusual in the other oocytes (Fig. 4E). In the wt-Aurora-A or K169R mutant injected oocytes, the aspect of the maturing oocytes differed from the control oocytes (Fig. 4E, compare panels b, c and d). The white spot was translucent, the outline of the white spot was shadowy, and the inside area deeply depigmented. The aspect of oocytes injected with the S349A mutant was even more perturbed with a discontinuous and blurry outline of the white spot and a depigmentation of the animal pole (Fig. 4E, panels h). In contrast to the other...

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**Fig. 4.** Analysis of oocyte maturation after injection of the different recombinant Aurora-A proteins: (A) Two equivalent oocytes from all injected oocytes at a time corresponding to 100% MII arrested oocytes were processed for western blot using the specific 6E3 antibody directed against the recombinant protein. (B) Stage VI oocytes arrested in prophase I were injected with the dilution buffer (Merriam buffer) or 368 ng of the recombinant wild type (empty rhomb) or K169R mutant (full square) proteins. After addition of progesterone, the GVBD was monitored by the appearance of the white spot. The results are expressed as means ± standard deviation of 4 different experiments. (C) Stage VI oocytes arrested in prophase I were injected with 368 ng of the different recombinant Aurora-A proteins. Control oocytes were injected with the dilution buffer of the protein (Merriam buffer). After addition of progesterone, the GVBD was monitored by the appearance of the white spot. The results indicating the time necessary for reaching 50% GVBD are expressed as means ± standard deviations of 4 separate experiments. (D) Cytosolic extracts were prepared from all injected oocytes at a time corresponding to 100% MII arrested oocytes observed for the dilution buffer injected oocytes. Injected Aurora-A (His)₆ proteins were immunoprecipitated with the specific 6E3 antibody and tested for kinase activity with GST-p17 as substrate (upper panel). Extracts from all injected oocytes were also tested for histone H1 kinase activity (middle panel). The presence of cdc6 was analysed by western blot (lower panel). (E) Morphology of the injected oocytes, at a time when control oocytes are arrested in metaphase II.
centrosome. progesterone-triggered meiosis progression occurs in the absence of association of Aurora-A to the centrosomes, since in Xenopus oocytes, Aurora-A, but also on the autophosphorylation sites Thr 288 (equivalent to the Ser342 residue (a residue equivalent to the Ser349 in Xl-GSK-3 [Sarkissian et al., 2004]). Altogether these data demonstrate that Ser349 is part of a cryptic auto-phosphorylation site that requires structural modifications induced by the phosphorylation of other residues. Our data also shows that the auto-phosphorylation of Thr295 does not unveil this cryptic site.

Recombinant Aurora-A kinase Ser349 has been found phosphorylated in vitro in presence of Xenopus oocyte metaphase extract (Littlepage et al., 2002). This may be the result of the autophosphorylation induced secondarily to the phosphorylation of the kinase by Xl-GSK3, as described above. Nevertheless, it cannot be excluded that Ser349 is a primary phosphorylation site for other kinases present in the extract. The sequence around Ser349 is similar to a consensus domain \( ^{349} \text{RRT}^{352}, \) found in Histone H3 (Ser10) and Raf-1 (Ser338). PAK1 (p21 activating kinase) serves as a physiological upstream kinase phosphorylating these two serine residues (Li et al., 2002; Zang et al., 2002). Like Aurora-A, PAK1 has been shown to be localized on and around the spindles poles in the centrosomal region (Li et al., 2002), and as Aurora-A, deregulation of PAK1 induces the formation of abnormal mitotic spindle (Vadamuduri et al., 2000). Altogether, these observations lead us to envisage that Xl-Aurora-A could be phosphorylated by xPAK1. Our results demonstrate that in vitro Aurora-A Ser349 can be directly phosphorylated by xPAK1, without other priming modifications. xPAK1 has been shown to be present in Xenopus oocyte and to control oocyte meiotic maturation (Faure et al., 1997; Faure et al., 1999). But the physiological interaction between xPAK1 and Aurora-A in Xenopus oocytes remains to be proved.

Ser349 is not a residue essential for the kinase activity of Aurora-A. Indeed, the mutation of this residue into an alanine do not affect the activity of the enzyme, as previously observed (Littlepage et al., 2002; Sarkissian et al., 2004). In contrast, the phosphorylation of this residue had some impact on the kinase activity. In agreement with a previous report (Sarkissian et al., 2004), Aurora-A autophosphorylated on Ser349 consecutively to the GSK-3-induced phosphorylation of Ser290/Ser291 displayed a reduced kinase activity. A similar drop of activity was provoked by the direct phosphorylation of Ser349 by xPAK1. This last result singularly contrasts with a recent observation made in human cells (Zhao et al., 2005). In NIH3T3 fibroblasts, PAK1 has been described to be a potent activator of Aurora-A at the centrosomes (Zhao et al., 2005). The authors showed that PAK1 binds efficiently to the inactive Aurora-A and catalyzes its phosphorylation on the Ser342 residue (a residue equivalent to the Ser349 in Xl-Aurora-A), but also on the autophosphorylation sites Thr 288 (equivalent to the Thr295 residue in Xl-Aurora-A) causing an activation of Aurora-A. The discrepancy between the 2 studies may rely upon the association of Aurora-A to the centrosomes, since in Xenopus oocytes, progesterone-triggered meiosis progression occurs in the absence of centrosome.

Aurora-A plays various functions during Xenopus oocyte meiotic maturation, including meiotic spindle control (Castro et al., 2003; Kinoshita et al., 2005; Liu and Ruderman, 2006; Peset et al., 2005; Sattinover et al., 2004) and translation regulation (Mendez et al., 2000; Pascreau et al., 2005; Sarkissian et al., 2004). During progesterone induced oocyte maturation, Aurora-A is neo-synthesized at the time of GVBD, then Aurora-A protein levels remain constant between meiosis I and meiosis II. During this transition however, Aurora-A follows a biphasic activation that is regulated by the phosphorylation of the kinase (Ma et al., 2003). The transient inactivation was correlated with a dephosphorylation of the enzyme while inversely, its hyperphosphorylation lead to its reactivation. In the present report, we focused on Ser349 phosphorylation. This phosphorylation has been observed in recombinant Aurora-A kinase incubated in presence of metaphase extracts. Using a specific anti-phospho-Ser349 antiserum, we demonstrate that Ser349 is phosphorylated in Xenopus oocytes and that its level of phosphorylation fluctuates during oocyte maturation. In oocytes blocked in prophase of first meiosis, the kinase appears to be highly phosphorylated. The phosphorylation level drops after progesterone stimulation and reincreases transiently 1 h after GVBD at a time when a drop of Aurora-A activity is observed. Because Ser349 phosphorylation is a negative regulator of Aurora kinase activity, these results suggests that this event may participate to the transient inactivation of Aurora-A observed during the meiotic transition.

To question the physiological function of Ser349 phosphorylation during meiosis, we followed the maturation of oocytes injected with the S349A Aurora-A mutant, a mutant missing the phosphorylatable Ser349. When compared to oocytes injected with a similar amount of wild-type recombinant Aurora-A, the maturation kinetics was similar in oocytes injected with the S349A mutants. The maturation was complete in both cases as evidenced by the activation of H1 kinase and the expression of Cdc6. However, the oocytes injected with the S349A mutant showed a different pattern of pigmentation and degenerated very quickly. In contrast, the oocytes injected with the T294A–T295A–S349A mutant which also lacks the phosphorylatable Ser349 but which is devoid of any kinase activity, maturated quite normally without showing any sign of degeneration. These observations indicate that the maturation cannot be achieved properly with an excess of active Aurora-A lacking the phosphorylatable Ser349 residue. The absence of Ser349 phosphorylation may prevent the negative regulation of Aurora-A activity which occurs during the meiosis transition, leading to unwanted phosphorylated substrate proteins.

In conclusion, we showed that: (1) in the absence of other proteins, Ser349 is a site that is neither auto- nor trans-phosphorylated, (2) Ser349 can be directly phosphorylated by xPAK1, and (3) the phosphorylation of Ser349 leads to a partial inactivation of Aurora-A kinase. We also provide evidence that the phosphorylation of Ser349 could participate in the reduction of Aurora-A activity observed during the meiosis I to meiosis II transition, and that this phosphorylation is required for Xenopus oocytes to mature into fertilizable eggs.

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