Phosphorylation of Maskin by Aurora-A participates to the control of sequential protein synthesis during *Xenopus laevis* oocyte maturation.

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ABSTRACT

At the end of oogenesis, *Xenopus laevis* stage VI oocytes are arrested at the G2/M transition (prophase) waiting for progesterone to release the block and begin maturation. Progesterone triggers a cascade of phosphorylation events such as a decrease of pKA and an increase of MPF activity. Progression through meiosis is controlled by a sequential synthesis of several proteins. For instance, the MAP kinase kinase kinase c-Mos is the very first protein to be produced, while cyclin B1 appears only after meiosis I. After the meiotic cycles, the oocyte arrests at metaphase of meiosis II with an elevated c-Mos kinase activity (CSF-cytostatic factor).

Using a two-hybrid screen, we have identified maskin, a protein involved in the control of mRNA sequential translation, as a binding partner of Aurora-A, a protein kinase necessary for oocyte maturation. Here we show that, in vitro, Aurora-A directly binds to maskin and that both proteins can be co-immunoprecipitated from oocyte extracts suggesting that they do associate in vivo. We also demonstrate that Aurora-A phosphorylates maskin on a Ser residue conserved in TACC (Transforming acidic coiled coil) proteins from Drosophila to human. When the phosphorylation of this Ser was inhibited in vivo by microinjection of synthetic peptides that mimic maskin phosphorylated sequence, we observed a premature maturation. Under these conditions, proteins such as cyclin B1 and Cdc6 that are normally detected only in meiosis II, were massively produced in meiosis I before the occurrence of the nuclear envelope breakdown. This result strongly suggests that phosphorylation of maskin by Aurora-A prevents meiosis II proteins to be produced during meiosis I.
INTRODUCTION:

In *Xenopus* oocytes, progesterone induces meiotic maturation by triggering the conversion of pre-MPF, stockpiled as an inactive form in prophase I oocyte, into MPF at the time of Germinal Vesicle Breakdown (GVBD) (1,2). The stage VI of the first meiotic division is a step of growth and synthesis of mRNA that will be used later for progression of prophase I arrested oocyte through meiosis I. After GVBD, continued protein synthesis is still necessary to successfully complete meiosis I and to allow the reappearance of the MPF at the onset of meiosis II.

The dormant mRNAs, which are synthesized and stored in the growing oocyte, become translationally activated during the completion of meiosis (oocyte maturation). During meiotic maturation, oocytes are transcriptionally repressed, and all necessary proteins are translated from preexisting, maternally derived mRNAs (3). The translation of mos mRNA is necessary to produce the serine/threonine kinase Mos, a key regulator of oocyte maturation. Mos is a direct activator of mitogen-activated protein kinase (MAPK) kinase (MEK), which in turn activates MAPK (4). Mos protein levels are tightly regulated in vivo during oocyte maturation (5,6) to induce the MAP kinase cascade that directly activates the MPF. Mos translational control is exerted through the regulation of Mos mRNA (A) tail in immature oocytes, to which further adenyl residues are added during oocyte maturation (7,8). This early translation of c-Mos mRNA was reported to be essential for the progesterone stimulated maturation (9). However, it was recently demonstrated that c-Mos synthesis is not necessary for the entry in meiosis I, but is required after meiosis I to proceed to meiosis II in order to induce the CSF arrest (10).
In addition to Mos, a number of other *Xenopus* maternal mRNAs are translationally regulated by cytoplasmic polyadenylation during progesterone-stimulated oocyte maturation. Furthermore, the control of temporal order of the mRNA translation is crucial for oocyte maturation to proceed normally (11,12). Maternal mRNAs are not translated at the same time, but selectively and sequentially elongated and translated all along oocyte maturation to insure a timely progression through meiosis I and II and to arrest oocytes at metaphase of meiosis II (13-16). The mRNAs encoding c-Mos, several cyclins (cyclin B1, cyclin B4), Cdk as well as enzymes required for DNA replication and chromatin assembly, are concerned by this translational control (17,18).

Prior to the onset of meiotic maturation, these mRNAs possess a relatively short poly(A) tail. Progesterone-stimulated translational control may be divided into three processes: (i) progesterone-initiated signal transduction, (ii) signal amplification, and (iii) mRNA cytoplasmic polyadenylation and translation. After progesterone treatment, several of these messages are translationally activated by poly(A) elongation, the poly(A) tail elongates up to around 100 nucleotides, and the mRNAs can thereafter be recruited by the translational machinery (19,20). Dynamic changes in poly(A) tail length of some of these maternal mRNAs play a crucial role in their ribosome recruitment and temporally regulated pattern of translation (7).

Cytoplasmic polyadenylation is directed by two types of sequence specific elements in the 3’ untranslated region (UTR) of these mRNAs: a uracil-rich cytoplasmic polyadenylation element (CPE) and the polyadenylation hexanucleotide sequence AAUAAA (3,7,21,22,23,24). CPEB is a protein that binds CPE at the 3’ end of the mRNA. CPEB interacts with maskin, a factor which also associates with the mRNA 5’ cap binding factor eIF4E. The
binding of maskin to CPEB and eIF4E induces a loop formation in the mRNA. In *Xenopus*, the CPEB-maskin-eIF4E complex prevents the translation of mRNAs such as cyclin B1, c-Mos or Cdc6.

When masked, these mRNAs typically have short poly(A) tails. In immature oocytes the CPE of mRNA such as cyclin B is bound to the cytoplasmic polyadenylation element binding protein (CPEB), a zinc-finger- and RRM-containing protein, and the poly(A) tail of the mRNA is typically short. The translation inhibition before maturation results from the interaction of maskin and eIF4E, this last interaction inhibits the binding of eIF4G to eIF4E that would trigger the initiation of mRNA translation. The association of CPEB with maskin assures that only CPE-containing mRNAs are repressed.

In *Xenopus* oocytes, polyadenylation is initiated when progesterone interacts with its receptor leading to the activation of Aurora-A kinase and the phosphorylation of CPEB. Recent studies suggested that this phosphorylation event provokes the recruitment of CPSF resulting in an addition of a poly(A) tail to mRNA. The phosphorylation of CPEB induces CPEB to bind and recruit CPSF possibly stabilizing it on the AAUAAA sequence which in turn attracts poly(A) polymerase to the end of the mRNA. The activation of mRNA translation needs the dissociation of the complex eIF4E, maskin and CPEB (25,26). This conformation loop of the mRNA prevents the recruitment of eIF4G on eIF4E and the subsequent 40S ribosomal subunit positioning at the mRNA 5’ end, inhibiting the mRNA translation (27,28).

In a search for *Xenopus laevis* Aurora-A substrates using a two-hybrid screen, we identified maskin (*Xenopus* TACC) as an Aurora-A binding partner. We demonstrate that both proteins interact *in vitro* and *in vivo*, and that Aurora-A phosphorylates maskin *in vitro*. 
Maskin is a phosphoprotein which phosphorylation is partially dependant on Aurora A in vivo. We found that injection in *Xenopus* stage VI oocytes of peptides mimicking the maskin sequence phosphorylated by Aurora-A accelerates the appearance of GVBD (meiosis I) induced by progesterone by allowing early translation of proteins normally expressed only for meiosis II.
MATERIALS AND METHODS:

Material:

*Xenopus laevis* adult females (CNRS-Rennes-France) were bred and maintained under laboratories conditions. They were anaesthetized under 0.2% phenoxyethanol and surged in order to take half of an ovary to obtain stage VI oocytes.

Antibodies:

Monoclonal antibodies against XI-Aurora-A (1C1 and 6E3) were produced in the laboratory, polyclonal antibodies against cyclin B1 and cyclin B2 were gifts from T. Hunt (Cancer Research UK, South Mimms, UK). The polyclonal anti-XI-Aurora-A antibodies was a gift from T. Lorca (CRBM-Montpellier-France), the polyclonal antibodies against maskin was a gift from J. Raff (Cancer Research UK, Cambridge, UK) and the polyclonal antibodies anti-Cdc6 a gift from M. Mechali (IGH, Montpellier, France). The polyclonal antibodies against the phospho-Ser626 of the protein maskin was performed by Eurogentec (Searing Belgium) using the following peptide ESVLRKQ(S)LYKFDP. The GST and β-tubulin antibodies were from Sigma (G-7781 and T-4028, respectively) and the anti c-Mos antibody was from Santa-Cruz Biotechnology (SC086).

Synthetic Peptides:

The synthetic peptides mp1 (SFKESVLRKQSLYLKFDP) and smp1 (scramble mp1 LSLYFEKSQLKVLRPDK) were synthesized on a Milligen 9050 Pepsynthesizer in the SESO-laboratory-UMR6510-CNRS Rennes. The peptides were then purified by HPLC
(Hewlett Packard) dissolved in appropriate buffer (2.5 % DMSO in H₂O) and stored at –20°C until used.

**Detection of Aurora-A/maskin interaction through the two-hybrid system:**

The HpaI/XhoI XI-Aurora-A fragment cloned in pET21 (29) was subcloned in pGBT11. The cDNA *Xenopus* egg library was obtained from Moreau J. (CRBM-Paris 7). The PCR fragment was digested by BamHI and XhoI, then subcloned in a pGADGH vector in the BamHI/SalI restriction sites. Yeast strain SFY 526 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, can¹, gal4-54, gal80-538, URA3 : GAL1-lacZ*) was transformed by means of pairwise combination of both two-hybrid vectors and grown on a medium without leucine or tryptophan. Galactosidase activities were assayed using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside staining on filter replicates according to Breeden and Nasmyth (30). Both pGADGH and pGBT9 vectors without insert were used as negative controls.

**Cloning of the DNA encoding p17, maskin, p17 S626A and maskin S626A:**

*Xenopus laevis* maskin cDNA was obtained from MRC Geneservice (#14227-h18), then subcloned into a pGEX-4T-1 vector (Amersham Biosciences, #27-4580-01) in order to produce the recombinant GST-maskin Full length (Fl) protein. P17 cDNA was subcloned in the same vector. Two rounds of PCR amplification obtained the mutant proteins GST-p17 S626A and GST-maskin FlS626A.

The mutation S626A was performed by PCR amplification in the presence of the following oligonucleotides for GST-p17 and GST-maskin:
(Sens)GTGCTCAGAAAGCAGGCTCTCTATCTGA’;(Antisens)TCAGATAGAGAGCCTGCTTTCTGAGCAC. The second PCR performed to amplify the GST-p17 and GST-maskin mutated sequence were realized in the presence of the following oligonucleotides.

GST- p17 :(Sens)-CGCGGATCCGTGGTTTTAAGTTATGCTGACC;
   (Antisens)CGGGAATTCGGAGCTCGAAACTCGTCTTC;
GST-maskin: (Sens)-CGCGGATCCATGAGCCTTCA;
   (Antisens)-CCGCTCGAGTCAGATCTTCCC.

All constructs were sequenced in full (Genome Express).

**Purification of recombinant proteins:**

All recombinant proteins were prepared from E. coli strain BL21(DE3)pLysS. The bacteria induced to produce Aurora-A(His)$_6$ were lysed in the IMAC 5 buffer (20 mM Tris-Hcl pH 7.5; 500 mM NaCl; 10 % glycerin and 5 mM imidazol) containing 1 mg/ml lysozyme and 1 mM PMSF for 1 hour at 4°C (29). The bacteria lysates were then centrifuged for 30 min at 12,000 g at 4°C (JA-20 rotor, Beckman) and the supernatants were filtered. The proteins were then purified by Ni-NTA-agarose affinity chromatography following the manufacturer’s instructions (Qiagen SA). The beads were washed twice with 10 vol of IMAC 5 and were then incubated in 5 % BSA in IMAC 5 for 1 hour at 4°C. The supernatants were incubated with the beads for 3 hours at 4°C and the beads were washed twice with 10 vol of IMAC 5 and three times with 10 vol of IMAC20 (20 mM Tris-HCl pH 7.5; 500 mM NaCl; 10 % glycerol; 20 mM imidazole). The His-tagged proteins were eluted with IMAC 250 (IMAC-250 mM imidazol). The eluted fractions were controlled in concentration by Bradford analysis and for purity by electrophoresis onto a 12.5% SDS gel (31).
The recombinant GST-p17, GST-p17S626A, GST-maskin and GST-maskinS626A were prepared as described by (32). The bacteria were lysed in PBS (138 mM NaCl; 2.7 mM KCl; 14 mM Na₂HPO₄; 1.8 mM KH₂PO₄; pH 7) with 1 mg/ml lysozyme and 1 mM PMSF for 1 hour at 4° C. The glutathione-sepharose 4B beads (Amersham-Biosciences-) were washed twice with PBS and then incubated in 5 % BSA in PBS for 1 hour at 4° C. The bacteria lysate was mixed with the beads for 3 hours on a wheel at 4° C. The column was washed 2 times with PBS and 3 times with washing buffer (WB300; 50 mM Tris-HCl pH 8; 300 mM NaCl; 0.02 % Tween-20; 1 mM PMSF). The proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl pH 8. The eluted fractions were controlled in concentration by Bradford analysis and for purity by electrophoresis onto a 12.5% SDS polyacrylamide gel (31).

Affinity Chromatography on a Nickel-NTA-Agarose : Ni-NTA pull-down assay

Fifty µl of nickel-agarose beads were washed in IMAC 5 and saturated with 10 vol of IMAC 5 containing 3 % BSA for 1 hour at 4° C. In parallel, 4 µg of purified recombinant GST-p17 were incubated with 4 µg of recombinant Aurora-A(His)₆ in 500 µl of interaction buffer (IB: 50 mM Tris-HCl pH 8; 100 mM KCl; 5 mM MgCl₂; 0.1 % Triton X-100; 20 % glycerol) for 3 hours at 4° C on a wheel. Then the mixture was loaded on the beads and incubated at 4° C for 3 hours. The beads were washed twice during 10 min at 4° C in 10 vol of IMAC 5 and 3 times in 10 vol of IMAC 20..

Histidine-tagged proteins and proteins bound to histidine-tagged proteins were eluted with 20 µl of IMAC 250. The samples were mixed with 20 µl of 2X-Laemmli buffer and the proteins were separated on a 12.5 % SDS polyacrylamide gel and submitted to a western blot using the 1C1 antibody (dil : 1/200) and GST antibody (dil : 1/50,000).
**Affinity chromatography on a GST-4B Agarose : GST-pulldown assay**

50 µl of glutathione-sepharose 4B beads (Amersham Biosciences 17-0756-01) were washed in PBS and saturated with PBS containing 3% BSA for 1 hour at 4° C. Four µg of purified recombinant GST-p17 were incubated with 4 µg of recombinant Aurora-A(His)$_6$ in 500 µl of binding buffer (BB : 50 mM Tris-HCl pH 8 ; 50 mM NaCl ; 0,02 % BSA ; 0,02 % Tween-20 ; 1 mM PMSF) for 3 hours at 4° C. Then the mixture was loaded on the beads and incubated at 4° C for 3 hours. The beads were washed 5 times with WB300 during 10 min at 4° C. The proteins were eluted with 50 mM Tris-HCl pH 8 containing 10 mM glutathione, mixed with 20 µl of 2X-Laemmli sample buffer and separated on a 12.5 % SDS polyacrylamide gel. Nitrocellulose membranes were then submitted to western blot with the 1C1 antibody (dil : 1/200).

**In vivo co-immunoprecipitation :**

Ten µl of dried Affiprep protein-A (BioRad) were washed with 500 µl of Immunopure (A) IgG binding buffer (IBB-Pierce) and were incubated for 2 hours at 4° C with 500 µl of 1C1 antibody (dil :1/20) in IBB (dil: 1/2), and then washed twice with 500 µl TBS (50 mM Tris HCl pH 7.5 ; 150mM NaCl). Beads, were then incubated with a 10 equivalent oocytes extract (MII) for 2 hours at 4° C on a wheel. The beads were washed once in 500 µl of NaCl 0.5% and 5 times with 500 µl TBST (TBS-0.05% Tween 20).Bound proteins were eluted in 10 µl of 2X-Laemmlili sample buffer and the proteins were separated on a 12.5 % SDS polyacrylamide gel and then immunoblotted. The western blot analyses were performed with the anti-GST antibody (dil : 1/50,000) and the 1C1 (dil : 1/200).
**Protein kinase assay:**

Two µg of Aurora-A(His)$_6$ were incubated in 15 µl of the kinase buffer (50 mM Tris-HCl pH 7.5 ; 25 mM NaCl ; 1 mM DTT ; 10 mM MgCl$_2$) in the presence of 5 µCi [$\gamma$-32P] ATP at 3,000 Ci/mmol and 10 µg of GST-p17, or GST-p17 S626A, GST-maskin or GST-maskin S626A. The reactions were incubated for 10 min at 30° C, stopped by addition of 5 µl of 5x Laemmli sample buffer, separated on a 12.5 % SDS-PAGE and analyzed by autoradiography.

**Phosphatase treatment:**

A two oocytes *Xenopus laevis* extract was treated with four hundred units of λ−PPase for 60 min at 30° C (New Englands Biolabs). The proteins were then separated onto a 12.5 % polyacrylamide gel and transferred onto nitrocellulose membrane before western blotting with the antibody against *Xenopus laevis* maskin (dil : 1/5000).

**In vivo analysis:**

Denuded oocytes (stage VI) were prepared in Merriam buffer (88 mM NaCl ; 0.33 mM Ca(NO$_3$)$_2$ ; 1 mM KCl ; 0.41 mM CaCl$_2$ ; 0.82 mM MgSO$_4$ ; 10 mM Hepes pH 7.4) (33). Groups of 100 oocytes were injected with 35 ng of mp1 (35 nl at 1 mg/ml) or with the same volume of dilution buffer (2.5% DMSO in H$_2$O). Oocytes were stimulated for maturation with 3.10$^{-6}$ M of progesterone at 21° C and GVBD was monitored by the appearance of the white spot. Every hour after stimulation, oocytes were collected by groups of 10 and extracts were prepared with the extraction buffer (EB : 80 mM β-glycerophosphate, pH 7.5 ; 20 mM EGTA ; 15 mM MgCl$_2$ ; 1 mM DTT) as described in (34). The lysate was added with proteases
inhibitors and centrifuged for 30 min at 15,000 x g and frozen at –80° C until process. The extracts were then used to measure the histone H1 kinase activity with 0.25 oocyte equivalent.

**Western Blotting**

Oocytes extracts were electrophoresed on a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane using the Biorad system. The membranes were washed with TBST and saturated with 5% low fat milk in TBST for 2 hours at room temperature. Western blot analysis were performed for identification of *Xenopus laevis* Aurora-A, cyclin B2, cyclin B1, c-Mos and Cdc6 overnight at 4° C in 2.5 % low fat milk. The polyclonal antibodies raised against Aurora-A (dil : 1/5,000), cyclin B2 (dil : 1/5,000), cyclin B1 (dil : 1/5,000), c-Mos (dil : 1/5,000), Cdc6 (dil : 1/2,000) anti Phospho-Ser626-maskin (dil : 1/5,000) and the monoclonal antibody against Aurora-A 1C1 (dil : 1/200) were detected with appropriate horseradish peroxidase conjugated secondary antibodies (dil : 1/50,000 ; Jackson Immuno-Research Laboratories) and the western blot chemiluminescence Super Signal kit from Pierce (Perbio). Samples loadings in the different western blots were controlled with the polyclonal anti-β-tubulin (dil : 1/2,500).
RESULTS

Aurora-A interacts with maskin in vivo

A search for proteins interacting with XI-Aurora-A was performed by two-hybrid screen using Aurora-A as bait and a *Xenopus laevis* oocyte cDNA library. The strongest binding partner found (Fig. 1A) was encoded by a 435-bp cDNA that shared 88% identity with a portion of the CPEB-associated factor maskin cDNA (nucleotides 1545 to 1980). The isolated cDNA encoded a 145 amino acids peptides corresponding to a part of the original clone, hereafter-called p17 (Fig. 1B) (27). The p17 sequence was subcloned into a pGEX-4T-1 vector, the fusion protein GST-p17 was expressed in *E.coli*, then purified on a glutathione-Sepharose column. The recombinant GST-p17 protein run as a 56 kDa protein in SDS-PAGE (Fig. 1C lane 2, upper panel) and was recognized by a polyclonal antibody directed against GST-tag (Fig. 1C lane 1, lower panel). The recombinant GST-p17 protein (Fig. 1C lane 2) as well as the endogenous full length maskin from oocytes extracts (migration at 150 KDa but theoretically 110 KDa when calculated with the molecular weight of the amino acids) were both recognized with an anti-maskin antibody (Fig. 1C Lane 2 and 3). Comparison of the p17 sequence with those of XI-maskin and TACC from drosophila or human reveals a consensus sequence …[R]x[S*][L]… in which the serine residue (S*) is a site of phosphorylation for the TACC proteins (Fig. 1D).

As the two proteins interact in the two hybrid system, we asked whether maskin was phosphorylated in oocytes and whether it was an Aurora-A substrate.
**Aurora A and maskin interact in vitro.**

To investigate whether the protein maskin was a partner of Aurora-A, we performed two pull-down assays using recombinant tagged proteins. In a first series of experiments, Aurora-A(His)_6 was incubated with or without GST-p17 or GST, then the mixture was loaded onto glutathione-Sepharose beads. Aurora-A(His)_6 was found to bind only to the GST-p17 containing beads (Fig. 2A). In a second round of experiments, the protein GST-p17 previously incubated with or without Aurora-A(His)_6 was mixed with nickel-agarose beads (Ni-NTA-agarose) (Fig. 2B lane 1 and 2). GST-p17 was found to bind only the Aurora-A(His)_6 containing column (Fig. 2B lane 1). These results indicated that both proteins interacted directly in vitro.

**Maskin is phosphorylated in vivo**

To test whether maskin was phosphorylated in vivo, protein extracts from oocytes at different stage of maturation (prophase, GVBD an metaphase arrest) were separated on SDS-PAGE and we analyzed the phosphorylation status of the endogenous protein by western blot with an antibody anti maskin. The electrophoretic migration of maskin was compared in the extracts prepared from *Xenopus* oocytes arrested in prophase I (stage VI) and metaphase II (matured) before and after treatment with λ-PPase. The upper shift of the protein was not observed in the prophase extract consequently the treatment with the λ-PPase did not induce any electrophoretic mobility shift suggesting that, in prophase I oocytes, maskin might not be phosphorylated. (Fig. 2C lanes 1 and 2). In contrast, an upward shift of maskin was observed in GVBD and metaphase II arrested compared to prophase I arrested oocytes (Fig. 2C, compare lanes 1, 3 and 5). However a modification of the mobility was observed in both
GVBD and metaphase extract after treatment with the λ-PPase. (Fig. 2C, lanes 4 and 6). The shift was due to protein phosphorylation demonstrating that maskin is phosphorylated in vivo.

As maskin is phosphorylated in vivo, we asked whether Aurora A could participate to this phosphorylation. We therefore assess whether the interaction between Aurora-A and maskin occurs in oocytes. When endogenous Aurora-A was immunoprecipitated from prophase and metaphase II Xenopus oocytes extracts using anti-Aurora-A 1C1 antibody, maskin was found to co-immunoprecipitate (Fig. 2D lane 1, upper panel). Maskin was not detected in an immunoprecipitate performed with a monoclonal antibody (6E3) directed against the recombinant protein Aurora-A(His)₆ that does not recognize the endogenous Aurora-A protein (Fig. 2D lane 2). To determinate whether the interaction evolved during maturation, the presence of maskin in Aurora-A immunoprecipitate was compared between prophase I, GVBD and metaphase arrested oocytes (metaphase II). In both extracts, while the amount of immunoprecipitated Aurora-A increased between prophase I and GVBD oocytes as previously described (35) (Fig. 2D, lanes 3, 4 and 5, lower panel), the amount of maskin protein bound to Aurora-A did not increased suggesting that both proteins remain associated throughout oocyte maturation, from prophase I to metaphase II and that newly synthesized Aurora-A did not bind to maskin (Fig. 2D lanes 3,4 and 5). As previously observed in fig 2C, maskin presented an uppershift electrophoretic mobility in the GVBD and metaphase II stage suggesting that Aurora-A might be responsible for this shift.

**Aurora-A phosphorylates maskin in vitro**

Since we demonstrated a physical interaction between Aurora-A and maskin in vitro, we investigated whether the kinase could phosphorylate maskin. The Aurora-A(His)₆ protein
was incubated with the recombinant GST-p17 protein in the presence of $[\gamma-^{32}\text{P}]\text{ATP}$. Aurora-A did not phosphorylate GST alone as previously reported (Fig. 3A lane 3) (36). The incorporation of radioactivity in GST-p17 was observed in the presence of Aurora-A(His)$_6$ indicating that the kinase phosphorylated p17 \textit{in vitro} (Fig. 3A lane 2 upper panel). This experiment shows that the recombinant protein Aurora-A phosphorylates a fragment of maskin \textit{in vitro}.

**Maskin is an activating partner of Aurora-A:**

It has been reported that binding of Aurora-A to its physiological substrates such as TPX2 and Ajuba, results in the activation of the kinase (37,38). We thus investigated whether maskin was also an Aurora-A activator. Phosphorylation reactions were performed in the presence of $[\gamma-^{32}\text{P}]\text{ATP}$ with fixed amounts of Aurora-A(His)$_6$ and GST-H3 tail that contains the Ser (S10) phosphorylated by Aurora-A \textit{in vitro} (39,40). Increasing amounts of GST-maskin were added to the reaction and the activity of Aurora-A was measured on GST-H3 tail (Fig. 3B, middle panel). As expected, incorporation of $^{32}\text{P}$ onto the GST-maskin followed the increase of GST-maskin concentration in the reaction mix. Interestingly, the phosphorylation of GST-H3 tail was found to be more efficient as the amount of GST-maskin increased in the mixture (Fig. 3B, upper panel). The control performed in the presence of GST alone revealed that the GST protein is not able to amplify the kinase activity of the Aurora-A(His)$_6$ onto the substrate GST-H3 tail (data not shown). GST-maskin induced a stimulation of Aurora-A kinase activity on GST-H3 tail comparable to the stimulation observed in the presence of TPX2 (Fig. 3B lane 10). A quantification of the radioactivity incorporated in GST-H3
indicated that maskin provoked a 7 fold increase in Aurora-A(His)₆ activity (Fig. 3C). Maskin would then appear to be a third substrate activator of Aurora-A as Ajuba, and TPX2.

**Aurora-A phosphorylates Ser626 in maskin**

The maskin fragment p17 sequence was aligned with the maskin full-length sequence, human-TACC3 and D-TACC, two proteins previously shown to be phosphorylated by Aurora-A (41). A conserved Ser was found in a consensus sequence for Aurora kinase…[R]x[S][L]… in the three proteins as well as in p17 and maskin (Fig. 1D) (42). In maskin, the Ser is located at position 626 (Fig. 1B).

We mutated the amino acid corresponding to the Ser626 in the GST-p17 and GST-maskin sequence and asked whether Aurora-A could phosphorylate this mutant. When GST-p17-S626A protein was incubated in the presence of Aurora-A, the amount radioactivity-incorporated was very low compared to the radioactivity incorporated in the wild type protein (Fig. 4A, lane 2 and 3 upper panel). An antibody prepared against phosphorylated Ser626 revealed that the amino acid was fully phosphorylated in the GST-p17 protein while it was not in the GST-p17S626A.

We then synthesized two peptides of 18 amino acids mimicking the sequence surrounding maskin Ser626. The peptide mp1 corresponded to the wild type sequence with a Ser residue at position 626. The peptide Smp1 was the scramble peptide of mp1 (Fig. 4B). The two peptides were incubated with Aurora-A(His)₆ in the presence of [γ-³²P]ATP. The peptide mp1 was readily phosphorylated by Aurora-A(His)₆ whereas Smp1 was not phosphorylated as shown by a low level of radioactivity incorporated in a ten-fold amount of substrate (Fig. 4C histogram). These results indicated that Aurora-A phosphorylated the Ser in
the mp1 peptide that mimics the sequence surrounding Ser626 in the full-length sequence of maskin. There was no phosphorylation onto the Ser in the scramble peptide.

If Ser626 was phosphorylated by Aurora-A in GST-p17, the addition of an excess amount of peptide in the kinase reaction should compete with the phosphorylation of GST-p17 or GST-maskin. The inhibitory effect of both mp1 and Smp1 peptides was tested in vitro on the phosphorylation of GST-maskin by Aurora-A(His)₆. Ten µg of the different peptides were added to a phosphorylation reaction containing Aurora-A(His)₆, GST-maskin and [γ-³²P]ATP (Fig. 5A lane 1). Unlike the scramble peptide (Fig. 5A lane 3), the mp1 peptide inhibited the incorporation of the radioactivity into GST-maskin (Fig. 5A lane 2). The phosphorylated form of GST-maskin was tested for the presence of phosphorylated Ser626. The antibody detected Ser626 phosphorylated onto GST-maskin incubated in the presence of Aurora-A(His)₆ alone or together with the Smp1 peptide (Fig. 5A lanes 1 and 3 lower panel). In contrast, no signal was observed in the presence of the mp1 peptide (Fig 5A, lane 2 lower panel). The same experiment was carried out with the GST-maskin-S626A (Fig 5A lanes 4 and 5). As expected the antibody directed against phosho-Ser626 did not detect GST-maskin S626A when phosphorylated by Aurora-A, however maskin mutated on Ser626 remained heavily phosphorylated by Aurora-A indicating that the kinase phosphorylated several sites on maskin. In contrast Aurora-A(His)₆ could not phosphorylate GST-maskin wt or GST-maskin-S626A in the presence of mp1 indicating that the peptide inhibited Aurora-A(His)₆ activity.
**The peptide mp1 inhibits the phosphorylation of maskin in vitro:**

In order to examine the effect of the mp1 synthetic peptide in the phosphorylation of the maskin full length protein, we incubated the recombinant protein GST-maskin in a phosphorylation assay in the presence of a metaphase extract that contains Aurora-A active kinase, with or without the synthetic peptides mp1 and Smp1 (Fig. 5B). Upon incubation in the presence of $\gamma^{32}$P]ATP and metaphase oocyte extracts, we observed an incorporation of radioactivity in the recombinant GST-maskin (Fig. 5B, lane 1 and 3 upper panel). The antibody directed against phospho-Ser626 detected maskin on Western Blot indicating that Ser626 was phosphorylated. Incorporation of radioactivity was also detected when GST-maskin-S626A was incubated in metaphase extracts. It was only in the presence of mp1, that using an antibody directed against the phospho-Ser626, that a decrease in Ser626 phosphorylation was observed in wild-type maskin (Fig. 5B lane 2).

As expected the same antibody did not detect the GST-maskin-S626A mutant when phosphorylated by metaphase extracts. However maskin mutated on Ser626 remained heavily phosphorylated by metaphase extracts suggesting that one or several kinase phosphorylated maskin on other residue than Ser626 (Fig. 5B, lane 1 and 5 lower panel). However, a metaphase extract still phosphorylated GST-maskin-S626A in the presence of the mp1 peptide. The same level of radioactivity incorporation was detected indicating that, indeed, maskin was phosphorylated on other residues than Ser626 by kinases different from Aurora-A considering that mp1 inhibited Aurora-A in vitro.

Our results suggest (1) that oocyte extracts phosphorylated maskin onto Ser626 (2) that addition of mp1 peptide specifically reduced the phosphorylation of the Ser626 by Aurora-A in the extract and (3) that the Ser626 was phosphorylated by Aurora-A even if the
protein was phosphorylated onto other amino acids by other kinases present in oocyte extracts.

**Injection of the peptide mp1 in oocytes induces early maturation**

We injected the peptide mp1 into prophase I arrested *Xenopus* oocytes and then induced the oocyte maturation with progesterone. We monitored the oocyte maturation by analyzing the appearance of the white spot. No maturation was observed when the injected oocytes were not treated with progesterone (data not shown). The oocytes injected with the Ser626 containing peptide mp1 reproducibly underwent GVBD earlier than the oocytes injected with the dilution buffer (Fig. 6A).

To get deeper insight into the understanding of mp1 effect on GVBD, we investigated the pre-MPF into MPF conversion by measuring histone H1 kinase activity (Fig. 6B and C first panels) and cyclin B2 phosphorylation (Fig. 6B and C second panels). The MPF was activated 3 hours earlier in the presence of mp1 than in the presence of the dilution buffer. A star on the Fig. 6 indicated the GVBD time. Consistent with the premature resumption of meiosis in mp1 injected oocytes, an early synthesis of c-Mos protein was also observed (Fig. 6 B and C fourth panels). When we measured the appearance of cyclin B1, a protein synthesized for meiosis II (13), we found an early synthesis of the protein in the oocytes injected with the mp1 peptide (Fig. 6B and C, third panels). To confirm this finding, we analyzed the appearance of Cdc6, a protein that is normally synthesized only after meiosis I. Just like cyclin B1, Cdc6 synthesis was detected much earlier in the mp1 injected oocytes (Fig. 4B and C fifth panels). Though, the appearance of both Cyclin B1 and Cdc6 occurred at the same time after GVBD (3 hours after GVBD). C-Mos, Cyclin B1 and Cdc6 mRNAs
contain CPE sequences (28,43) that are presumably used to regulate translation during oocyte maturation (16). Our results indicate that the injected oocytes enter prematurely in metaphase II stage.
DISCUSSION

The present study explores the role of the interaction of Aurora-A with the post-transcriptional regulator factor "maskin" during *Xenopus* oocyte maturation. Using the two-hybrid system analysis and recombinant proteins, we show evidence of a physical interaction between the two proteins *in vitro*. An *in vivo* analysis during oocyte maturation revealed that the interaction is necessary to prevent the entry of oocyte into the meiosis II before the completion of the meiosis I.

In *Xenopus*, oocytes maturation is regulated in part by mRNAs that are synthesized and stored in prophase arrested oocytes. These mRNAs are not translated in the same time but at specific times along meiotic division. In immature oocytes, dormant CPE-containing mRNAs are bound to CPEB, itself bound to maskin, preventing the translation.

Recent papers have described the cloning in drosophila and mammals of a centrosomal protein that co-purify with microtubules. This protein called TACC transforming acid coiled coil protein has been shown to interact with centrosomes and microtubules. Among the three TACC proteins identified in mammals, each of them interacts in a different and specific way. (44, 45). In *Xenopus*, a TACC protein called maskin has been identified (27). Maskin is part of a complex that associates with the 3'UTR via CPEB in order to regulate the translation of specific mRNAs. As described for human or drosophila TACC, maskin is localized onto the mitotic apparatus (25). In *Xenopus* embryos, immunostaining studies clearly showed that maskin and Aurora-A colocalize. The same observation has been made in *Xenopus* somatic cells, where maskin, as Aurora A, is localized to the centrosome and along the spindle during the metaphase (25). The picture is not as clear in *Xenopus* oocytes although maskin has been detected moderately concentrated at the animal pole while Aurora-A was specifically...
localized on the spindle. Recent papers have described a potential role Aurora-A in the separation of chromosomes and the spindle rotation during meiosis in *Xenopus* oocytes (46). There is no information on these two proteins in *Xenopus* oocytes and a role of maskin in the meiotic process has not been documented.

Recently, in drosophila, a physical interaction between D-TACC and Aurora A has been shown to be needed for the recruitment of D-TACC onto the mitotic apparatus (41). Consistent with these previous observations, we wondered whether maskin and Aurora-A interact in *Xenopus* oocytes and what is the physiological significance of this possible interaction. In a first round of experiments, we demonstrate for the first time *in vitro* that Xl-Aurora-A phosphorylates GST-p17, probably onto the amino acid Ser626. These results lead us to hypothesize that Aurora-A could interact with and phosphorylate maskin *in vivo* in order to recruit maskin onto the centrosome (25). Immunoprecipitation experiments revealed that Aurora-A and maskin interact *in vivo*. The interaction is detectable in prophase I and metaphase II oocytes in accord with the accumulation of the two proteins throughout oocyte growth (25,35). We observed that the protein maskin is phosphorylated during meiosis, only after GVBD as shown by the differential action of the λ–PPase. This phosphorylation has never been reported before but concur with the activation of Aurora-A at the same period (35). While it is too premature to affirm that this phosphorylation is owed to Aurora-A, we can hypothesize that Aurora-A is partially responsible of the upper shift observed in our assays.

Such hypothesis raises the question whether maskin is a physiological substrate for Aurora-A. Other physiological substrates of Aurora-A such as TPX2 (Targeting protein for *Xenopus* kinesin like protein 2) and Ajuba, were identified as binding activator partner of
Aurora-A (37,38). As supported by the \textit{in vitro} phosphorylation assay in the presence of the substrate H3, maskin is also an Aurora-A activating factor. In the absence of GST-maskin, Aurora-A phosphorylated moderately the GST-H3 tail and this activity increased in a GST-maskin concentration dependent manner. It remains possible that a priming phosphorylation of Aurora-A may occur in order to initiate a basal activity, then the association with the physiological substrate will mediate a hyper-activation of the kinase (47). As the detection of maskin in the two-hybrid screen was performed with the catalytic domain of Aurora-A, we could hypothesize that, as TPX2, maskin binds to the catalytic domain of Aurora-A. In contrast, Ajuba interacts directly with the N-terminal non-catalytic domain (38,47).

What is the role of the phosphorylation of maskin? The identification of maskin as a substrate of Aurora-A, clearly places the kinase upstream the translation of mRNA coding for proteins that are necessary for oocyte maturation. The mRNAs initiating the process of oocyte maturation are those encoding c-Mos or cyclin B. In \textit{Xenopus}, c-Mos protein kinase is an efficient activator of the MAPK kinase pathway and is able to induce meiotic maturation when injected in prophase I oocyte (5). \textit{Xenopus} mos also induces oocyte maturation in the absence of progesterone, but it is not clear whether its synthesis is sufficient, and required, to initiate the meiotic maturation. The exact role of mos during oocyte maturation is still a matter for debate (10,18,48). Injection of morpholinos designed to inhibit the expression of the protein mos, into prophase I oocyte, does not affect the entry into meiosis I, but the role of mos in meiosis could be to prevent the mitotic cycle after meiosis I, and to trigger the cell cycle arrest in oocytes, in order to attempt fertilization (10). On the other hand, injection of mRNA encoding mutant CPEB completely blocked endogenous c-Mos synthesis and oocyte
maturation (9).

In *Xenopus* oocytes, the masking of mRNAs encoding c-Mos or cyclin B is mediated by the association of maskin with CPEB and eIF-4E making a loop that inhibits the association of the polyA polymerase onto the 3’UTR (27). Progesterone stimulation of maturation induces the activation of Aurora-A and the phosphorylation of the protein CPEB on the Ser174. This phosphorylation is the first step that controls the polyadenylation of mRNA encoding cyclin B1, which is necessary for the translation of the protein. This polyadenylation depends on the adenylation and translation of c-Mos (49). The phosphorylated CPEB protein recruits the CPSF protein and helps it to bind to the hexanucleotide and then recruits the polyA polymerase to the end of the mRNA (24).

Our study clearly demonstrates that Aurora-A phosphorylates maskin *in vitro*. Peptides mimicking the phosphorylated sequence of maskin, act as competitors in the kinase assay and inhibit the phosphorylation of the substrate GST-p17. These peptides, which also inhibit Aurora-A catalyzed phosphorylation of GST-H3 tail, or MBP, appears to be useful tools to inhibit Aurora-A kinase activity. The peptide mp1 is a substrate of Aurora-A. It is phosphorylated by Aurora-A and can inhibit by competition, the phosphorylation of other substrates.

As demonstrated *in vitro*, we expected that, *in vivo*, the phosphorylation of maskin depend on Aurora-A. So in order to determine if the phosphorylation of maskin by Aurora-A is involved in the oocyte maturation process, we studied the consequence of the injection of the mimicking peptides on the oocyte maturation. The mp1 peptide induced an early entry in metaphase I when injected in *Xenopus* oocytes before their stimulation with progesterone.
The injection of a non peptidic specific inhibitor of Aurora A capable of preventing the \textit{in vitro} phosphorylation of maskin and other substrates by Aurora A (data not shown-confidential) had a similar effect on oocyte maturation. This strongly suggests that \textit{in vivo}, the peptides mp1 interfere with Aurora-A kinase activity. Our results clearly show that the inhibition of Aurora-A activity in oocytes stimulated with progesterone, induces a precocious maturation by affecting mRNA translation.

How does the inhibition of Aurora A lead to advance progesterone induced maturation of oocytes? Based on the appearance of the white spot on the oocytes, and confirmed by the premature synthesis of mos, cyclin B1 and Cdc6, the inhibition of Aurora-A provokes an advance of GVBD. This advance never exceeds 2 or 3 hours, which corresponds to the amount of time necessary for the oocytes to complete meiosis I after GVBD. Our hypothesis is that Aurora-A phosphorylates maskin to ensure that it stays associated with CPEB between GVBD and meiosis I, and that, consequently, a specific population of mRNA remains masked. The inhibition of Aurora-A which would prevent maskin phosphorylation, would eliminate the 2 hours gap between GVBD and the end of meiosis II, and trigger the translation of mRNAs that are normally translated in meiosis II, or at least when meiosis I is complete. It is thus possible that Aurora-A phosphorylates maskin to prevent synthesis of meiosis II proteins during meiosis I. Aurora-Activity would then protect meiosis I from protein required for meiosis II.

The exact role of Aurora-A in meiotic maturation is still not clear. On one hand, the ectopic expression of the active kinase is known to accelerate GVBD in \textit{Xenopus} (50). On the other hand, our results show that the inhibition of the kinase activity also induces an advance in
oocyte maturation (51). However, in contrast to control oocytes, the synthesis of both c-Mos and cyclin B1 is initiated at the same time after GVBD in mp1 injected oocytes. Also, Cdc6 that controls the competence of the oocyte to replicate its DNA, must be repressed in meiosis I, and the repression must be removed in meiosis II to allow DNA replication after fertilization (26,52). This protein is synthesized earlier in mp1 injected oocytes too. Aurora-A exhibits a biphasic pattern of phosphorylation and activation that coincides with that of MPF, and the expression of a constitutively active Aurora-A appears to be incompatible with the meiosis I to meiosis II transition (53). In vivo, the phosphorylation of maskin by Aurora-A could protect the oocytes going through meiosis from entering mitotic cycle without DNA synthesis. As the Aurora-A activity decreases at the meiosis I to meiosis II transition the two proteins would temporarily dissociate. The consequence of this dissociation at the end of the meiosis I would be the loss of the masking function of the complex eIF4E/maskin/CPEB. Consequently, the polyadenylation and then translation of the mRNAs encoding c-Mos and cyclin B1 could happen.

In conclusion, we evidence the direct interaction of Aurora-A and maskin both in vitro and in vivo. We show that Aurora-A phosphorylates maskin in vitro on Ser626 and further demonstrate that maskin is a new substrate/activator of Aurora-A. The function of maskin phosphorylation by Aurora-A during oocyte maturation could be to ensure the masking of specific mRNAs to prevent, during meiosis I, the synthesis of proteins required solely for meiosis II, and to prevent the oocyte to enter metaphase II prematurely.
FIGURE LEGENDS

Fig. 1:

A: Identification of p17 as a new Aurora-A binding protein in a two-hybrid screen

The β-galactosidase activity of the yeast colonies co-transfected with both two-hybrid vectors pGADGH and pGBT9 (upper panel); pGADGH-p17 and pGBT9-pAurora-A (lower panel).

B: Scheme of maskin and p17 identified in the two-hybrid system

C: Expression of a recombinant GST-p17 protein

Upper panel: GST-p17 recombinant protein was overexpressed in bacteria and purified. Protein from bacterial extract (“NI” : no induction with IPTG and “I” after induction) were analyzed by SDS-PAGE and stained with Coomassie blue. Recombinant GST-p17 purified protein (lane 1 and 2) or proteins from *Xenopus* oocytes extract (stage VI, lane 3) were analyzed by Western blot with an anti-GST antibody (dil : 1/50,000 lane 1) or an anti-maskin antibody (dil : 1/5,000 lane 2-3).

D: Comparison of p17 sequence with maskin, Hs-TACC3 and D-TACC

The three sequences share a common …[KR]x[ST][ILV]… sequence (boxed in grey), a consensus site for Aurora kinase, around Ser626 in maskin. The p17 protein sequence displays 88% of homology with maskin protein sequence. The fragment p17 is a sequence comprise between the amino acid 515 and 660 of maskin.
**Fig. 2:**

**GST-p17 interacts with Aurora-A(His)₆ in vitro and in vivo**

**A:** GST-p17 (lane 1), GST (lane 2) or binding buffer (BB) alone (lane 3) were incubated with Aurora-A(His)₆ and then bound to glutathione-sepharose 4B beads. The presence of Aurora-A(His)₆ was tested with anti-IC1 antibody (dil : 1/200).

**B:** Aurora-A(His)₆ (lane 1) or Interaction buffer (BB) alone (lane 2) were incubated with GST-p17 and then bound to Ni-NTA-agarose beads. The presence of GST-p17 was tested with anti-GST antibody (dil : 1/50,000).

**C: Maskin is phosphorylated in vivo during oocyte maturation**

The protein maskin extracted from *Xenopus* oocytes arrested in prophase I (lanes 1 and 2) or in metaphase II (lanes 3 and 4) was analyzed by SDS-PAGE before (-) and after λ–PPase treatment (+) and immunoblotted with an antibody against maskin (dil : 1/5,000).

**Maskin and Aurora-A interact in vivo**

**D:** Aurora-A was immunoprecipitated from metaphase II oocyte extract with the 1C1 monoclonal antibody (lane 1) or with the 6E3 antibody (lane 2). Aurora-A and maskin were immunodetected with a polyclonal antibody against Aurora-A (dil : 1/200 lower panel) or with a polyclonal antibody against maskin (dil : 1/5,000 upper panel) respectively.

Aurora-A was immunoprecipitated from prophase I oocyte extract (stage VI, lane 3) GVBD oocytes extracts (lane 4) and metaphase II oocyte extract (lane5) with the 1C1monoclonal antibody (lane 5). Aurora-A and maskin were immunodetected with a polyclonal antibody against Aurora-A (dil : 1/200 lower panel) or with a polyclonal antibody against maskin (dil : 1/5,000 upper panel) respectively.
**Fig. 3:**

**A:** **GST p17 is phosphorylated by Aurora-A *in vitro***

Aurora-A(His)_6 was incubated alone (lane 1) or with GST-p17 (lane 2) in presence of \([\gamma^{32}\text{P}]\text{ATP}\). GST-p17 alone was subjected to the same reaction (lane 3). Samples were separated by SDS-PAGE and the gel was stained with Coomassie blue (lower panel). The GST-p17 phosphorylation was analyzed by autoradiography (upper panel).

**B:** **GST-maskin is an activator of the Aurora-A(His)_6 kinase***

A phosphorylation reaction involving constant amount of Aurora-A(His)_6 (excepted for lane 6 and 7, no kinase) and GST-H3 tail (excepted for lane 6 and 8, no GST-H3 tail) was performed in the presence and increasing amounts of GST-maskin (0, 1, 2.5, 5 µg, lanes 2-5) and \([\gamma^{32}\text{P}]\text{ATP}\). The reaction was stopped by adding Laemmli sample buffer and analyzed on a 12.5% SDS-polyacrylamide gel. The gels were stained by Coomassie blue (lower panel) and the phosphorylation of the GST-H3 tail (middle panel) and GST-maskin (upper panel) was analyzed by autoradiography.

The same phosphorylation reaction was performed with constant of Aurora-A(His)_6 and GST-H3 tail in the absence (lane 9) or presence (lane 10) of GST-TPX2 (10 µg,) and \([\gamma^{32}\text{P}]\text{ATP}\). The reaction was stopped by adding Laemmli sample buffer and analyzed on a 12.5% SDS-polyacrylamide gel. The gels were stained by Coomassie blue (lower panel) and the phosphorylation of the GST-H3 tail (middle panel) and GST-TPX2 (upper panel) was analyzed by autoradiography.
C: Quantification of Aurora-A activation

Autoradiograms of the experiments performed as described in fig 3C were quantified by Image Quant analysis and plotted as a function of the GST-maskin added in the incubation mixture.

Fig. 4:

A: Aurora-A phosphorylation site on GST-p17

Aurora-A(His)₆ was incubated alone (lane 1) or with GST-p17 (lane 2) in presence of [γ³²P]ATP. GST-p17 S626A was subjected to the same reaction (lane 3). The reaction was stopped by adding Laemmli and samples were separated by SDS-PAGE and the gel was stained with Coomassie blue (lower panel). The GST-p17 phosphorylation was analyzed by autoradiography (upper panel). The gels were stained by Coomassie blue (middle panel) and the phosphorylation of the GST-p17 (lower panel) was analyzed by western blot with the polyclonal antibody against the phospho-Ser626 (dilution 1/5,000).

B: Sequences of the two synthetic peptides

mp1 is a 18 amino-acid peptide that contains the consensus sequence …RxxSL… for Aurora-A. The Smp1 was the scramble peptide of mp1.

C: The mp1 is phosphorylated by Aurora-A(His)₆ on the Ser626

Two µg of mp1 (lane1), 2 µg of Smp1 (lane 2) 20 µg of Smp1 (lane 3) were used in a phosphorylation assay together with 2 µg of Aurora-A-(His)₆. Incorporation of radiolabeled phosphate was measured by Cerenkov scintillation counting.
Fig. 5:

A: The peptide mp1 inhibit phosphorylation of GST-maskin but not Smp1

The peptides mp1 and Smp1 were added to a kinase assay performed in the presence of Aurora-A(His)$_6$ and GST-maskin and $[^{32}P]ATP$ (lane 1). The effect of 10 µg of mp1 (lane 2) and 10 µg of Smp1 (lane 3) was tested on GST-maskin phosphorylation by Aurora-A(His)$_6$. The same experiment was performed with Aurora-A(His)$_6$ and GST-maskinS626A (lane 4) in the presence of mp1 (lane 5). Samples were analyzed by electrophoresis on a 12.5% SDS-PAGE. The gel was stained by Coomassie blue (middle panel) and autoradiographed (upper panel). A Western blot was performed with the antibody against phospho-Ser626 (dilution 1/5,000)

B: Effect of mp1 and Smp1 on the phosphorylation of GST-maskin by metaphase oocyte extract

The peptides mp1 and Smp1 were added to a kinase assay performed in the presence of a metaphase extract and GST-maskin $[^{32}P]ATP$ (lane 1). The effect of 10 µg of mp1 (lane 2) and 10 µg of Smp1 (lane 3) was tested on GST-maskin phosphorylation by the extract. The same experiment was performed with the metaphase extract and GST-maskin-S626A (lane 4) in the presence of mp1 (lane 5). Samples were analyzed by electrophoresis on a 12.5% SDS-PAGE. The gel was autoradiographed (upper panel) stained by Coomassie blue (middle panel). A Western blot was performed with the antibody against phospho-Ser626 (dilution 1/5,000)
Fig. 6:

Effect of the injection of the peptide mp1 on the kinetic of oocyte maturation

**A:** Prophase I oocytes were injected with 35 ng of mp1 or 2.5% DMSO in H₂O (dilution buffer), and were then stimulated with 3 µM progesterone. Scoring the appearance of the white spot monitored GVBD.

**B and C:** Oocyte extracts were prepared from ten injected oocytes every hour following progesterone addition. Stage VI oocyte were injected with either the buffer alone (Fig. 6B), mp1 (Fig. 6C). The extracts were analyzed for their MPF kinase activity (Histone H1 kinase activity) and for oocyte maturation markers behavior by Western blot (cyclin B2, cyclin B1, c-Mos and Cdc6). The β-tubulin revealed by Western blot was used as loading control.
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Pascreau et al. 2004 Fig 1 A, B, C & D
Pascreau et al. 2004 Fig 2 A, B & C
Pascreau et al. 2004 Fig 2 D
Pascreau et al. 2004 Fig 3 A

Aurora-A(His)$_6$ + + −
GST-p17 − + +

$^{32}$P GST-p17 →

GST-p17 →
Aurora-A(His)$_6$ →

1 2 3
Pascreau et al. 2004 Fig 3 B

Aurora-A(His)$_6$ + + + + + − − − +
GST-H3 + + + + − − + −
GST-maskin − + + − −

$^{32}$P-GST-maskin

$^{32}$P-GST-H3

GST-maskin

Aurora-A(His)$_6$

GST-H3

1 2 3 4 5 6 7 8

Aurora-A(His)$_6$ + +
GST-H3 + +
GST-TPX2 − +

$^{32}$P GST-H3

GST-TPX2

Aurora-A(His)$_6$

GST-H3

9 10

Pascreau et al. 2004 Fig 3 B
A

| Aurora-A(His)$_6$ | + | + | + | - |
|-------------------|---|---|---|---|
| GST-p17           | - | + | - | + |
| GST-p17 S626A     | - | - | + | - |

^{32}P GST-p17

GST-p17

Aurora-A(His)$_6$

Phospho Ser$_{626}$ GST-p17

1 2 3 4

B

mp1: SFKESVLRKQSLYLKFDP

Smp1: LSLYFEKSQSKVLRPDK

C

\[\text{32P incorporation (cpm \times 10^3)}\]

\[\text{1 2 3 4} \]

\[\text{2 \mu g peptide} \]

\[\text{20 \mu g peptide} \]

Pascreau et al. 2004 Fig 4 A, B & C
|                | 1 | 2 | 3 | 4 | 5 |
|----------------|---|---|---|---|---|
| Aurora-A(His)$_6$ | + | + | + | + | + |
| GST-Maskin       | + | + | + | - | - |
| GST-Maskin S626A | - | - | - | + | + |
| mp1              | - | + | - | - | + |
| Smp1             | - | - | + | - | - |

Pascreau et al. 2004 Fig 5 A
|                | MII extract | GST-Maskin | GST-Maskin S626A | mp1 | Smp1 |
|----------------|-------------|------------|------------------|-----|------|
|                | +           | +          | −                | −   | −    |
|                | +           | +          | −                | +   | +    |
|                | −           | −          | +                | +   | +    |
|                | −           | −          | −                | +   | +    |

**Passcreau et al. 2004 Fig 5 B**

- **32P GST-Maskin**
- **GST-Maskin**
- **β-tubulin**
- **P-Ser$_{626}$ Maskin**

1  2  3  4  5
Pascreau et al. 2004 Fig 6 A, B & C
Phosphorylation of maskin by aurora-A participates to the control of sequential protein synthesis during Xenopus laevis oocyte maturation
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