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PUBLISHER'S NOTE

Publisher's Note: Progesterone regulates the accumulation and the activation of Eg2 kinase in *Xenopus* oocytes (*J. Cell Sci.* 113, 1127-1138)

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Journal of Cell Science was made aware of several issues raised by readers concerning duplication of data in Fig. 6A, Fig. 10B, Fig. 7A and Fig. 8A,B in *J. Cell Sci.* (2000) **113**, 1127-1138.

After discussion with the corresponding author, Catherine Jessus, the journal referred this matter to Université Pierre et Marie Curie (UPMC, now Sorbonne Université), who investigated and cleared the authors of any wrongdoing. The UPMC committee concluded that no correction was necessary (full reports available at: http://www2.cnrs.fr/sites/communique/fichier/rapport_conclusions.pdf and http://www2.cnrs.fr/sites/communique/fichier/rapport_analyse_detaillee.pdf).

The editorial policies of Journal of Cell Science state that: "Should an error appear in a published article that affects scientific meaning or author credibility but does not affect the overall results and conclusions of the paper, our policy is to publish a Correction…" and that a Retraction should be published when "…a published paper contain[s] one or more significant errors or inaccuracies that change the overall results and conclusions of the paper…". Journal of Cell Science follows the guidelines of the Committee of Publication Ethics (COPE), whose advice on this case was: "…if the findings as described in the text are unchanged and the conclusion is unchanged, then it is entirely reasonable for the journal to issue a note, alerting readers, from now on, to the issues with the blots." As the investigating committee at UPMC cleared the authors of wrongdoing and concluded that no Correction was necessary, the appropriate course of action – according to the advice from COPE – is to publish a Publisher's Note, which the journal has made as detailed as possible.

The authors were unable to locate original data for this paper. The policy of the UPMC is that authors should retain original data for 10 years and the paper falls outside this period.

The authors justify the use of the same control data in blots in Fig. 6A and Fig. 10B, and Fig. 7A and Fig. 8A,B since the conditions were exactly the same in each case. They use two alternative phrases, a precise one ('2 hours later') and a more global one ('when 100% GVBD was reached'), to indicate the same time of collection of oocytes and state that: "The re-use of Cyclin B2 and Eg2 blots is fully justified as they arise from one single experiment, using the same oocytes and same conditions, from which different parts are illustrated in different figures. The same controls were re-used each time and are identical despite the slightly different wording [in the figure legends]".

Readers should also note that there are unmarked splices on some of the blots in the paper. Although such splicing is not acceptable by today's standards, it was a common practice when the paper was published, which was before the journal's policy on figure manipulation was implemented (early in 2013).

Journal of Cell Science refers readers to other notices related to the UPMC investigation:

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Progesterone regulates the accumulation and the activation of Eg2 kinase in *Xenopus* oocytes

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SUMMARY

***Xenopus* prophase oocytes reenter meiotic division in response to progesterone. The signaling pathway leading to Cdc2 activation depends on neosynthesized proteins and a decrease in PKA activity. We demonstrate that Eg2 protein, a *Xenopus* member of the Aurora/Ipl1 family of protein kinases, accumulates in response to progesterone and is degraded after parthenogenetic activation. The polyadenylation and cap ribose methylation of Eg2 mRNA are not needed for the protein accumulation. Eg2 protein accumulation is induced by progesterone through a decrease in PKA activity, upstream of Cdc2 activation. Eg2 kinase activity is undetectable in prophase and is raised in parallel with Cdc2 activation. In contrast to Eg2 protein accumulation, Eg2 kinase activation is under Cdc2 control. Furthermore, by using an anti-sense strategy, we show that Eg2 accumulation is not required in the transduction pathway leading to Cdc2 activation. Altogether, our results strongly suggest that Eg2 is not necessary for Cdc2 activation, though it could participate in the organization of the meiotic spindles, in agreement with the well-conserved roles of the members of the Aurora family, from yeast to man.**

Key words: Eg2, Cdc2, PKA, Progesterone, *Xenopus* oocyte, Aurora kinase

INTRODUCTION

In the *Xenopus* ovary, oocytes are arrested at the G_2/M boundary of the first meiotic division. This G_2/M block is released by progesterone that triggers after a lag period of 4 to 6 hours the activation of the Cdc2/cyclin B2 complex or MPF (M-phase promoting factor), the universal inducer of the G_2/M transition (reviewed by Jessus and Ozon, 1993). The progesterone-transduction pathway leading to MPF activation is not yet elucidated, as well as the majority of the Cdc2 targets required for the completion of meiotic divisions. Cdc2 is activated through the dephosphorylation of its two inhibitory residues, Thr14 and Tyr15. The Cdc25 phosphatase, which is itself activated by complex phosphorylation reactions involving the Plx1 kinase, catalyzes this activation reaction (Jessus and Ozon, 1995; Karaiskou et al., 1999; Kumagai and Dunphy, 1996; Qian et al., 1998).

Two controls involved in the pathway leading to Cdc2/Cdc25 activation are known. The first one depends on the synthesis of new proteins (Wasserman and Masui, 1975), among them is c-Mos (Sagata et al., 1988), which indirectly leads to the activation of MAP kinase (reviewed by Sagata, 1997). However, it is not yet clear how the MAP kinase pathway and the MPF activation pathway are linked. The second control is achieved through the inhibition of the cAMP-dependent protein kinase, PKA (Maller, 1990; Maller and Krebs, 1977). However, the in vivo targets of PKA remain unknown. The possibility that PKA could regulate the neosynthesis and/or the stabilization of proteins needed for MPF activation is an attractive hypothesis. A major objective is therefore to look for proteins whose synthesis and/or stability are needed for MPF activation and that are controlled by PKA.

Cdc2 activation in *Xenopus* oocytes correlates with a burst in protein phosphorylation, resulting from the activation of multiple Ser/Thr kinases (Jessus and Ozon, 1993; Maller et al., 1977). Whether the kinase activation represents an upstream or downstream event of Cdc2 activation remains to be determined. A kinase that is activated before Cdc2 might be involved in the transduction pathway that leads to Cdc2 activation whereas a kinase whose activation depends on Cdc2 activity might represent a component of the phosphorylation cascade required for meiotic divisions. Eg2 is an attractive candidate as a protein whose translation is controlled during maturation and as a kinase playing a role in meiotic divisions. Eg2 protein is a member of the Eg family, whose mRNAs are adenylated during meiotic maturation (Paris et al., 1991). Eg2 also belongs to the Aurora/AIRK emerging family of protein kinases (Giet and Prigent, 1999; Roghi et al., 1998). The highest levels of expression of the various members of this family are found in the gonads (Gopalan et al., 1997; Kimura et

al., 1997; Roghi et al., 1998; Schumacher et al., 1998a,b; Terada et al., 1998; Yanai et al., 1997), and all of them are expressed in germ cells, IAK3 being exclusively germinal (Gopalan et al., 1999). An interesting characteristic of the members of the Aurora family resides in the oscillations of their protein level during the cell cycle, peaking in M-phase and being destroyed as the cells reenter interphase (Gopalan et al., 1997; Kimura et al., 1997; Terada et al., 1998). Based on the adenylation regulation of Eg2 mRNA during maturation and the oscillations of the Aurora proteins during the mitotic cell cycle, one can expect that Eg2 translation is also highly regulated during meiotic division.

Aurora family members regulate microtubule-based mitotic events (Bischoff and Plowman, 1999; Giet and Prigent, 1999). They could function at distinct stages of mitosis, some of them controlling early mitotic events, i.e. centrosome separation and bipolarity of the spindle, while other ones would play an essential role in late mitotic or meiotic events, controlling cytokinesis and extrusion of the polar body in oocytes. Since Eg2 kinase, like the other members of the Aurora family, controls spindle function (Giet et al., 1999; Roghi et al., 1998), it could therefore play a role downstream of MPF activation, and participate in the establishment of meiotic spindles.

Surprisingly, Andresson and Ruderman (1998) reported that Eg2 protein electrophoretic migration is retarded within 30 minutes after progesterone addition in *Xenopus* oocytes, suggesting that Eg2 kinase is phosphorylated and could be an early component of the progesterone-activated signaling pathway. This observation raises at least three interesting questions:

(1) Is Eg2 kinase activated during the early steps of the meiotic maturation process, as reported by Andresson and Ruderman (1998), or downstream of Cdc2 activation, as suggested by the well-conserved function of the Aurora family members in eukaryotic cells?

(2) Is the Eg2 protein level controlled by progesterone upstream or downstream of Cdc2 activation?

(3) Is Eg2 necessary for meiotic maturation?

To answer these questions, we took advantage of $p21^{cip1}$, a well-known inhibitor of cdk/cyclin complexes (Xiong et al., 1993). It has been recently reported that $p21^{cip1}$, when microinjected into *Xenopus* prophase oocytes, binds endogenous Cdc2/cyclin complexes and prevents their activation in ovo (Frank-Vaillant et al., 1999). Therefore, $p21^{cip1}$ represents a powerful tool to study in vivo proteins that are regulated in response to progesterone and independently of Cdc2 activation, i.e. regulated upstream of Cdc2 activation in the maturation induction pathway. In the present paper, we report that Eg2 protein accumulates in response to progesterone, independently of Cdc2 activation. Eg2 accumulation is regulated by PKA and is independent of the polyadenylation and of the cap ribose methylation of its mRNA. In contrast to the regulation of Eg2 protein accumulation, Eg2 kinase activity is dependent on Cdc2 activation. Neither accumulation nor activation of Eg2 are required for Cdc2 activation.

MATERIALS AND METHODS

Materials

Xenopus laevis adult females (CNRS, Rennes, France) were bred and maintained under laboratory conditions. [γ-^{32}P]ATP was purchased from DuPont NEN (Boston, MA) and [α-^{32}P]dCTP from Amersham (Saint Quentin, France). Okadaic acid was from ICN (Orsay, France). Reagents, unless otherwise specified, were from Sigma (Saint Quentin Fallavier, France).

Oligonucleotides

Oligonucleotides were made by Eurogentec (Seraing, Belgium). The anti-sense oligodeoxynucleotide was 5′-GGAGATTTCCTA-3′, modified by a C6 amine at the 3′position. This sequence is complementary to the end of the coding sequence and the beginning of the 3′ untranslated region of Eg2 cDNA. The control non-sense oligodeoxynucleotide was 5′-ATCCTTTAGAGG-3′, modified by a C6 amine at the 3′position. They were resuspended in distilled water at a final concentration of 3 mg/ml and stored at –20°C.

Xenopus oocyte treatments

Fully grown *Xenopus* oocytes were isolated and prepared as described by Jessus et al. (1987). Oocytes were injected with various proteins: recombinant $p21^{cip1}$ at 1 μM intracellular concentration, 8 pmoles recombinant rabbit muscle PKI per oocyte, 10 ng PKAc (Promega, Charbonnieres, France) per oocyte, 50 ng recombinant *Xenopus* MBP-c-Mos protein per oocyte, 25 ng recombinant human Cdc25A per oocyte and 150 ng of oligodeoxynucleotides per oocyte. Oocytes were also incubated in the presence of various reagents: 1 μM progesterone, 750 μM S-isobutylthioadenosine (SIBA), 100 μg/ml cycloheximide, 1 mM of 3-isobutyl-1-methylxanthine (IBMX), 50 μM roscovitine (kind gift of Dr L. Meijer, CNRS, Roscoff, France), 1 mM 6-DMAP and 200 μM E-64. Maturation of oocytes was monitored by the appearance of a white spot at the animal pole of the oocyte. Oocytes were referred to as at the germinal vesicle breakdown stage (GVBD) when the first pigment rearrangement appeared; at that time, the basal part of the germinal vesicle starts to break down, as judged by cytological analysis (Huchon et al., 1981a). Oocytes were referred as to ‘metaphase II-arrested oocytes’ when they were collected at least 2 hours after GVBD. Activation of metaphase-II arrested oocytes was induced by electric shock (Karsenti et al., 1984).

Xenopus oocyte extracts

Oocytes were lysed at 4°C in 4 volumes of EB (80 mM β-glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol and protease inhibitors: 25 μg/ml leupeptin and aprotinin, 10 μg/ml pepstatin, 1 mM benzamidine, 1 μM AEBSF from Pentapharm AG, Basel, Switzerland), supplemented or not with 1 μM okadaic acid. Lysates were centrifuged at 15,000 ***g*** at 4°C for 15 minutes (Sigma 302K centrifuge). The supernatant was collected and centrifuged at 100,000 ***g*** at 4°C for 1 hour in a TL-100 Beckman centrifuge (TL-100-2 rotor). The 100,000 ***g*** supernatant was collected and then analyzed.

Purification of bacterially expressed recombinant proteins

PKI and $p21^{cip1}$ were purified as described by Frank-Vaillant et al. (1999), MBP-c-Mos as described by Roy et al. (1996) and Cdc25A as described by Rime et al. (1994).

Western blotting

Samples equivalent to 3 to 5 oocytes in Laemmli buffer (Laemmli, 1970) were electrophoresed either on 12.5% SDS-PAGE Anderson gels (Anderson et al., 1973) or on 12% SDS-PAGE Laemmli gels (Laemmli, 1970) and transferred to nitrocellulose filters (Schleicher and Schuell, Ecquevilly, France) using a semi-dry blotting system (Millipore, Saint Quentin, France) as described by Jessus et al. (1991). The following antibodies were used: mouse monoclonal anti-Eg2 antibody (1:100 dilution; Roghi et al., 1998), rabbit polyclonal anti-ERK1 antibody (1:3000 dilution, Santa Cruz Biotechnologies, Santa Cruz, USA), rabbit polyclonal anti-c-Mos antibody (1:500 dilution, Santa Cruz Biotechnologies), sheep polyclonal anti-cyclin B2 antibody (1:10

dilution of the purified retro-eluted antiserum; Gautier et al., 1990). The secondary antibodies (Jackson Immunoresearch, West Grove, USA) were HRP-conjugated anti-mouse antibody, HRP-conjugated donkey anti-rabbit antibody and HRP-conjugated anti-sheep antibody. The chemiluminescence detection system was from NEN.

Eg2 kinase assays

Eg2 kinase activity was measured in Eg2 protein immunoprecipitates. *Xenopus* oocyte extracts were incubated for 2 hours in the presence of the mouse monoclonal anti-Eg2 antibody (1:100 dilution) at 4°C, and then for 1 hour at 4°C in the presence of Protein G-Sepharose beads (Pharmacia Biotech, Saint Quentin, France). The beads were then washed four times alternately with kinase buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) and EB. Addition of high concentrations of salt (from 150 mM to 1 M NaCl) and/or detergent (from 0 to 1% of Triton X-100 or NP-40) did not modify the results of the immunoprecipitation and the kinase activity. To assay the kinase activity, the beads were incubated for 15 minutes at 30°C in the presence of kinase buffer containing 10 μM ATP, 2 μCi [γ-^{32}P]ATP (DuPont NEN) and either 1 mg/ml β-casein or 1 mg/ml myelin basic protein (MBP). The reaction was stopped by adding Laemmli buffer (Laemmli, 1970) and boiling. After electrophoresis and autoradiography, the bands corresponding to β-casein or MBP were excised and the associated-radioactivity was measured in a Wallac 1409 scintillation counter.

Histone H1 kinase assays

To measure Cdc2 kinase activity, histone H1 kinase assays were performed on p13 Sepharose-bound extracts (3 oocytes equivalent) in the presence of [γ-^{32}P]ATP (DuPont NEN) and 0.1 mg/ml histone H1 according to the method of Jessus et al. (1991).

RNA preparation and northern blot analysis

Ten oocytes were homogenized in 0.25 M Tris-HCl, pH 8, 0.2 M NaCl, 60 mM EDTA and 2% SDS (50 μl per oocyte) and nucleic acids were extracted by the proteinase K-phenol method (Harland and Misher, 1988). After phenol extraction, the aqueous solution was precipitated with isopropanol. The pellet was diluted in 4 M LiCl and stored overnight at 4°C. The final pellet of RNA was dried and diluted in distilled water at a concentration of 1 μg/μl.

For northern analysis of Eg2 mRNA, 10 μg equivalent RNA were loaded on a 1.2% formaldehyde agarose gel. Electrophoresis and transfer of RNA by capillary action to Hybond N nylon were performed as described by Sambrook et al. (1989). To detect the RNA, a double strand DNA probe complementary to the entire open reading frame of Eg2 was labelled with [α-^{32}P]dCTP. The probe was made using the Rediprime DNA labeling system (Amersham) and 5 μl of [α-^{32}P]dCTP (6000 Ci/mmol). Membranes were pre-hybridized for 1 hour at 62°C in hybridization solution (50 mM Tris-HCl, pH 7.5, 1 M NaCl, 1% SDS, 1% PPI, 50% formamide, 10% Denhardt's). Hybridization was performed for 12 hours at 62°C in the presence of 2×10^6 cpm/ml of probe in hybridization solution. After incubation, membranes were washed twice in 2× SSC at room temperature and 30 minutes in 2× SSC plus 0.5% SDS at 42°C. The membranes were scanned with a Phosphorimager (Molecular Dynamics).

RESULTS

Establishment of specific experimental conditions allowing the detection of Eg2 accumulation, phosphorylation and kinase activity

It has been reported that Eg2 protein of *Xenopus* oocytes undergoes an electrophoretic retardation in response to progesterone, resulting from a phosphorylation event and correlated with the activation of the kinase (Andresson and Ruderman, 1998). In order to confirm this important observation and to further analyze this process, we undertook experiments to study Eg2 protein levels and activity during meiotic maturation. Prophase and metaphase II oocytes were homogenized in the absence of okadaic acid, and the Eg2 protein was analyzed by western blotting using the Laemmli

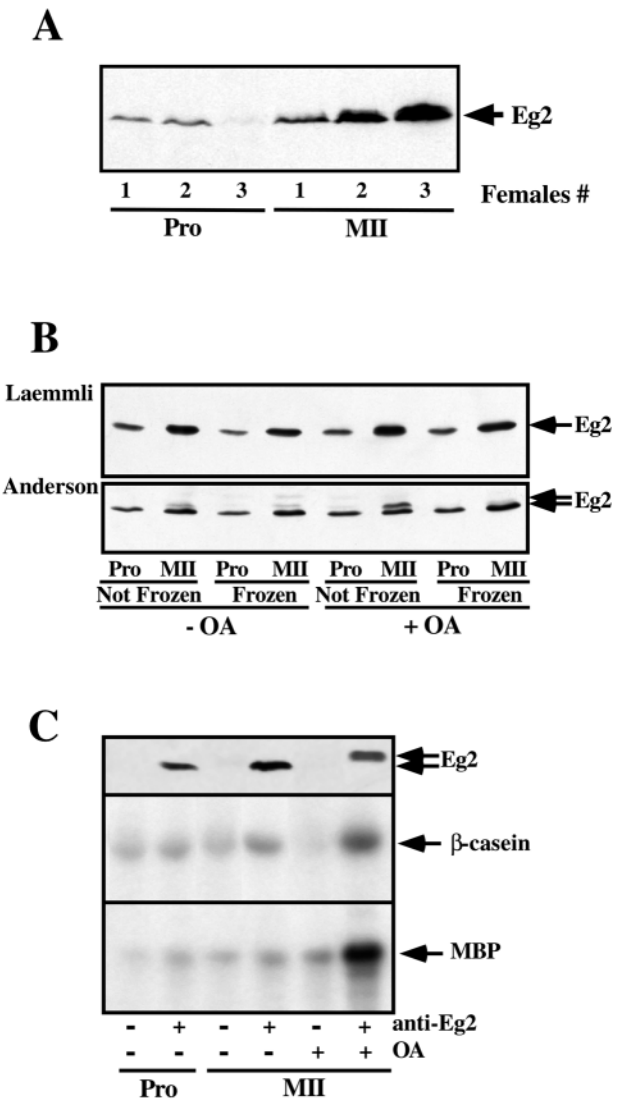

Fig. 1. Eg2 protein is accumulated, phosphorylated and activated during progesterone-induced meiotic maturation. (A) Prophase or metaphase II oocytes (MII) from three females were homogenized in the absence of okadaic acid (OA). Lysates were subjected to western blot with the anti-Eg2 antibody (Laemmli SDS-PAGE procedure). (B) Prophase (Pro) and metaphase II oocytes (MII) were frozen at –80°C or not prior to homogenization in the presence or absence of OA. The same lysates were subjected to electrophoretic separation under either Laemmli or Anderson SDS-PAGE conditions and western blotted with the anti-Eg2 antibody. (C) Prophase oocytes (Pro) were homogenized in the absence of OA and metaphase II oocytes (MII) were homogenized in the absence or in the presence of OA. Lysates were immunoprecipitated with the anti-Eg2 antibody. Controls were subjected to the same immunoprecipitation procedure in the absence of the antibody. Precipitates were immunoblotted with the anti-Eg2 antibody (Anderson SDS-PAGE system, upper panel) and assayed for Eg2 kinase activity using either β-casein (middle panel) or MBP (lower panel) as substrate.

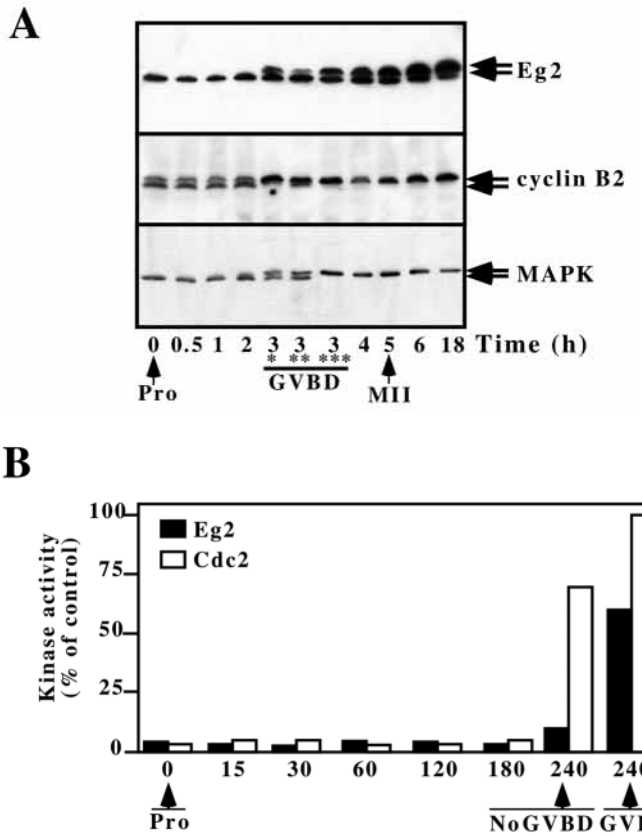

Fig. 2. Time-course of Eg2 protein accumulation, phosphorylation and activation during progesterone-induced meiotic maturation. (A) Prophase oocytes were incubated in the presence of progesterone and groups of 20 oocytes were collected at indicated times after progesterone addition. Lysates were subjected to western blot (Anderson SDS-PAGE procedure) with the anti-Eg2 antibody (upper panel), with the anti-cyclin B2 antibody (middle panel) and with the anti-MAP kinase antibody (lower panel). GVBD started 3 hours after progesterone addition, and metaphase II stage (MII) was reached 2 hours after GVBD. At 3 hours, three types of oocytes were selected: a mixture of oocytes taken randomly before or at GVBD (*), oocytes collected before GVBD (**), and oocytes collected at GVBD (***). (B) Prophase oocytes were incubated in the presence of progesterone, collected at indicated times after progesterone addition and homogenized. Lysates were immunoprecipitated with the anti-Eg2 antibody and assayed for Eg2 kinase activity (β-casein as substrate) or pulled down on p13-beads and assayed for Cdc2 kinase activity (histone H1 as substrate). GVBD started 4 hours after progesterone addition, and the metaphase II stage (MII) was reached 2 hours after GVBD. Two types of oocytes were selected at 240 minutes: some having achieved GVBD (GVBD) and others prior to GVBD (no GVBD). Eg2 and Cdc2 kinase activities are expressed as % of their maximal level.

SDS-PAGE system of protein separation (Laemmli, 1970). The monoclonal anti-Eg2 antibody used in this study was the same as the one used by Andresson and Ruderman (1998). Under these experimental conditions, the electrophoretic mobility of Eg2 protein from prophase and metaphase oocytes was unchanged (Fig. 1A). However, the Eg2 protein level is higher in matured oocytes. Fig. 1A illustrates various levels of increase in Eg2 protein in oocytes from 3 different females. In prophase oocytes, the protein is present at a low level (Fig. 1A), sometimes barely detectable by western blotting (Figs 1A, 4E and 5). Its level is increased in metaphase II oocytes (Fig. 1A). This observation was reproducibly observed in more than 30 females.

The visualization of the electrophoretically retarded form of Eg2 requires specific experimental conditions (Fig. 1B): first, the oocytes have to be homogenized directly, without a preliminary freezing step at −80°C. The commonly used procedure that consists of freezing the oocytes before homogenization leads to the disappearance of the shifted form of Eg2 (Fig. 1B). Second, the presence of 1 μM okadaic acid, a specific inhibitor of types 1 and 2A Ser/Thr phosphatases (Bialojan and Takai, 1988), is required in the lysis buffer to observe the electrophoretic retardation of Eg2 (Fig. 1B). This strongly suggests that Eg2 is modified by phosphorylation during meiotic maturation. Third, the Anderson SDS-PAGE system of electrophoretic separation (Anderson et al., 1973) has to be used. The shifted form is not detectable with the Laemmli procedure (Laemmli, 1970; Fig. 1B). Conversely, the Laemmli SDS-PAGE system allows a better visualization of the global accumulation of Eg2 protein. Depending on the females, the alteration of Eg2 electrophoretic mobility results either in a doublet (partial shift, Fig. 1B, also see Figs 2A, 3E, 7A, 8 and 9) or in a single upper band (full shift, see Figs 1C, 3E and 7B).

In order to determine whether the shifted form of Eg2 correlates with its activation, Eg2 kinase activity was assayed. The unshifted form of Eg2 protein was immunoprecipitated from either prophase or metaphase oocytes homogenized in the absence of okadaic acid. The retarded form of Eg2 was immunoprecipitated from metaphase oocytes homogenized in the presence of okadaic acid. The immunoprecipitation leads to the depletion of Eg2 protein from the extracts (our unpublished results). Eg2 kinase activity was then assayed in the immunoprecipitates described above. Either myelin basic protein (MBP) or β-casein were used as substrates. Those are the two commonly used in vitro substrates for the Aurora kinases (Andresson and Ruderman, 1998; Bischoff et al., 1998; Gopalan et al., 1997; Kimura et al., 1997; Roghi et al., 1998; Tseng et al., 1998; Zhou et al., 1998). Both gave similar results (Fig. 1C), as already reported by Zhou et al. (1998). Identical levels of Eg2 kinase activity were also recovered when higher concentrations of salt (from 150 mM to 1 M NaCl) and/or detergent (from 0 to 1% of Triton X-100 or NP-40) were used during immunoprecipitation (data not shown). Eg2 kinase activity was undetectable in prophase oocytes (Fig. 1C). In contrast, Eg2 kinase was active in metaphase oocytes, if experimental conditions maintaining the shifted form were used. The absence of okadaic acid in the lysis buffer resulted in the disappearance of the electrophoretic retardation and a lack of kinase activity (Fig. 1C). It can therefore be concluded that Eg2 is activated during meiotic maturation and that the phosphorylation of Eg2, visualized by the electrophoretic retardation, is required for its kinase activity. This conclusion is in agreement with the results reported by Andresson and Ruderman (1998), which also show that Eg2 phosphorylation is necessary for its kinase activity.

Altogether, these experiments show that Eg2 is subjected to two distinct regulations during meiotic maturation; firstly it accumulates, and secondly its kinase activity is turned on. To further investigate the molecular basis of this dual regulation

of Eg2, we adopted in the subsequent experiments illustrated in this paper the optimal experimental conditions, as follows: Eg2 accumulation was analyzed in the Laemmli SDS-PAGE system, which favors the detection of the protein accumulation but does not allow the visualization of the shifted form. Eg2 activity was either estimated by an Eg2 mobility shift assay in the Anderson system or directly measured by kinase assay after Eg2 immunoprecipitation. The average activity of Eg2 from metaphase oocytes towards MBP is 1 fmol P incorporated/minute/oocyte at 30°C. This is much less than the activities measured for Cdc2 and MAP kinase from metaphase towards the same substrate (550 fmol P incorporated/minute/oocyte at 30°C for both kinases). We therefore selected β-casein to measure Eg2 kinase activity since, in contrast to MBP, it is not an in vitro substrate for Cdc2 and MAP kinase.

Time-course of Eg2 protein accumulation, phosphorylation and activation during progesterone-induced maturation

We then determined more precisely the time-course of Eg2 accumulation and activation during meiotic maturation. Prophase oocytes were incubated in the presence of progesterone. The first morphological event characterizing the entry into M-phase is germinal vesicle breakdown (GVBD), starting 3 hours after progesterone addition in this typical experiment (Fig. 2A). The metaphase II stage was reached 2 hours after GVBD. Oocyte extracts were prepared at different times during meiotic maturation and the Eg2 protein level was analyzed by western blotting, using the Anderson SDS-PAGE system of protein separation (Anderson et al., 1973; Fig. 2A). The increase in Eg2 protein level and the appearance of the electrophoretic retardation were both detected at around GVBD time (Fig. 2A), when Cdc2 and MAP kinase are also activated. This was visualized by the electrophoretic retardation of these proteins (Fig. 2A).

To measure Eg2 kinase activation during meiotic maturation, the protein was immunoprecipitated from oocytes at different times following progesterone addition and the Eg2 kinase activity was assayed in the immunoprecipitates, using β-casein as substrate. As expected, Eg2 phosphorylation (Fig. 2A) and Eg2 kinase activity (Fig. 2B) are strictly correlated. Eg2 kinase activity was undetectable during the lag period preceding GVBD and raised at GVBD time (Fig. 2B). Cdc2 kinase activity was also assayed in the same extracts. As expected, it was activated just before GVBD and was fully active at GVBD (Fig. 2B). Eg2 kinase activation shortly followed Cdc2 activation (Fig. 2B).

Eg2 protein turn-over and kinase activity during meiotic maturation and parthenogenetic activation

It is well known that the protein synthesis rate and the turn-over of numerous proteins, including mitotic cyclins and c-Mos, are increased at GVBD (Taieb et al., 1997). To determine whether Eg2 protein turn-over is regulated at this period, cycloheximide was added to the maturing oocytes at GVBD time for 2 hours. As expected, protein synthesis inhibition led to the inactivation of Cdc2 kinase activity and to the disappearance of cyclin B2 and c-Mos (Fig. 3B-D), two proteins exhibiting a high turn-over at this period (Furuno et al., 1994; Kobayashi et al., 1991; Thibier et al., 1997). In contrast, the Eg2 protein level at GVBD remained stable in the absence of protein synthesis (Fig. 3A). However, although the protein level was unaffected, the kinase activity was turned off, as judged by the reversibility of the migration shift observed in the Anderson system (Fig. 3E).

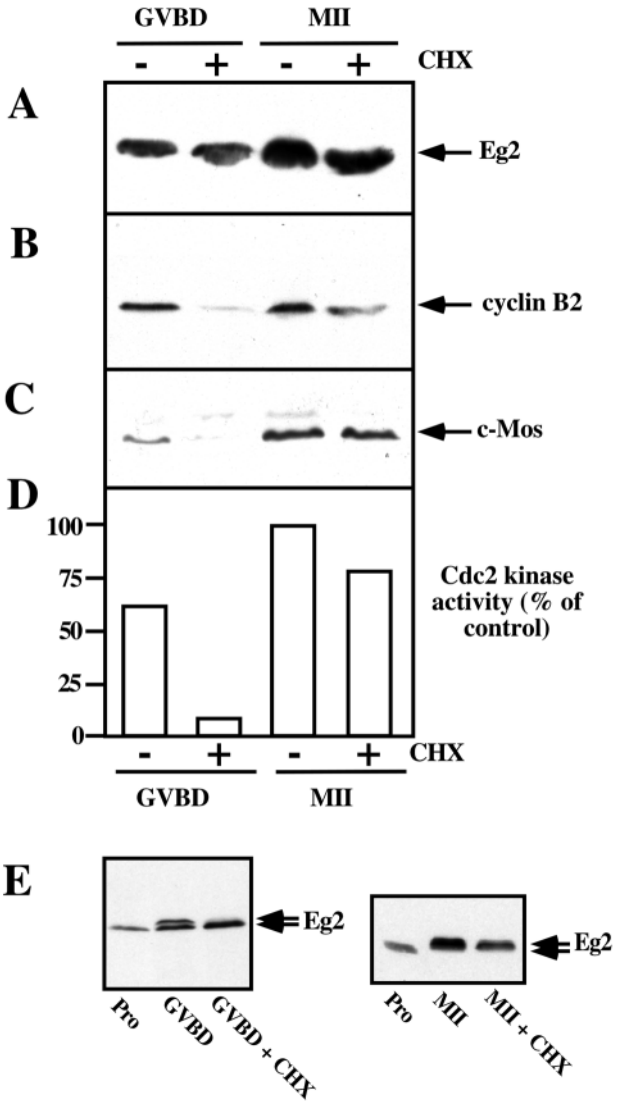

Fig. 3. Eg2 regulation at GVBD and at metaphase II. Oocytes were incubated in the presence of progesterone and collected either at GVBD or at the metaphase II (MII) stage. They were either immediately homogenized, or incubated for 2 more hours in the presence of cycloheximide (CHX) before homogenization. Lysates were subjected to western blots with the anti-Eg2 antibody (Laemmli SDS-PAGE procedure) (A) or with the anti-cyclin B2 antibody (B) or with the anti-c-Mos antibody (C) or to Cdc2 kinase assay (D). (E) Lysates were subjected to western blots with the anti-Eg2 antibody in the Anderson SDS-PAGE system.

Protein synthesis inhibition at metaphase II did not affect the level of c-Mos, and only slightly affected Cdc2 activity and the level of cyclin B2, as already described by Nishizawa et al. (1992) and Thibier et al. (1997) (Fig. 3B-C), since these proteins are stabilized during the metaphase I-metaphase II transition. Eg2 protein level and activity were not altered by this treatment (Fig. 3A,E).

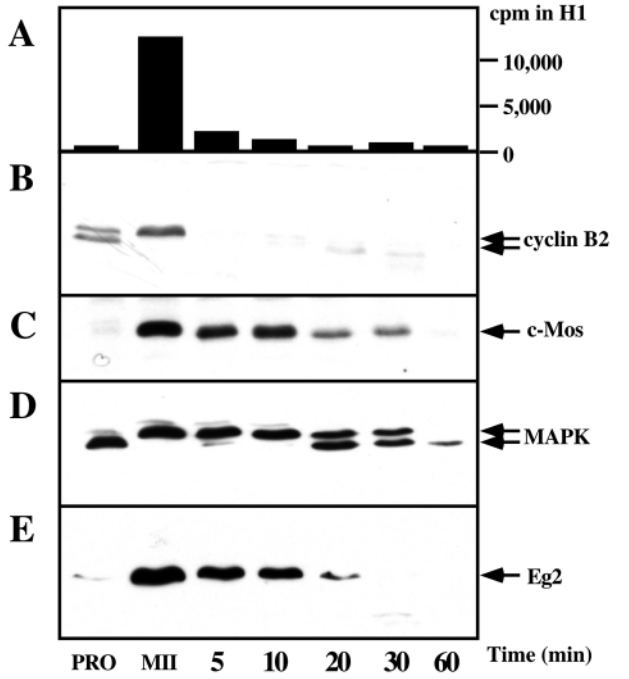

Fig. 4. Eg2 protein is degraded after the activation process. Metaphase II oocytes, matured in vitro after addition of progesterone, were activated by an electric shock. Activated oocytes were collected at indicated times after the electric shock and homogenized. Prophase oocytes (PRO) and metaphase II oocytes (MII) were also collected. Lysates were subjected to Cdc2 kinase assay (A) or western blots with the anti-cyclin B2 antibody (B), with the anti-c-Mos antibody (C), with the anti-MAP kinase antibody (D) or with the anti-Eg2 antibody (E, Laemmli SDS-PAGE procedure).

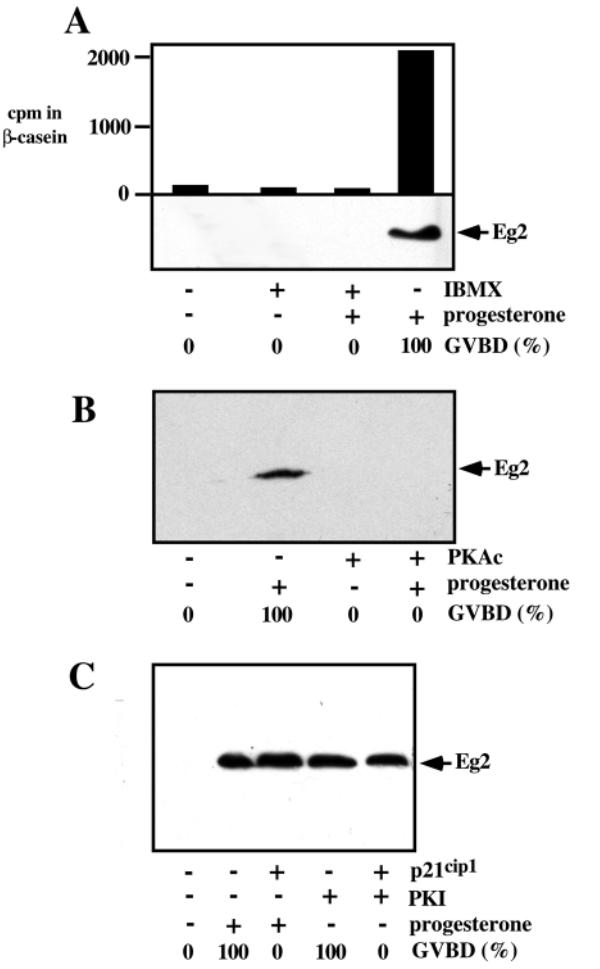

Fig. 5. PKA negatively regulates Eg2 protein accumulation and activation. (A) Prophase oocytes were incubated for 2 hours in the presence of IBMX. Progesterone was then added or not. Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes and homogenized. Lysates were either immunoprecipitated with the anti-Eg2 antibody and assayed for Eg2 kinase activity using β-casein as substrate, or subjected to western blot with the anti-Eg2 antibody (Laemmli SDS-PAGE procedure). (B) Prophase oocytes were injected or not with PKAc. Two hours after, progesterone was added or not. Oocytes were then collected 2 hours after 100% GVBD was reached in progesterone control oocytes and homogenized. Lysates were subjected to western blot with the anti-Eg2 antibody (Laemmli SDS-PAGE procedure). (C) Prophase oocytes were injected or not with $p21^{cip1}$. One hour later, oocytes were injected or not with PKI or treated by progesterone. Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes and homogenized. Lysates were subjected to western blot with the anti-Eg2 antibody (Laemmli SDS-PAGE procedure).

C-Mos and cyclins A, B1 and B2 are degraded when metaphase II oocytes reenter interphase following fertilization or in vitro activation. To determine whether Eg2 protein obeys a similar rule, metaphase II oocytes were activated in vitro by an electric shock and extracts were prepared at different times following activation. As shown in Fig. 4A, Cdc2 kinase was inactivated within the 5 minutes following the electric shock. As expected, the degradation of cyclin B2 did parallel Cdc2 inactivation (Fig. 4B). C-Mos proteolysis was slightly delayed when compared to cyclin B2 and was completed at between 30 and 60 minutes (Fig. 4C). MAP kinase activity was estimated by its electrophoretic retardation due to its phosphorylation. The inactivation of MAP kinase followed the destruction of c-Mos (Fig. 4D). Similarly, Eg2 protein was destroyed between 20 and 30 minutes post-activation (Fig. 4E). This time-course of Eg2 disappearance was slower than cyclin B2 destruction and closer to the c-Mos degradation pattern (Fig. 4). It was ascertained that Eg2 kinase activity decreases after activation, following the same time-course as Eg2 protein disappearance. Eg2 kinase activity was undetectable 30 minutes after the electric shock (our unpublished results).

A decrease in PKA activity is necessary and sufficient to induce Eg2 accumulation and activation

Progesterone induces a drop in the cAMP level and a subsequent decrease in PKA activity required to transduce the hormonal effects (reviewed by Jessus and Ozon, 1993). We investigated whether the Eg2 protein accumulation and activation induced by progesterone occur upstream or downstream of the cAMP step. In a first approach, prophase oocytes were incubated for 2 hours in the presence of IBMX, a phosphodiesterase inhibitor, known to increase the cAMP level in the *Xenopus* oocyte (Mulner et al., 1979); then, progesterone was added. As previously reported (Mulner et al., 1979), the induction of meiotic maturation by progesterone was blocked. Under these conditions, Eg2 protein did not

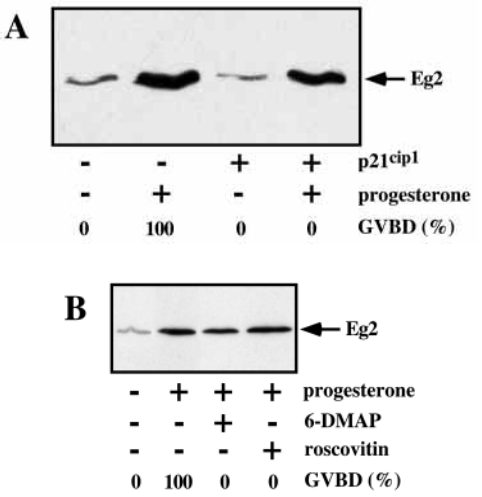

Fig. 6. Eg2 protein accumulates in response to progesterone independently of Cdc2. (A) Prophase oocytes were injected or not with $p21^{cip1}$. One hour later, progesterone was added or not. Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes. Lysates were subjected to western blot with the anti-Eg2 antibody (Laemmli SDS-PAGE procedure). The percentage of oocytes that underwent GVBD is indicated below. (B) Prophase oocytes were incubated or not in the presence of either 6-DMAP or roscovitine for 18 hours, then progesterone was added. Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes. Lysates were subjected to western blot with the anti-Eg2 antibody (Laemmli SDS-PAGE procedure). The percentage of oocytes that underwent GVBD is indicated below.

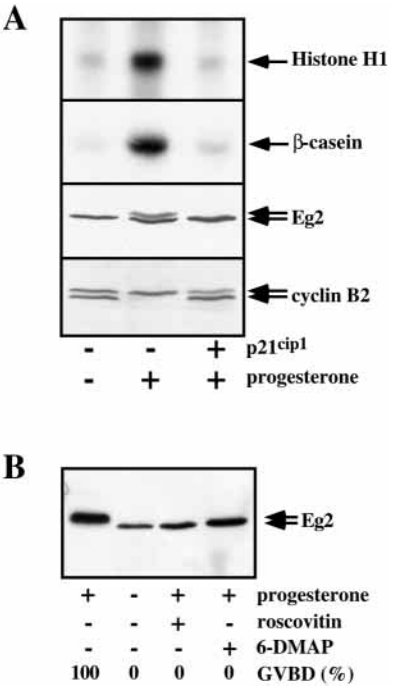

Fig. 7. Eg2 kinase activation depends on Cdc2 activity. (A) Prophase oocytes were injected or not with $p21^{cip1}$. One hour later progesterone was added or not. Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes. Lysates were either precipitated on p13-beads and assayed for Cdc2 kinase activity (histone H1 as substrate) or immunoprecipitated with the anti-Eg2 antibody and assayed for Eg2 kinase activity (β-casein as substrate), or subjected to western blot with the anti-Eg2 antibody (Anderson SDS-PAGE procedure) and with the anti-cyclin B2 antibody. (B) Prophase oocytes were incubated or not in the presence of either 6-DMAP or roscovitine for 18 hours. Progesterone was then added. Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes. Lysates were subjected to western blot with the anti-Eg2 antibody (Anderson SDS-PAGE procedure). The percentage of oocytes that underwent GVBD is indicated below.

accumulate and the kinase was not activated (Fig. 5A), demonstrating that the cAMP drop normally induced by progesterone is required for Eg2 protein accumulation and activation.

We then analyzed the consequences of directly modifying PKA activity. PKAc, the catalytic subunit of PKA, was microinjected in prophase oocytes. One hour later, progesterone was added. As expected, meiotic maturation was blocked (Maller and Krebs, 1977), and Eg2 protein did not accumulate (Fig. 5B), demonstrating that the decrease in PKA activity is necessary for Eg2 protein accumulation.

To determine whether a PKA down-regulation is sufficient to lead to Eg2 protein accumulation and activation, PKI, a specific inhibitor of PKA activity, was microinjected into prophase oocytes. As already reported (Huchon et al., 1981b), meiotic maturation was induced by PKI microinjection. Eg2 protein was accumulated (Fig. 5C) and activated (see Fig. 8C) under these conditions.

When Cdc2 is locked in an inactive state by the microinjection of $p21^{cip1}$, a protein inhibitor of most known Cdks when used at high concentration (1 μM; Xiong et al., 1993), PKI microinjection was no longer able to induce meiotic maturation (Fig. 5C), as previously reported (Frank-Vaillant et al., 1999). Under these conditions, Eg2 protein still accumulated (Fig. 5C), demonstrating that the decrease in PKA activity is not only necessary, but also sufficient to induce the accumulation of Eg2 protein. This accumulation of Eg2 is therefore a response to PKA inhibition and is not mediated through Cdc2 activation. This observation prompted us to analyze in more detail whether Eg2 accumulation occurs following progesterone stimulation, but independently of Cdc2 activation.

Eg2 protein accumulation occurs in response to progesterone independently of Cdc2 activation

When microinjected into prophase oocytes at an intracellular concentration of 1 μM, $p21^{cip1}$ blocks the progesterone-induced meiotic maturation by binding and inhibiting specifically oocyte Cdc2 (Frank-Vaillant et al., 1999). This allows the analysis of the events induced by progesterone independently of Cdc2 activation. $P21^{cip1}$ was microinjected into prophase oocytes. One hour later, progesterone was added to the external medium. GVBD did not occur within the following 8 hours, while the control progesterone-treated oocytes underwent 100% GVBD within this period (Fig. 6A). Interestingly, the Eg2 protein level was increased after 6 hours of progesterone-treatment in the oocytes injected or not by $p21^{cip1}$ (Fig. 6A). Identical results were obtained by using two other inhibitors of Cdc2 activation in *Xenopus* oocyte, roscovitine (Meijer et al., 1997) and 6-DMAP (Jessus et al., 1991; Fig. 6B). It therefore appears that Eg2 protein

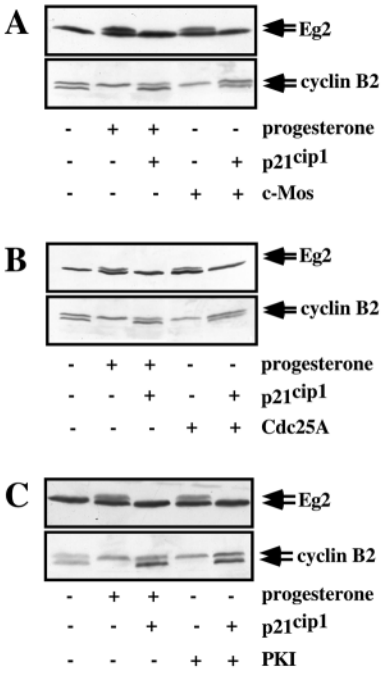

Fig. 8. Eg2 kinase activation does not require progesterone. C-Mos protein (A), Cdc25A (B) and PKI (C) were injected into prophase oocytes in the absence of progesterone. Prophase oocytes and progesterone-treated oocytes were used as controls. $P21^{cip1}$ was microinjected or not 1 hour before the injection of c-Mos, or Cdc25A or PKI. Oocytes were collected when 100% GVBD was reached in progesterone control oocytes. Lysates were subjected to western blot with the anti-Eg2 antibody (Anderson SDS-PAGE procedure) and with the anti-cyclin B2 antibody.

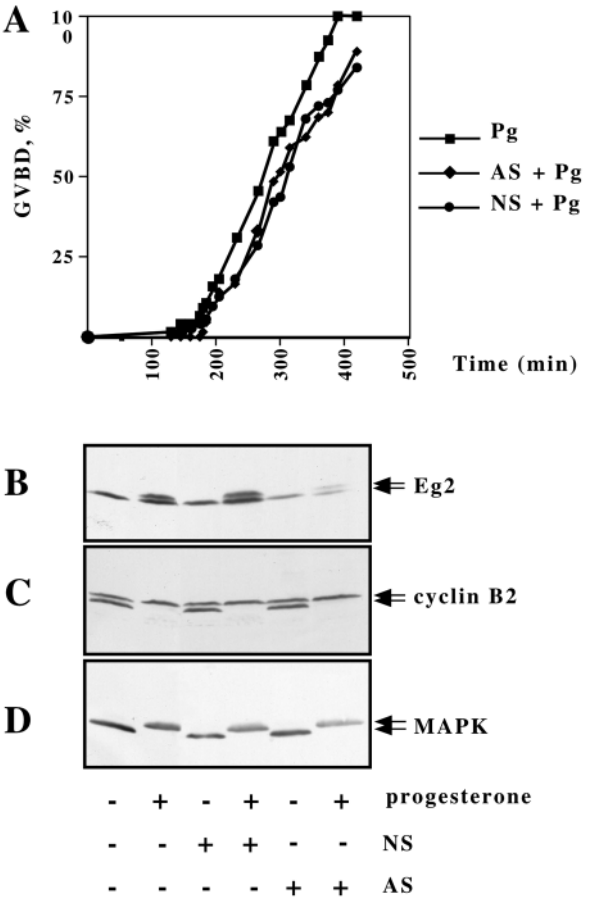

Fig. 9. Eg2 accumulation is not required for Cdc2 activation. Prophase oocytes were injected or not with anti-sense oligonucleotides (AS) or non-sense oligonucleotides (NS). 18 hours after the injection, progesterone (Pg) was added or not. The time-course of GVBD appearance was monitored (A). Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes. Lysates were subjected to western blot with the anti-Eg2 antibody (Anderson SDS-PAGE procedure) (B), or with the anti-cyclin B2 antibody C) or with the MAP kinase antibody (D).

accumulation occurs in response to progesterone, independently of Cdc2 activity and re-entry into M-phase.

Eg2 kinase activation is dependent on Cdc2

The same strategy was used to analyze whether Eg2 activation is under the control of Cdc2 activity. $P21^{cip1}$ was microinjected into prophase oocytes and progesterone was added one hour later. GVBD never occurred and Cdc2 was not activated, as judged by the electrophoretic mobility of cyclin B2, and Cdc2 kinase assay using histone H1 as substrate (Fig. 7A). In sharp contrast to Eg2 protein accumulation (Fig. 6A), Eg2 kinase activation, measured using β-casein as substrate, was totally prevented in the presence of $p21^{cip1}$ (Fig. 7A). Eg2 protein was not shifted under these conditions (Fig. 7A), in agreement with the absence of kinase activity. Identical results were obtained with two other Cdc2 inhibitors, roscovitine and 6-DMAP (Fig. 7B).

To confirm that Eg2 activation is dependent on Cdc2 activation and does not require the presence of progesterone, three inducers of MPF activation, the c-Mos protein kinase (Sagata et al., 1989), the Cdc25A phosphatase (Rime et al., 1994) and the PKA inhibitor PKI (Huchon et al., 1981b), were injected into prophase oocytes in the absence of progesterone. Cdc2 activation was monitored by the mobility retardation of cyclin B2, and Eg2 activity was estimated after the shift of the protein. As already known, the injection of either c-Mos, or Cdc25A or PKI led to GVBD and Cdc2 activation (Fig. 8). Under these conditions, Eg2 was phosphorylated and activated (Fig. 8). Therefore the activation of Cdc2 is sufficient to lead to Eg2 activation. When $p21^{cip1}$ is microinjected one hour before microinjection of either c-Mos or Cdc25A or PKI, Cdc2 activation was totally prevented (Fig. 8), as reported (Frank-Vaillant et al., 1999). The shifted form of Eg2 was undetectable under these conditions (Fig. 8), again indicating that Cdc2 activation is necessary for Eg2 activation.

Altogether, these results demonstrate that, in contrast to Eg2 protein accumulation, Eg2 kinase activity depends on Cdc2 activation and does not appear to be a component of the progesterone-signaling pathway lying upstream of Cdc2.

Eg2 accumulation is not required for Cdc2 activation

Eg2 accumulation is controlled by progesterone independently of Cdc2. We therefore investigated whether the accumulation of the protein is involved in the transduction pathway leading to Cdc2 activation. Antisense oligodeoxynucleotides were designed to inhibit the accumulation of the protein. Non-sense oligodeoxynucleotides were used as control. The

oligonucleotides were microinjected into prophase oocytes. 16 hours after, the level of Eg2 protein was slightly reduced in the anti-sense-microinjected oocytes whereas it was not modified in the non-sense-injected control oocytes (Fig. 9B). Progesterone was then added and the time-course of GVBD appearance was monitored. Eg2 accumulation was never induced by progesterone in anti-sense microinjected oocytes, whereas it occurred normally in non-sense-injected control oocytes (Fig. 9B). However, the remaining amount of Eg2 present in the oocytes after anti-sense oligonucleotide injection was phosphorylated in response to progesterone (Fig. 9B). The injection of anti-sense or non-sense oligonucleotides did not modify the time-course of progesterone-induced GVBD (Fig. 9A) and did not prevent Cdc2 and MAP kinase activation (Fig. 9C-D). The accumulation of Eg2 is therefore not required in the signaling pathway induced by progesterone and leading to Cdc2 activation.

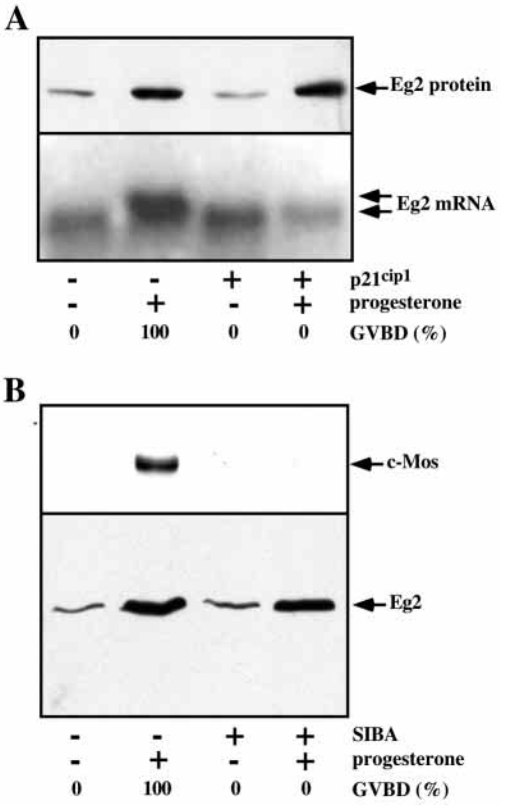

Fig. 10. Polyadenylation and cap ribose methylation of the Eg2 mRNA are not required for the progesterone-dependent accumulation of the protein. (A) Prophase oocytes were injected or not with $p21^{cip1}$. One hour later, progesterone was added or not. Oocytes were collected 2 hours after 100% GVBD was reached in progesterone control oocytes and homogenized for western blot analysis with the anti-Eg2 antibody (upper panel, Laemmli SDS-PAGE system) and northern blot analysis of total RNA (lower panel). Analysis of the adenylation change in oocyte RNA was revealed by a cDNA probe to Eg2 coding region. (B) Prophase oocytes were incubated in the presence or in the absence of SIBA. Two hours later, progesterone was added or not. Oocytes were collected after 100% GVBD was reached in control progesterone-treated oocytes and homogenized for western blot analysis with the anti-Mos antibody (upper panel) and the anti-Eg2 antibody (lower panel, Laemmli SDS-PAGE procedure).

Eg2 protein accumulation is not controlled by mRNA polyadenylation and cap ribose methylation

Eg mRNAs are polyladenylated during meiotic maturation and deadenylated after fertilization (Paris et al., 1991). The role of the polyadenylation process in translation depends on the mRNA. For example, the polyadenylation of mos mRNA is necessary for its translation (Sheets et al., 1995), whereas polyadenylation of cyclin B1 mRNA is not required for the synthesis of the protein (Ballantyne et al., 1997; Barkoff et al., 1998; Frank-Vaillant et al., 1999; Nebreda et al., 1995). To determine whether the polyadenylation of Eg2 mRNA controls its translation, we investigated the polyadenylation status of Eg2 mRNA in oocytes exhibiting various levels of Eg2 protein. As previously reported, Eg2 mRNA is polyadenylated during meiotic maturation induced by progesterone, paralleling the accumulation of the protein (Fig. 10A). When progesterone-induced meiotic maturation is blocked by the injection of $p21^{cip1}$, Eg2 mRNA is not polyadenylated, although the Eg2 protein accumulates under these conditions (Fig. 10A). This result shows that Eg2 mRNA polyadenylation is not required for the accumulation of the protein, which can occur in the absence of polyadenylation of the mRNA.

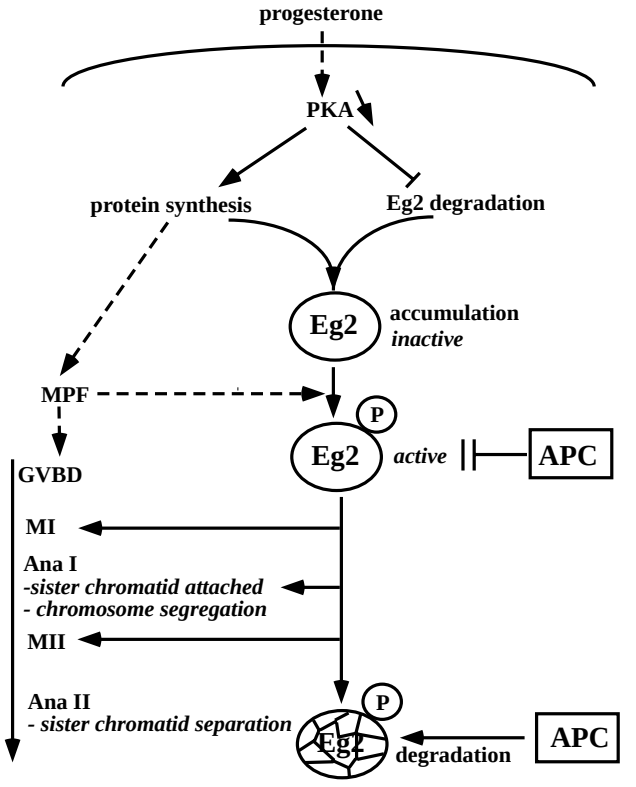

Fig. 11. Regulation of Eg2 protein accumulation and Eg2 kinase activation in response to progesterone in *Xenopus* oocyte. Progesterone leads to Eg2 protein accumulation through the decrease in PKA activity and independently of Cdc2 activation. PKA could negatively regulate protein synthesis and positively regulate Eg2 protein degradation, explaining how a decrease in PKA activity leads to Eg2 protein accumulation. In contrast, Eg2 kinase activation depends on Cdc2. Eg2 protein is stable at metaphase I where it could control the microtubular structures ensuring the first meiotic division. In contrast, it is degraded at the exit from meiosis II.

S-isobutylthioadenosine (SIBA) is a methyltransferase inhibitor that prevents mos synthesis and inhibits *Xenopus* oocyte meiotic maturation (Kuge et al., 1998). We investigated the effects of this inhibitor on Eg2 protein accumulation. Prophase oocytes were incubated in the presence of 750 μM SIBA for 2 hours and progesterone was then added. Meiotic maturation normally induced by progesterone was totally prevented by SIBA and mos did not accumulate (Fig. 10B), as previously reported (Kuge et al., 1998). However, the accumulation of Eg2 protein was still observed (Fig. 10B). This result demonstrates that Eg2 protein accumulation does not depend on mRNA cap ribose methylation and confirms that it is independent of Cdc2 activation.

DISCUSSION

The transduction pathway induced by progesterone in *Xenopus* oocyte is not completely understood. Since direct Cdc2 activation is able to induce through positive feedback loop a number of the effects triggered by progesterone stimulation, such as c-Mos synthesis, MAP kinase, Cdc25 and polo kinase activation, and even the drop of cAMP level and PKA activity (Haccard et al., 1993; Qian et al., 1998; Rime et al., 1994), it is difficult to distinguish the primary effects induced by the hormone from those that are induced or retrocontrolled by Cdc2. By microinjecting high levels of $p21^{cip1}$, an inhibitor able to block meiotic maturation and Cdc2 activation normally induced by progesterone (Frank-Vaillant et al., 1999), we were able to demonstrate that progesterone induces Eg2 protein accumulation independently of Cdc2 activation. In contrast, Eg2 kinase activation remains dependent on Cdc2 activation.

How does progesterone lead to Eg2 protein accumulation? In prophase oocyte, the Eg2 protein level results from an equilibrium between synthesis (it decreases after protein synthesis inhibition) and degradation (it accumulates in the presence of E-64, a cysteine protease inhibitor; Barrett et al., 1982; our unpublished results). The accumulation occurring in response to progesterone might then be achieved not only by a specific up-regulation of Eg2 translation, but also by down-regulation of Eg2 degradation. In contrast to the c-Mos mRNA, whose polyadenylation and cap ribose methylation are required for the translation (Kuge et al., 1998; Sheets et al., 1995), both these modifications of Eg2 mRNA are not required for the accumulation of the protein induced by progesterone. PKA downregulation is clearly necessary and sufficient for Eg2 protein accumulation, demonstrating that progesterone acts through a decrease in PKA activity to control Eg2 protein accumulation. PKA negatively regulates the general rate of protein synthesis (unpublished data). Interestingly, the sole injection of PKAc induces a decrease in the low amount of Eg2 protein present in prophase oocytes (unpublished data). Therefore, the accumulation of Eg2 protein induced by progesterone could result from both a global stimulation of protein synthesis and a specific inhibition of Eg2 protein degradation, both brought about by the drop in PKA activity (Fig. 11).

Eg2 kinase activation and Eg2 accumulation are regulated by two independent pathways. In contrast to the regulation of the protein level, Eg2 kinase is activated post-transductionally and downstream of Cdc2 (Fig. 11). Eg2 accumulation is not sufficient to lead to the kinase activation, since the protein accumulates in response to progesterone under conditions where the kinase is inactive ($p21^{cip1}$, roscovitine, 6-DMAP, SIBA). Conversely, Eg2 kinase can be activated in the absence of protein accumulation (anti-sense experiments), demonstrating that the increase in Eg2 level is not necessary for its kinase activity. Eg2 kinase is activated at around GVBD time by phosphorylation; this is visualized by an electrophoretic retardation of the protein. It has been recently shown that a retardation of Eg2 protein electrophoretic mobility is induced within the 15 minutes following progesterone addition (Andresson and Ruderman, 1998), well before GVBD and Cdc2 activation. We are not able to explain the origin of the discrepancy between the results of Andresson and Ruderman (1998) and ours, all of them performed with the same monoclonal anti-Eg2 antibodies. The *Xenopus* females used by Andresson and Ruderman (1998) were primed with gonadotropin while ours were not. Priming usually results in a shorter in vitro time-course of oocyte meiotic maturation, suggesting that some early steps of the progesterone-signaling pathway have been initiated in the ovary in response to the gonadotropin treatment. This could possibly explain the discrepancy between their results and ours. The appearance of the phosphorylated and active form of Eg2 at GVBD time, an event strictly depending on Cdc2 activation, has been reproducibly observed in our hands in more than 30 females, strongly supporting the view that Eg2 kinase activation is not an early event induced by progesterone, but a consequence of Cdc2 activation at GVBD time. The identification of the kinase and of the okadaic-sensitive phosphatase involved in Eg2 kinase regulation (Cdc2, c-Mos, MAP kinase, $p90^{rsk}$, among other attractive kinase candidates, PP2A and PP1 as phosphatase candidates) is currently under investigation in our laboratory.

Is Eg2 an active component of the transduction pathway initiated by progesterone and leading to Cdc2 activation? The data of Andresson and Ruderman (1998) suggested that Eg2 kinase could play a role upstream of Cdc2 activation. Our present findings eliminate the hypothesis of a role for Eg2 kinase upstream of Cdc2. Indeed, Eg2 kinase is not activated when Cdc2 activation is specifically prevented by $p21^{cip1}$, demonstrating that Eg2 kinase activation depends, directly or indirectly, on Cdc2 activity, and therefore cannot be a component of the transduction pathway leading to Cdc2 activation. These experiments do not eliminate the possibility that the accumulation of the protein could be involved in the transduction pathway leading to Cdc2 activation. However, microinjection of Eg2-specific anti-sense oligonucleotides into oocytes inhibits Eg2 protein accumulation without preventing Cdc2 and MAP kinase activation induced by progesterone. It may therefore be concluded that the accumulation of Eg2 and its kinase activity are not required in the signaling pathway induced by progesterone to initiate Cdc2 activation.

What role could Eg2 protein play during the meiotic maturation process? As suggested by the functions of the Aurora/Ipl1 family members (Bischoff and Plowman, 1999; Giet and Prigent, 1999), Eg2 kinase could contribute to the microtubular rearrangements occurring during meiotic divisions. At the time of GVBD, a giant microtubular structure, resembling a monopolar spindle and associated with the chromosomes, organizes at the basal part of the germinal vesicle (Gard, 1992; Huchon et al., 1981a; Jessus et al., 1986). It migrates towards the animal pole, where it is reorganized into the two successive

metaphase spindles (Gard, 1992; Jessus et al., 1986). These original microtubular structures allowing the chromosome and chromatid segregation could be regulated by the Eg2 kinase (Fig. 11). Supporting this hypothesis, Eg2 and Eg5, a kinesin-related protein required for spindle assembly and a substrate of Eg2 (Giet et al., 1999; Sawin and Mitchison, 1995), associate at GVBD (our unpublished results). It is also remarkable that the highest expression levels of the mammalian homologues of Eg2 are observed in the gonads (Gopalan et al., 1997, 1999; Kimura et al., 1997; Roghi et al., 1998; Schumacher et al., 1998a,b; Terada et al., 1998; Yanai et al., 1997), supporting a role for these kinases in the organization of the meiotic spindle.

Eg2 protein is degraded as the oocytes reenter interphase in response to an electric shock mimicking the activation process. This observation is in agreement with the degradation of the Aurora/Ipl1 kinases that occurs when cells exit mitosis (Gopalan et al., 1997; Kimura et al., 1997; Terada et al., 1998; Yanai et al., 1997). This favors the suggestion that Eg2 protein plays a microtubular role in spindle function and is destroyed when anaphase begins, as are a number of the anaphase-promoting complex (APC) substrates (Peters, 1998). It is therefore tempting to speculate that Eg2 protein is also an APC substrate, as supported by the association between Cdc20/fizzy, a protein required for APC activation, and aurora2/Aik in human cells (Farruggio et al., 1999). The Eg2 sequence contains a putative destruction box, located in the C-terminal domain of the protein (Andresson and Ruderman, 1998). The microinjection of the Eg2 mutant deleted in this putative D-box would indicate whether this domain is a functional D-box required for the degradation of the protein. Interestingly, Eg2 protein is stable at the metaphase I-metaphase II transition. The proteins that are ubiquitinated and degraded by the proteasome are normally subjected to a high turn-over at this period. This includes cyclin A, cyclin B1, cyclin B2 and c-Mos (Huchon et al., 1993; Nishizawa et al., 1992; Roy et al., 1991; Sagata et al., 1989; Watanabe et al., 1989), since all these proteins rapidly disappear when protein synthesis is inhibited during the metaphase I-metaphase II period, a treatment allowing the degradation machinery to work in the absence of protein synthesis. In contrast to cyclins and c-Mos, Eg2 protein is stable under these conditions, suggesting that it is protected against the degradation machinery. How and why is Eg2 protein protected from APC at anaphase I and not at anaphase II? It is known that the phosphorylation of c-Mos on Ser3 stabilizes the protein as the oocyte enters metaphase II (Nishizawa et al., 1992, 1993). We are currently investigating whether the phosphorylation state of Eg2 protein could explain the differential sensitivity of Eg2 protein towards degradation at early prometaphase I and at activation. One striking difference between anaphase I and anaphase II is that homologous chromosome segregation operates during anaphase I and sister chromatid segregation during anaphase II. Therefore, the cohesive proteins that hold together the sister chromatids have to be preserved during anaphase I and have to be removed during anaphase II. Whether Eg2 protein is involved in this specifically meiotic regulation occurring at anaphase I is an attractive hypothesis (Fig. 11).

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