# Human retinoblastoma protein (Rb) is phosphorylated by cdc2 kinase and MAP kinase in *Xenopus* maturing oocytes

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**Abstract** ***Xenopus*** **oocyte meiotic maturation combines features of G0/G1 and G2/M transitions of the cell cycle. To study the in ovo Rb kinase activity, we have microinjected human Rb into oocytes. Microinjected human Rb localizes into the nucleus, is hypophosphorylated in prophase oocytes, becomes hyperphosphorylated during meiotic maturation and is dephosphorylated as the cell reenters interphase. Inactivation or overexpression of the cyclin D-cdk4/6 complex in an oocyte extract does not affect the Rb kinase activity. This kinase activity could be attributed to both cdc2-cyclin B and MAP kinase, opening new perspectives of investigation in somatic cells.**



*Key words:* *Xenopus*; Meiotic maturation; Rb kinase; cdk; Cyclin; Mitogen-activated protein kinase

## 1. Introduction

The *Xenopus* oocyte is arrested in meiotic prophase I. The prophase release is dependent on extracellular signals: both progesterone and insulin-like growth factor 1 (IGF-1) are able to induce meiotic divisions. These signals induce various biochemical pathways that ultimately activate the cdc2-cyclin B kinase. The oocyte then arrests at metaphase II [1]. Therefore, meiotic maturation clearly resembles a G2/M transition. During this process, kinases such as mitogen-activated protein (MAP) kinase [2] or c-raf kinase [3,4] are also activated. In somatic cells, the growth factor-induced transduction cascades recruiting these kinases will ultimately be tied to elements of the cell cycle. In the oocyte, kinases such as c-raf and MAP kinase are activated at the same time as the cdc2-cyclin B complex, just before breakdown of the nuclear envelope (GVBD or germinal vesicle breakdown) [3–6]. Oocyte prophase release therefore shares common features with a G0/G1 transition, a resemblance emphasized by involvement of G1 cyclins like cyclin A [7], cyclin E [8,9] and cyclin D2. *Xenopus* cyclin D2 has been cloned recently and appears to be the only D-type cyclin expressed at the mRNA level in the oocytes [10]. Similarly to their roles in G0/G1/S transitions, these cyclins could be involved in induction of meiosis reinitiation. The major target of G1 cdk-cyclins in somatic cells is the retinoblastoma gene product, Rb, which has been demonstrated to be present in *Xenopus* oocytes [11].

Rb has the ability to suppress cell proliferation and this activity is controlled by its cell cycle-dependent phosphorylation. Rb phosphorylation begins in late G1 and continues until M phase [12]. The hypophosphorylated forms of Rb found in G1 are active during growth suppression by binding and inhibiting the transcription factor E2F, while hyperphosphorylated forms found during other cell cycle phases are inactive [13]. At least three different cdks are involved in the phosphorylation of Rb during the G1 and S phase of the somatic cell cycle [12]. Cyclin D-cdk4/6 complexes are most prominently implicated in the phosphorylation of Rb. Involvement of cyclin E in the phosphorylation of Rb is also strongly suggested. Rb might also be a physiological substrate for cyclin A-dependent kinase. At G2 and M phases, cyclin B-cdc2 might also contribute to the phosphorylation of Rb [12].

Since all cdks involved in Rb phosphorylation and some kinases involved in mitogenic transduction pathways such as MAP kinase are present and activated during meiotic maturation, the oocyte model system could contribute to the molecular identification and characterization of the in vivo Rb kinases. We therefore used the oocyte as a test tube to study the in vivo activity levels of Rb kinase after microinjection of its substrate, Rb, into the oocyte. Microinjected human Rb is hyperphosphorylated during meiotic maturation at the time of nuclear envelope breakdown in parallel with the appearance of a measurable Rb kinase activity in oocyte extracts. Surprisingly, cyclins E and D2 and cdk2 and cdk4 do not contribute to the in ovo Rb kinase activity, which could rather be attributed to MAP kinase and the cdc2-cyclin B complex.

## 2. Materials and methods

### 2.1. Materials

*Xenopus laevis* adult females (CNRS, Rennes, France) were bred and maintained under laboratory conditions. [$\gamma$-$^{32}$P]ATP (>3000 Ci/mmol) was obtained from NEN. Reagents were from Sigma, unless otherwise specified.

### 2.2. Preparation and treatments of oocytes

Isolated oocytes were prepared as in [5]. Prophase oocytes were induced to mature by addition of 1 μM progesterone. Activation of metaphase II oocytes was induced by electric shock as in [14] and oocytes were collected 30 min later. For microinjection, 50 nl of sample was microinjected into each oocyte. Enucleation of prophase oocytes and nucleus preparation were performed as described [15].

### 2.3. Extract preparation

Batches of 100 oocytes were homogenized in 400 μl EB [5] at 4°C and centrifuged at 15 000×*g* at 4°C for 30 min. The supernatant was used for assaying kinase activities or for immunoblotting. In some cases, this extract was then centrifuged at 100 000×*g* for 1 h at 4°C. GST-Rb was isolated from microinjected oocytes by incubating the 15 000×*g* extract for 60 min at 4°C in the presence of glutathione-Sepharose beads (Pharmacia). Beads were then washed and resuspended in Laemmli sample buffer [16] before immunoblotting. In the case of alkaline phosphatase treatment, Rb isolated from oocytes

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*Abbreviations:* GST, glutathione *S*-transferase; GVBD, germinal vesicle breakdown; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; Rb, retinoblastoma gene product


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and bound to glutathione-Sepharose beads was incubated for 20 min at 30°C in 50 μl phosphatase buffer (Tris pH 8.5, 0.1% β-mercaptoethanol) containing 5 U alkaline phosphatase before adding Laemmli sample buffer.

*2.4. In vitro activation of cdc2 and MAP kinase*

A 100 000×*g* extract from 100 prophase oocytes was supplemented with an ATP-regenerating system (1 mM ATP, 1 mM $MgCl_2$, 10 mM phosphocreatine, 50 μg/ml creatine kinase) and then incubated at 23°C for 3 h with either 1 μg/ml GST-cdc25A or 500 nM okadaic acid (ICN) or 500 nM okadaic acid and 10 μg/ml $p21^{CIP1}$ or control EB buffer. Samples were collected either for Western blot analysis with an anti-MAP kinase or for Rb kinase and histone H1 kinase assays.

*2.5. Kinase assays*

For p13 binding of cdc2-cyclin B and histone H1 kinase assays, extracts corresponding to 10 oocytes were diluted in 100 μl EB and then processed with p13-Sepharose beads according to [5]. Cdc2-cyclin B kinase activity was also assayed in anti-cyclin B2 immunoprecipitates. For cdc2-cyclin A and cdk2-cyclin A kinase assays, recombinant GST-cdc2 (33 μg/ml) or GST-cdk2 (33 μg/ml) was incubated with purified cyclin A (16 μg/ml) and phosphorylated on threonine-161 and 160 respectively by addition of pure civ1 kinase (50 μg/ml) (kindly provided by Dr. C. Mann, CEA, France [17]) in the presence of 100 μM ATP. Complexes were then isolated on glutathione-Sepharose beads (Pharmacia), washed in kinase buffer [5] and processed for kinase assays. Active thiophosphorylated bacterially expressed MAP kinase was purified as described in [18]. Kinase assays were performed in 50 μl kinase buffer containing 10 μM [γ-$^{32}$P]ATP (2 μCi) and either 10 μg histone H1 (Boehringer Mannheim) or 125 μg myelin basic protein (MBP) or 2 μg soluble Rb for 30 min at 30°C. The reactions were stopped by adding Laemmli sample buffer and by boiling at 100°C for 5 min before SDS-PAGE and autoradiography. The bands of histone H1, MBP and Rb were excised from the gel and counted. In some cases, Rb bound to glutathione-Sepharose beads (Pharmacia) was used as a substrate. Extracts were incubated in the presence of Rb bound to glutathione-Sepharose beads and 10 μM [γ-$^{32}$P]ATP (2 μCi) for 30 min at 30°C. Beads were then extensively washed before adding Laemmli sample buffer.

*2.6. Immunoblotting*

Proteins were electrophoresed and transferred to nitrocellulose filters as described [5]. The mouse monoclonal anti-human Rb antibody (X2160) was provided by Dr. E. Harlow (Harvard Medical School, USA) [19] and the rabbit polyclonal anti-GST antibody was provided by Dr. K. Galaktionov (CSHL, USA) [10]. Anti-GST (1:250 dilution) and anti-MAP kinase (1:3000 dilution, anti-ERK1, Santa Cruz) antibodies were revealed with HRP-conjugated swine anti-rabbit antibody (Dako). Anti-Rb antibody (1:1 dilution) was revealed with HRP-conjugated rabbit anti-mouse antibody (Dako). ECL Western blotting detection system was from NEN.

*2.7. Bacterial production of recombinant proteins*

Glutathione *S*-transferase (GST) fusion *Xenopus* cyclin D2 protein expression and purification have been described in [10]. An identical procedure was used to express GST-human $p16^{INK4}$ (pGEX-KG-p16 plasmid kindly provided by Dr. M. Serrano, CSHL, USA), GST-human $p21^{CIP1}$ (pGEX-KG/p21 plasmid kindly provided by Dr. H. Zhang, CSHL, USA), GST-human large pocket Rb (pGEX-GST-Rb plasmid kindly provided by Dr. B. Ducommun, CNRS, France) and GST-human cdc25A (pGEX-KG-human cdc25A provided by Dr. K. Galaktionov, CSHL, USA). GST-*Xenopus* cdk4, GST-*Xenopus* cdk2 and GST-*Xenopus* cdc2 (pGEX-*Xenopus* cdk4 plasmid, pGEX-KG-*Xenopus* cdc2 and pGEX-KG-*Xenopus* cdk2 plasmids kindly provided by Dr. T. Hunt, ICRF, UK) were purified according to [20]. Human cyclin A (pT7f1 plasmid kindly provided by Dr. C. Bréchot, INSERM, France) was produced as described in [21].

## 3. Results

*3.1. Microinjected Rb localizes into the nucleus*

Although *Xenopus* Rb protein was shown to be expressed at a low level in metaphase II oocytes by immunoblotting [11], we were unable to detect the endogenous *Xenopus* Rb protein by using the same monoclonal antibody, since the Rb concentration is below the detectable level and since the monoclonal anti-human Rb antibody has a low affinity for the *Xenopus* protein. To study the oocyte Rb kinase activity, we microinjected GST-Rb into prophase oocytes. To ascertain the relevance of Rb microinjection, we first investigated the intracellular localization of GST-Rb. In somatic cells, Rb localizes almost exclusively into the nucleus, where it controls transcription of various genes through binding to transcription factors [12]. Prophase oocytes were microinjected with GST-Rb and then enucleated. Nuclei, enucleated oocytes and entire oocytes were subjected to immunoblotting with an anti-GST antibody. In non-injected oocytes, the antibody recognizes a thin 25 kDa band, corresponding to *Xenopus* GST (Fig. 1, lane 1). In GST-Rb-injected whole oocytes, the antibody recognizes a 70 kDa band, the expected molecular weight of GST-Rb, and a 25 kDa band corresponding to a mixture of endogenous GST and bacterially expressed GST present in the GST-Rb preparation (Fig. 1, lane 2). In GST-Rb-injected enucleated oocytes, the amount of GST-Rb is diminished whereas the quantity of GST is unaffected (Fig. 1, lane 3). As expected, nuclei isolated from GST-Rb-injected oocytes exhibit a strong band of GST-Rb whereas GST appears excluded from the nucleus (Fig. 1, lane 4). Taking into account the volume of the nucleus ($8\times10^6$ μm$^3$) versus the volume of the cytoplasm ($900\times10^6$ μm$^3$ total oocyte volume including vitellus, $450\times10^6$ μm$^3$ of active cytoplasm) and by scanning the bands revealed by immunoblotting, the concentration of GST-Rb was found to be 37 times higher in the nucleus than in the cytoplasm.

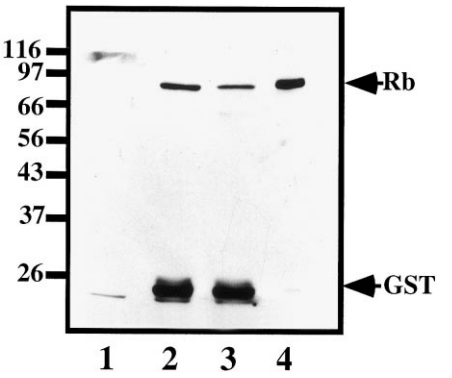


Fig. 1. Intracellular localization of microinjected GST-Rb into *Xenopus* prophase oocytes. Prophase oocytes were microinjected with bacterially expressed GST-Rb. Two hours after microinjection, they were enucleated. Immunoblotting with an anti-GST antibody of three non-injected prophase oocytes (lane 1), three GST-Rb-injected prophase oocytes (lane 2), three GST-Rb-injected prophase oocytes after enucleation (lane 3) and 11 nuclei from GST-Rb-injected prophase oocytes (lane 4). Arrows point to the position of human GST-Rb and GST. Positions of molecular weight markers are indicated on the left (kDa).

*3.2. In vivo phosphorylation of microinjected Rb during meiotic maturation*

To study the oocyte Rb kinase, GST-Rb was microinjected into prophase oocytes. Oocytes were then exposed to progesterone in order to induce meiotic maturation. Metaphase II oocytes were eventually activated by electric shock, a treatment mimicking the early events induced by fertilization: the

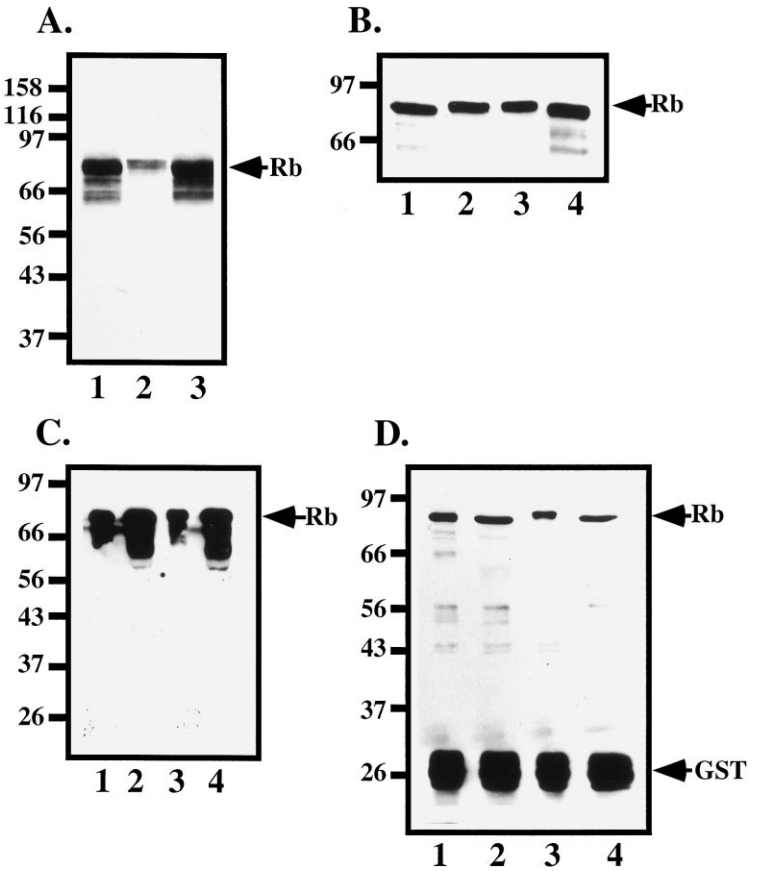


Fig. 2. Reversible phosphorylation of microinjected Rb during meiotic maturation. Prophase oocytes were microinjected with 0.1 mg/ml bacterially expressed GST-Rb and were incubated 2 h later in the presence of $10^{-6}$ M progesterone. Prophase, GVBD, metaphase II and activated oocytes were then collected and microinjected GST-Rb was isolated on glutathione-Sepharose beads. A: Immunoblotting with an anti-human Rb antibody of GST-Rb from prophase oocytes (lane 1), from metaphase II oocytes (lane 2) and from activated oocytes (lane 3). B: Immunoblotting with an anti-GST antibody of GST-Rb from prophase oocytes (lane 1), from GVBD oocytes (lane 2), from metaphase II oocytes (lane 3) and from activated oocytes (lane 4). C, D: Immunoblotting with an anti-human Rb antibody (C) and with an anti-GST antibody (D) of GST-Rb from prophase oocytes (lanes 1 and 2) and from GVBD oocytes (lanes 3 and 4). After extraction on glutathione-Sepharose beads, GST-Rb was incubated in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of 5 U alkaline phosphatase. Arrows point to the position of human GST-Rb and GST. Positions of molecular weight markers are indicated on the left (kDa).

cell reenters interphase. Microinjected GST-Rb was isolated from prophase, GVBD, metaphase II and activated oocytes by affinity binding on glutathione-Sepharose beads and detected by immunoblotting with two antibodies: an anti-GST antibody recognizing the GST tag of the protein, and a monoclonal anti-human Rb antibody. Both antibodies recognize a 70 kDa band in microinjected oocytes, corresponding to GST-Rb (Fig. 2). GST-Rb is much less recognized by the monoclonal anti-Rb antibody in metaphase II oocytes than in prophase and activated oocytes (Fig. 2A). In addition, the mobility of the poorly recognized metaphase form is retarded in comparison with both other forms (Fig. 2A). In contrast, the same amount of GST-Rb is recognized by the anti-GST antibody in prophase, GVBD, metaphase II and activated oocytes (Fig. 2B), demonstrating that the microinjected protein is stable all over the meiotic cell cycle. The forms isolated from oocytes at GVBD and metaphase II migrate more slowly than both other forms (Fig. 2B). These observations suggest that GST-Rb is modified transiently during meiotic maturation starting GVBD. The modification observed in GVBD and metaphase II oocytes decreases the affinity of the monoclonal anti-Rb antibody towards Rb (Fig. 2A) and retards the migration of the protein (Fig. 2A,B).

To investigate the possibility that this modification corre-

Table 1
Specific activity of various protein kinases towards histone H1, MBP and Rb in vitro

| | Cdk2-cyclin A | Cdc2-cyclin A | Cdc2-cyclin B | MAP kinase |
|---|---|---|---|---|
| Histone H1 | 1.250 | 0.354 | 6.941 | 0.107 |
| MBP | ND | ND | 5.253 | 15.530 |
| Rb | 0.116 | 0 | 0.514 | 7.605 |

The cdc2-cyclin A and cdk2-cyclin A complexes were reconstituted and phosphorylated on threonine-161 and 160 in vitro by using bacterially expressed recombinant proteins. The cdc2-cyclin B complex was immunoprecipitated from metaphase II oocytes with an anti-cyclin B2 antibody. MAP kinase is an active thiophosphorylated bacterially expressed *Xenopus* form. Activities are expressed as pmol P incorporated/μg of enzyme/min at 30°C. ND: not determined.

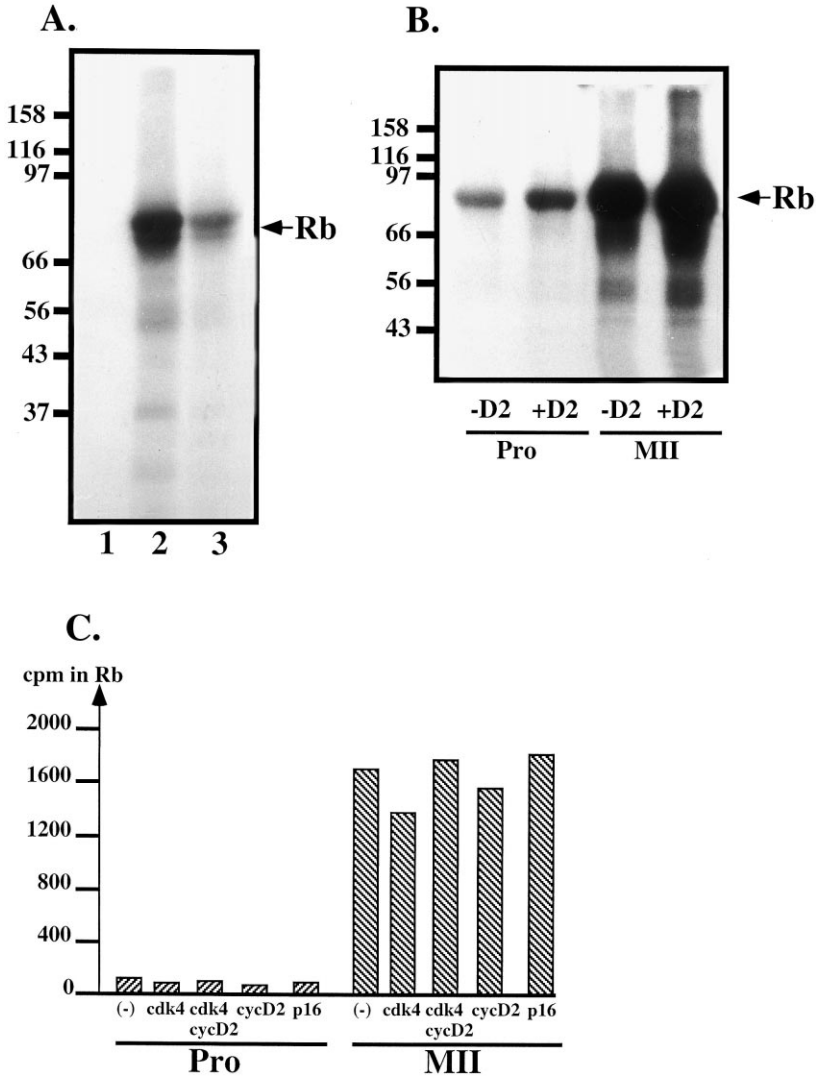


Fig. 3. Reversible activation of an Rb kinase during meiotic maturation. A: Prophase oocytes (lane 1), metaphase II oocytes (lane 2) and activated oocytes (lane 3) were homogenized and centrifuged at 15 000×*g*. The 15 000×*g* supernatants were incubated in the presence of [γ-$^{32}$P]ATP and GST-Rb bound on glutathione-Sepharose beads. After extensive washing, beads were resuspended in Laemmli sample buffer, loaded on SDS-PAGE and autoradiographed. B: Prophase oocytes were microinjected with 0.1 mg/ml bacterially expressed GST-cyclin D2 (+D2) or with control buffer (−D2) and were incubated 2 h later in the presence of $10^{-6}$ M progesterone. Prophase (Pro) and metaphase II (MII) oocytes were then collected and the Rb kinase activity was assayed. C: A 15 000×*g* supernatant from prophase (Pro) and metaphase II (MII) oocytes was preincubated in the presence of control buffer (−), or 30 μg/ml GST-cdk4 (cdk4) or 30 μg/ml GST-cdk4 and 30 μg/ml GST-cyclin D2 (cdk4/cycD2) or 30 μg/ml cyclin D2 (cycD2) or 25 μg/ml GST-p16$^{INK4}$ (p16) for 20 min at 23°C. The extracts were then assayed for Rb kinase activity. The position of GST-Rb is indicated on the right. Positions of molecular weight markers are indicated on the left (kDa).

sponds to a phosphorylation event, the same experiment was repeated with prophase and GVBD microinjected oocytes by introducing a phosphatase treatment after extraction of GST-Rb and before immunoblotting. After phosphatase treatment, the affinity of the monoclonal anti-Rb antibody towards GST-Rb extracted from GVBD oocytes strongly reincreases (Fig. 2C). Similarly, phosphatase treatment accelerates Rb mobility of GVBD oocytes as revealed with the anti-GST antibody (Fig. 2D). This result indicates that Rb is phosphorylated at GVBD and in metaphase oocytes, and that the phosphorylated form exhibits a lower affinity for the anti-Rb antibody and a retarded mobility in the gel.

Interestingly, the prophase form of Rb was also affected by the phosphatase treatment, to a lesser extent than the GVBD form: its affinity for the monoclonal anti-Rb antibody and its mobility are also increased (Fig. 2C,D). After phosphatase treatment, both prophase and GVBD Rb forms behave identically in terms of affinity for the monoclonal anti-Rb antibody and migration, although the non-treated forms are obviously different. This result strongly suggests that Rb is hypophosphorylated in prophase whereas Rb is hyperphosphorylated in GVBD oocytes.

### 3.3. A Rb kinase is transiently activated during meiotic maturation

To assay the Rb kinase activity, 15 000×*g* extracts were prepared from prophase, metaphase II and activated oocytes and incubated with GST-Rb bound on glutathione-Sepharose beads in the presence of [γ-$^{32}$P]ATP. Beads were then washed out and loaded on SDS-PAGE. Phosphorylation of GST-Rb by the various extracts was analyzed by autoradiography (Fig. 3A). GST-Rb is not phosphorylated by prophase oocyte ex-

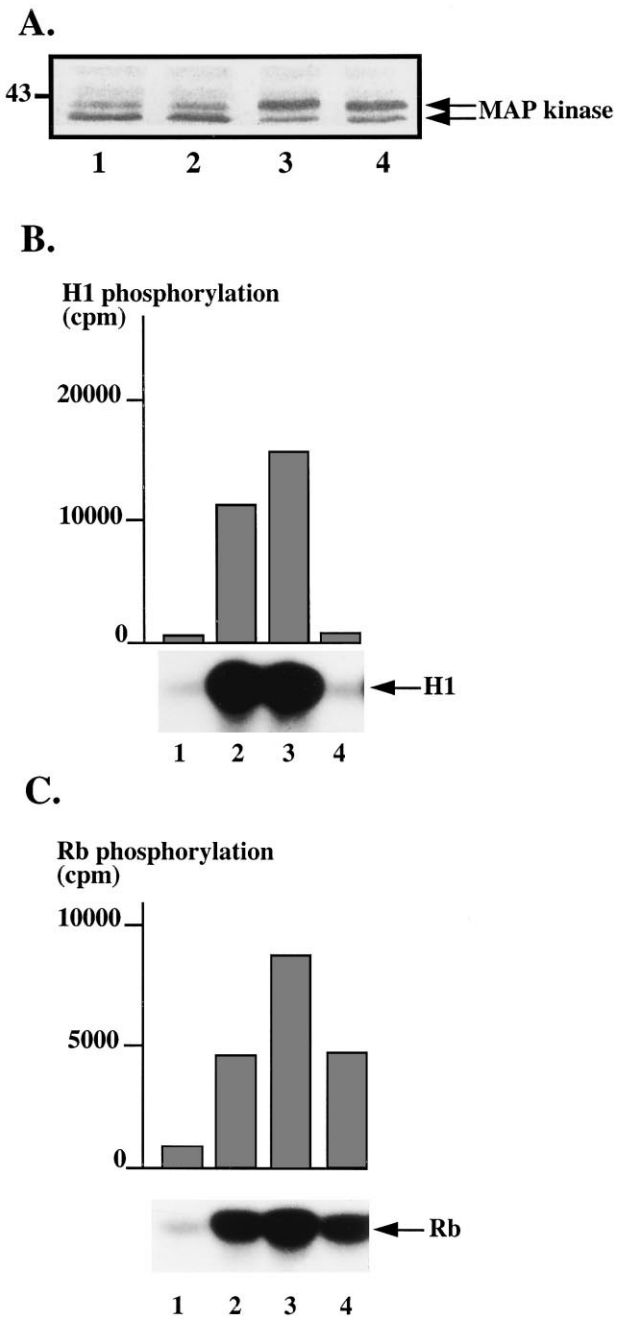


Fig. 4. MAP kinase activity and cdc2 kinase contribute to the Rb kinase activity. A 100 000×*g* supernatant from prophase oocytes was incubated for 3 h at 23°C in the presence of either control buffer (lane 1) or 1 μg/ml cdc25A (lane 2) or 500 nM okadaic acid (lane 3) or 500 nM okadaic acid and 10 μg/ml $p21^{CIP1}$ (lane 4). Samples were then collected for immunoblotting with an anti-MAP kinase antibody (A), for p13 binding of cdc2 and histone H1 kinase assay (B) and for Rb kinase assay on GST-Rb bound to glutathione-Sepharose beads (C). Arrows on the right point to the position of the two forms (active/inactive, respectively retarded/accelerated migration) of MAP kinase (A), of radioactive phosphorylated histone H1 (B) and of radioactive phosphorylated Rb (C).

tracts, and very poorly phosphorylated by activated oocyte extracts (Fig. 3A). In contrast, a strong phosphorylation occurs with the metaphase oocyte extract (Fig. 3A), suggesting that a Rb kinase is specifically activated during meiotic maturation and inactivated when oocytes reenter interphase after activation. GST is not phosphorylated by the various extracts (data not shown). By analyzing GST-Rb phosphorylation at various times after progesterone addition, we have shown that the Rb kinase is activated as soon as GVBD, and then remains stable until metaphase II arrest, confirming the previous analysis of the altered migration of microinjected GST-Rb (Fig. 2B).

### *3.4. Oocyte cdk2 and cdk4/6 kinases do not contribute to the Rb kinase activity*

Cyclin B-cdc2, cyclin A-cdc2 and cyclin D2-cdk4 complexes are activated during meiotic maturation [22] and are therefore potential candidates as Rb kinases. However, the cyclin E-cdk2 complex is activated at the time of metaphase II, much later than the Rb kinase activity.

In an attempt to identify the kinases responsible for Rb phosphorylation at GVBD, we studied the ability of the various candidates, cdc2-cyclin B, cdc2-cyclin A and cdk2-cyclin A, to phosphorylate Rb in vitro. Active cdc2-cyclin B was isolated by immunoprecipitation with an anti-cyclin B antibody from metaphase oocytes. *Xenopus* cdc2, *Xenopus* cdk2 and human cyclin A were recombinant proteins, associated and phosphorylated in vitro on threonine-161 and threonine-160 of cdc2 and cdk2 respectively, as described previously [17,20]. As shown in Table 1, these three complexes exhibit a lower specific activity for Rb than for histone H1 in vitro.

Prophase oocytes were microinjected with *Xenopus* cyclin D2 and then induced to mature by progesterone. The Rb kinase activity was then assayed in prophase or metaphase extracts from control or cyclin D2-injected oocytes. Cyclin D2 microinjection did not increase the Rb kinase activity of the oocyte (Fig. 3B). A similar experiment was performed in vitro. We used a 15 000×*g* extract from prophase and metaphase oocytes. The Rb kinase activity of these extracts is not affected by adding $p16^{INK4}$, a specific inhibitor of the cyclin D-cdk4/6 family (Fig. 3C), suggesting that cyclin D-cdk4/6, if present in the extracts, is not involved in Rb kinase activity. Moreover, addition of either *Xenopus* cyclin D2, *Xenopus* cdk4 or both proteins was unable to increase the Rb kinase activity of a metaphase extract or to generate Rb kinase activity in a prophase extract (Fig. 3C).

### *3.5. MAP kinase and cdc2-cyclin B complex could serve as oocyte Rb kinases*

Rb kinase is activated at GVBD, is stable until metaphase II and is inactivated during activation, as the cell reenters interphase. This activity pattern corresponds to the activity pattern of cdc2-cyclin B kinase, of c-mos kinase and of MAP kinase [1,23,24]. To determine whether these kinases are involved in the phosphorylation of ectopic Rb, we took advantage of a simplified in vitro system, consisting of a 100 000×*g* supernatant from prophase oocytes. This extract does not contain cdk2, c-mos kinase, cyclin E or cyclin A, which are synthesized during meiotic maturation in response to progesterone [22,24]. It contains inactive MAP kinase, as judged by its electrophoretic mobility that is strictly correlated with its phosphorylation state and its activity (Fig. 4A), inactive cdc2-cyclin B as judged by histone H1 kinase assay of p13-isolated cdc2 (Fig. 4B) and no Rb kinase activity (Fig. 4C). When the 100 000×*g* supernatant from prophase oocytes is supplemented with 500 nM okadaic acid, a specific inhibitor

of phosphatase 2A known to activate cdc2-cyclin B and MAP kinase after microinjection into prophase oocytes [25], both cyclin B-cdc2 complex and MAP kinase are activated in the extract (Fig. 4A,B). Under these conditions, a strong Rb kinase activity is generated (Fig. 4C).

We then manipulated the prophase extract to activate cdc2-cyclin B kinase without activating MAP kinase. Cdc25 is the specific phosphatase that directly dephosphorylates and activates the cdc2 kinase [26]. Addition in the prophase extract of a recombinant form of the cdc25 phosphatase leads to the specific dephosphorylation of cdc2 and activation of the cdc2-cyclin B complex (Fig. 4B). Under these conditions, MAP kinase is not activated (Fig. 4A). A Rb kinase is activated (Fig. 4C), indicating that the main cdk-cyclin complex of the oocyte, cdc2-cyclin B, contributes to the Rb kinase. However, the activation level of the Rb kinase activity is lower than after activation in the extract of both MAP kinase and cdc2-cyclin B kinase (Fig. 4C, lanes 2 and 3).

The reciprocal experiment was then conducted. MAP kinase can be activated in the extract in the absence of cyclin B-cdc2 kinase activity by adding 500 nM okadaic acid in the presence of $p21^{CIP1}$ that blocks cdc2 activation (Fig. 4A,B). Under these conditions, the Rb kinase is activated, but again, its level is decreased as compared to after okadaic acid addition alone (Fig. 4C). There is therefore an equal contribution of MAP kinase and cdc2-cyclin B to the Rb kinase activity under these conditions. The possibility that MAP kinase can phosphorylate Rb was confirmed by an in vitro approach. A constitutively active form of MAP kinase, a thiophosphorylated bacterially expressed histidine-tagged *Xenopus* MAP kinase, was purified as described previously [18] and its activity towards three different substrates, histone H1, MBP and GST-Rb, was assayed (Table 1). Rb is a good in vitro substrate of *Xenopus* MAP kinase (Table 1). GST is not phosphorylated by *Xenopus* MAP kinase in vitro (data not shown).

## 4. Discussion

### *4.1. A Rb kinase is activated during meiotic maturation*

The *Xenopus* Rb protein is present at a low level and exhibits only 56% overall identity with the amino acid sequence of human Rb [11]. These observations explain why the various monoclonal antibodies directed against human Rb that we used recognize *Xenopus* Rb very poorly. To study the Rb kinase activity, we took advantage of the possibility of microinjection in oocytes and we overexpressed in vivo a fusion protein consisting of the large pocket of human Rb and GST (GST-Rb). During meiotic maturation, GST-Rb is clearly modified at GVBD: its migration in SDS-PAGE is retarded and its affinity towards a monoclonal anti-human Rb antibody is decreased. These two modifications are abolished by in vitro phosphatase treatment, demonstrating that they correspond to a phosphorylation event. By the same approach, we demonstrated that GST-Rb is also phosphorylated in prophase non-stimulated oocytes, but to a much lesser extent than at GVBD. We therefore concluded that Rb is hypophosphorylated in prophase, a situation recalling the G0 or early G1 status of Rb, and becomes hyperphosphorylated during meiotic maturation, as it happens starting the late G1 until M phase of the somatic cell cycle [13]. When metaphase oocytes are activated and reenter interphase, Rb is dephosphorylated, as during the M/G1 transition of the cell cycle. Interestingly, the levels of Rb kinase activity occurring during meiotic maturation and activation mimic the phosphorylation events of Rb during a complete somatic cell cycle. *Xenopus* oocytes therefore represent a good tool to get new insights into the identity of Rb kinases.

### *4.2. The Rb kinase activated at GVBD does not correspond to cdk2 and cdk4/6 kinases*

In this report, we have shown that a Rb kinase activity is activated during meiotic maturation, in good correlation with the in vivo phosphorylation of the ectopic protein. Accumulating evidence indicates that phosphorylation of Rb during the somatic cell cycle is controlled by cyclin-cdk complexes. In an oversimplified scheme, Rb phosphorylation would be initiated by cyclin D-cdk4/6 in mid/late G1 and maintained through late G1, S and M successively by cyclin E-cdk2 and cyclin A-cdk2/cdc2. Since all these complexes are activated during meiotic maturation [22], they represent potential candidates as Rb kinases. However, inhibition of the cdk4/6 complexes by $p16^{INK4}$ inhibitor or overexpression of the cyclin D2-cdk4 complex in vivo or in the extracts did not lead to any variation of Rb kinase activity. Timing of synthesis and activation of cyclin E-cdk2 does not coincide with the appearance of the Rb kinase activity [8,9] and cdk7 kinase activity does not vary during oocyte maturation and early development [27], excluding the participation of these cdks to the oocyte Rb kinase activity.

### *4.3. MAP kinase and cdc2-cyclin B could contribute to the Rb kinase activity*

In parallel with the cdk pathway, progesterone induces the synthesis of the c-mos proto-oncoprotein, which acts as a MAP kinase kinase kinase and leads to the activation of MAP kinase [2]. However, the c-raf kinase, another member of the MAP kinase kinase kinase family, is also activated in response to progesterone [3,4].

The kinase responsible for Rb phosphorylation in the oocyte has to be activated at GVBD and inactivated after fertilization. The cdc2-cyclin B complex and the kinases involved in the MAP kinase cascade, such as c-mos, c-raf and MAP kinase, exhibit this type of activity pattern [1]. All of them could potentially serve as Rb kinases. C-mos, cyclin A, cyclin E and cdk2 proteins are absent in prophase oocytes and are synthesized in maturing oocytes in response to progesterone [24]. In contrast, we have shown that the Rb kinase is present in an inactive form in a high speed prophase extract and can be activated in the extract upon addition of okadaic acid or cdc25 phosphatase and independently of protein synthesis, excluding the possibility that c-mos and cyclin A- or cyclin E-dependent kinases could serve as Rb kinase in the oocyte. Similarly, the Rb kinase activity remains stable in metaphase extracts specifically depleted of c-raf kinase (data not shown), eliminating c-raf as Rb kinase.

In contrast, MAP kinase and the cdc2-cyclin B complex appear to be good candidates as Rb kinase in the oocyte. During progesterone-induced maturation, during fertilization and in prophase extracts supplemented with either cdc25 or okadaic acid, the activation of a Rb kinase correlates with the presence of active MAP kinase and cdc2 kinase. By activating both MAP kinase and cyclin B-cdc2 kinase in the prophase extracts, the Rb kinase activity was strongly activated. By selectively activating either MAP kinase or cdc2 kinase in

the extracts, we were able to generate an intermediate level of Rb kinase activity. These results strongly suggest that both kinases contribute to the phosphorylation of Rb in the extract. MAP kinase belongs to the family of proline-directed protein kinases, which also includes cdc2 and all cdks, and its substrate specificity therefore overlaps with cdks [28]. This could explain that a given protein can be phosphorylated either by MAP kinase or by cdks following the physiological status of the cell. Our results emphasize the parallel between the signal transduction pathways involved in the somatic cell responses and the hormonal triggering of maturation. They support the hypothesis that the cyclin B-cdc2 complex could serve as a Rb kinase during M phase. They open the possibility that MAP kinase contributes to Rb phosphorylation at the G0/G1 transition of the somatic cell cycle, a period when transduction cascades implicating MAP kinase are induced by growth factors and ultimately tie to elements of the cell cycle such as Rb.

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