

## CORRECTION

# Correction: Phosphatase 2A and polo kinase, two antagonistic regulators of Cdc25 activation and MPF auto-amplification (*J. Cell Sci.* 112, 3747-3756)

Anthi Karaiskou, Catherine Jessus, Thierry Brassac and René Ozon

Journal of Cell Science was made aware by a reader of several issues concerning duplication of data in Fig. 3B,C and Fig. 5B,D in *J. Cell Sci.* (1999) **112**, 3747-3756.

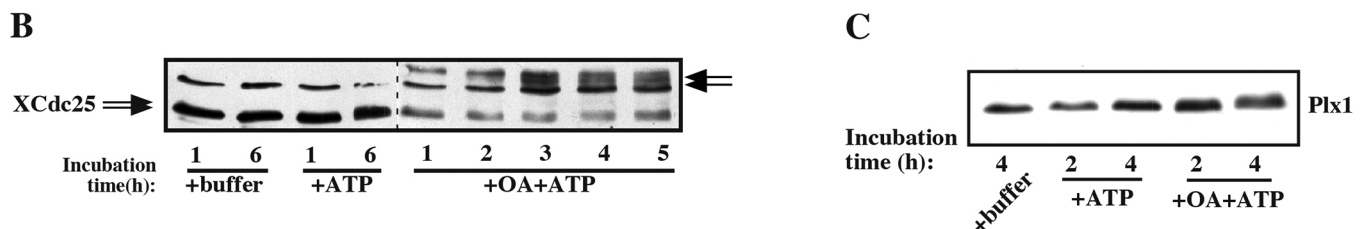
The first author, Anthi Karaiskou, subsequently also contacted the journal to say that during figure compilation, some of the bands in western blots in the figures were duplicated during figure assembly. After further discussion, the journal referred this matter to Université Pierre et Marie Curie (UPMC, now Sorbonne Université), who investigated and cleared the authors of any wrongdoing. The UPMC committee decided that the conclusions of the paper were not affected by the errors and recommended correction of the paper (full reports available at: [http://www2.cnrs.fr/sites/communique/fichier/rapport\\_conclusions.pdf](http://www2.cnrs.fr/sites/communique/fichier/rapport_conclusions.pdf) and [http://www2.cnrs.fr/sites/communique/fichier/rapport\\_analyse\\_detaillee.pdf](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 on Publication Ethics (COPE), which state: “Retraction should usually be reserved for publications that are so seriously flawed (for whatever reason) that their findings or conclusions should not be relied upon”. The standards of figure assembly and data presentation in this paper fall short of current good scientific practice. However, given that the investigating committee at UPMC declared that the conclusions of the paper were not affected by the errors, the appropriate course of action – according to COPE guidelines – is to publish a Correction, which the journal has made as detailed as possible.

Readers should note that the policy of the UPMC is that authors should retain original data for 10 years and that this paper falls outside this period.

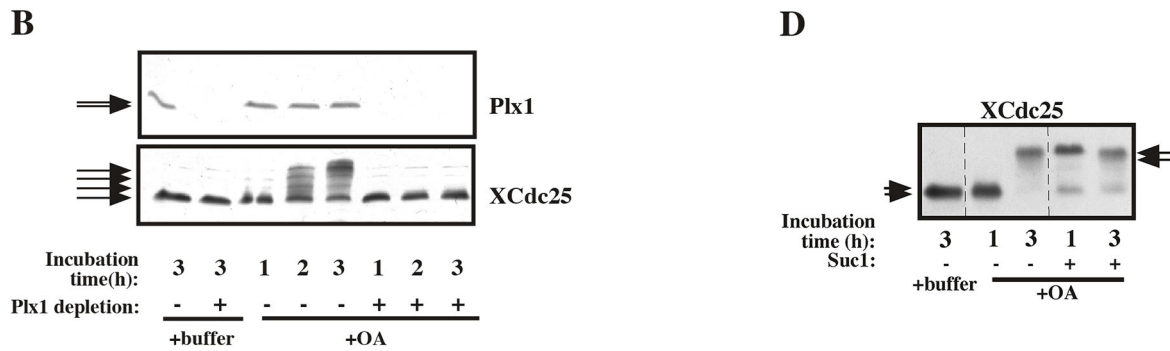
Although the authors were unable to locate all the original data, they did produce replicates of experiments carried out at the same time showing similar results for most blots; the authors have therefore assembled new figure panels. However, they were unable to find the original data for the pTyr Cdc2 blot in Fig. 3B or Plk1 blot in Fig. 3C and so have requested that these blots be disregarded. The authors state that as Cdc2 kinase activity is also shown in Fig. 3A, the conclusions are not affected by removal of the pTyr Cdc2 blot; the authors also state that the Plk1 panel in Fig. 3C does not bring any input to the scientific conclusion.

The new panels for Figs 3 and 5 and the corrected legends are shown.



**Fig. 3. Cdc2 kinase activation in the F40.** (B) The F40 was incubated in the absence of effectors (+buffer), or in the presence of an ATP-regenerating system (+ATP), or 500 nM okadaic acid and an ATP-regenerating system (+OA+ATP). After incubation for the times indicated (hours), samples were analyzed by western blotting using anti-*Xenopus* Cdc25 antibody. (C) The F40 was incubated in the absence of effectors (+buffer), or in the presence of an ATP-regenerating system (+ATP), or 500 nM okadaic acid and an ATP-regenerating system (+OA+ATP). Samples were collected at various times, as indicated, and analyzed by western blotting using an anti-*Xenopus* Plx1 antibody.

As a result of corrections to these figures, readers of *J. Cell Sci.* **112**, 3747-3756 should ignore text related to the blot removed from Fig. 3B on p. 3750 (second column): ‘This linear activation of Cdc2 kinase is correlated with a slight and progressive tyrosine dephosphorylation (Fig. 3B).’ Also, p. 3750 (bottom of second column) should now read: ‘After addition of ATP or ATP together with okadaic acid in the F40, Plx1 kinase is activated in parallel with Cdc2 kinase, as judged by its electrophoretic mobility and Plx1 kinase assay (Fig. 3C,D).’



**Fig. 5. Plx1 kinase and Suc1/Cks mediated Cdc2/Cdc25 association are necessary for the Cdc2/Cdc25 positive feed-back loop.** (B) The mock-depleted or Plx1-depleted F40 was incubated in the absence of effectors (+buffer) or in the presence of 500 nM okadaic acid and an ATP-regenerating system (+OA). Samples were collected at various incubation times, as indicated, and immunoblotted with the anti-Plx1 antibody (upper panel) and the anti-Cdc25 antibody (lower panel). (D) The F40 was incubated in the presence of an ATP-regenerating system, supplemented with buffer, with 500nM okadaic acid (+OA) or with okadaic acid and 40  $\mu$ g/ml p13Suc1 protein (Suc1). Samples were collected at various incubation times and submitted to western blotting with the anti-XCdc25 antibody. Arrows indicate the various migrating forms of XCdc25.

Readers should also note that there may be unmarked splicing of lanes from western blots in this paper, which although not acceptable by today's standards was prevalent in 1999 when the paper was published.

The authors apologise to the journal and readers for these errors.

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

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## Phosphatase 2A and Polo kinase, two antagonistic regulators of Cdc25 activation and MPF auto-amplification

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### SUMMARY

The auto-catalytic activation of the cyclin-dependent kinase Cdc2 or MPF (M-phase promoting factor) is an irreversible process responsible for the entry into M phase. In *Xenopus* oocyte, a positive feed-back loop between Cdc2 kinase and its activating phosphatase Cdc25 allows the abrupt activation of MPF and the entry into the first meiotic division. We have studied the Cdc2/Cdc25 feed-back loop using cell-free systems derived from *Xenopus* prophase-arrested oocyte. Our findings support the following two-step model for MPF amplification: during the first step, Cdc25 acquires a basal catalytic activity resulting in a linear activation of Cdc2 kinase. In turn Cdc2 partially phosphorylates Cdc25 but no amplification takes

place; under this condition Plx1 kinase and its activating kinase, Plkk1 are activated. However, their activity is not required for the partial phosphorylation of Cdc25. This first step occurs independently of PP2A or Suc1/Cks-dependent Cdc25/Cdc2 association. On the contrary, the second step involves the full phosphorylation and activation of Cdc25 and the initiation of the amplification loop. It depends both on PP2A inhibition and Plx1 kinase activity. Suc1-dependent Cdc25/Cdc2 interaction is required for this process.

Key words: Cdc2, Cdc25, Polo kinase, Phosphatase 2A, *Xenopus* oocyte

### INTRODUCTION

The entry into M phase of the first meiotic division of the *Xenopus* oocyte is initiated by the cyclin-dependent kinase Cdc2 or MPF (M-phase promoting factor). The protein phosphatase Cdc25 activates Cdc2 by dephosphorylating the Thr14 and Tyr15 inhibitory residues (Dunphy and Kumagai, 1991; Gautier et al., 1991; Jessus and Ozon, 1993).

In *Xenopus* oocyte, a unique property of MPF is its ability to undergo an autocatalytic activation, in the absence of protein synthesis and independently of the oocyte nucleus or GV (germinal vesicle), during the first meiotic division (Gerhart et al., 1984; Masui and Markert, 1971; Wasserman and Masui, 1975). A positive feed-back loop between Cdc2 and Cdc25 allows the abrupt activation of MPF just before GVBD (germinal vesicle breakdown) (Izumi et al., 1992; Kumagai and Dunphy, 1992). A major question is to understand how this positive feed-back loop is initially triggered and which molecular partners are involved in this process. Despite numerous studies, our knowledge of the biochemical regulation of this crucial event, which regulates not only the oocyte meiotic division but also the mitotic cell cycle, remains limited.

Two main characteristics of MPF auto-amplification have been established using the *Xenopus* oocyte: first, Cdc2 and Cdc25 form a transient complex around GVBD time (Gautier et al., 1991; Jessus and Beach, 1992); second, abrupt Cdc2

activation parallels a hyperphosphorylation of Cdc25, that is visualized by a large electrophoretic shift and is required for its activation (Izumi et al., 1992; Kumagai and Dunphy, 1992).

It is therefore of primary importance to understand how the association between Cdc25 and Cdc2 is regulated and to identify the protein kinases and phosphatases that control the phosphorylation level of Cdc25.

The *Xenopus* p9 protein (Xe-p9), the homologue of the Suc1/Cks proteins, interacts physically with Cdc2 in *Xenopus* egg extracts and appears to govern the interaction of Cdc2 with both positive and negative regulators; interestingly, it was shown that overexpression or depletion of Xe-p9 inhibits Tyr15 dephosphorylation and activation of Cdc2 in *Xenopus* egg extracts (Jessus et al., 1990; Patra and Dunphy, 1996, 1998). It may therefore be an important regulator of the transient interaction between Cdc2 and Cdc25.

Besides Cdc2, which is able to interact physically with regulatory docking proteins such as Suc1/Cks, Cdc25 can also associate with regulatory proteins like 14-3-3 or Pin-1 (Conklin et al., 1995; Kumagai et al., 1998b; Lu et al., 1996; Sanchez et al., 1997; Shen et al., 1998). In *Xenopus* egg extracts, the interaction of Cdc25 with 14-3-3  $\epsilon$  is mediated by phosphorylation of Ser287 of Cdc25, that is catalyzed by a *Xenopus* Chk1 kinase. The binding of 14-3-3  $\epsilon$  to Cdc25 delays its activation (Kumagai et al., 1998a,b). The WW domain of the peptidyl-prolyl isomerase Pin1 associates with the

hyperphosphorylated mitotic form of Cdc25 (Crenshaw et al., 1998; Lu et al., 1996, 1999; Yaffe et al., 1997), through the MPM2 epitope of Cdc25 (Yaffe et al., 1997). In vitro studies suggest that binding of either of these proteins, 14-3-3 or Pin1, does not appear to significantly affect the phosphatase activity of Cdc25 (Crenshaw et al., 1998; Kumagai et al., 1998b). Nevertheless, 14-3-3 and Pin-1 play a role in the timing of mitosis through mechanisms that remain to be elucidated.

Cdc25 is an in vitro substrate of numerous protein kinases. A major kinase that phosphorylates and activates Cdc25 is Cdc2 itself (Hoffmann et al., 1993; Izumi and Maller, 1993; Strausfeld et al., 1994). The phosphorylation of Cdc25 by Cdc2 is necessary for MPF auto-amplification but it is not sufficient (Izumi and Maller, 1993; Karaïskou et al., 1998). Moreover, although the hyperphosphorylated mitotic form of Cdc25 is an MPM2 epitope, it has been reported that *Xenopus* kinases phosphorylating MPM-2 are distinct from Cdc2 (Kuang and Ashorn, 1993). The phosphorylation of Cdc25 catalyzed by the *Xenopus* homologue of *Drosophila* Polo kinase, Plx1 kinase, has been implicated both in vitro and in vivo in Cdc2 activation. In contrast with Cdc2, Plx1 kinase converts Cdc25 into a MPM2 epitope in vitro (Kumagai and Dunphy, 1996; Qian et al., 1998a). Recently, a new kinase, Plkk1, able to phosphorylate and activate Plx1, has been characterized in *Xenopus* (Qian et al., 1998b). How is this kinase activated and whether it participates to the feedback loop between Cdc2 and Cdc25 remains to be determined. The possibility that other kinases phosphorylate Cdc25 to regulate positively or negatively its enzymatic activity or its subcellular localization cannot be excluded (Izumi and Maller, 1993, 1995; Qian et al., 1998a).

The identity of the protein phosphatases that catalyze the dephosphorylation of the different phosphorylated residues of Cdc25 is still uncertain. In vitro, high concentration of PP2A dephosphorylates the hyperphosphorylated form of Cdc25. Furthermore, okadaic acid, a specific inhibitor of PP2A and PP1 phosphatases, induces MPF auto-amplification, at concentration not sufficient to inhibit PP1 (Felix et al., 1990; Goris et al., 1989; Izumi et al., 1992; Karaïskou et al., 1998). However, the direct physiological target of PP2A has not been unequivocally identified.

In order to better understand the mechanism of the MPF autocatalytic activation during meiotic maturation, we have recently developed a cell-free system prepared from prophase-arrested *Xenopus* oocytes, that reproduces in vitro the all-or-none MPF activation (Karaïskou et al., 1998). In the present study, we have used this in vitro system, as well as a simplified fractionated one, to further investigate the different molecular partners that are implicated in the network that allows Cdc2 activation. We show here that the initiation of the Cdc2/Cdc25 feed-back loop, leading to an abrupt Cdc2 kinase activation, requires two sets of phosphorylation reactions on Cdc25; one is Cdc2 kinase-dependent and the second requires Plx1 activity and PP2A inhibition. Only this later step requires Suc1-mediated Cdc2/Cdc25 interaction.

## MATERIALS AND METHODS

### Materials

[ $\gamma$ -<sup>32</sup>P] ATP was purchased from DuPont NEN. Okadaic acid was from ICN. Reagents, unless otherwise specified, were from Sigma.

### Preparation of *Xenopus* oocyte lysates

Fully-grown *Xenopus* prophase oocytes were obtained as in the method of Jessus et al. (1987) and lysed in 4 volumes of EB (80 mM  $\beta$ -glycerophosphate, 20 mM EGTA, 15 mM MgCl<sub>2</sub>, 1 mM DTT, pH 7.3), supplemented with protease inhibitors: 25  $\mu$ g/ml leupeptin and aprotinin, 10  $\mu$ g/ml pepstatin, 1 mM benzamide, 1 mM AEBSF (Pentapharm AG).

### Fractionation of *Xenopus* oocyte extracts

*Xenopus* oocyte lysates were centrifuged at 15,000 g at 4°C for 15 minutes (Sigma 302K centrifuge). The cytoplasmic layer was then centrifuged at 100,000 g at 4°C for 1 hour in a TL-100 Beckman centrifuge (TL-100-2 rotor). The supernatant (cytosolic extract) was collected.

Further fractionation was performed by saturation of the cytosolic extract with 40% ammonium sulfate. After an overnight incubation at 4°C, the solution was centrifuged at 15,000 g at 4°C for 30 minutes (Sigma 302K centrifuge). The pellet was resuspended in EB buffer and ultrafiltrated by 3000 D MWCO Centriscart I units (Sartorius), to a final ratio of 1 oocyte in 1  $\mu$ l of EB, giving rise to the 0%-40% ammonium sulfate fraction or F40. The supernatant was further saturated to a final 60% ammonium sulfate concentration. Incubation, centrifugation, resuspension and ultrafiltration were performed as for the 0%-40% ammonium sulfate fraction, giving rise to the 40%-60% ammonium sulfate fraction or F60.

### In vitro MPF amplification assay

The cytosolic extract or the F40 were supplemented with either an ATP-regenerating system (10 mM creatine phosphate, 80  $\mu$ g/ml creatine phosphokinase, 1 mM ATP, 1 mM MgCl<sub>2</sub>) or with EB buffer. The extract or the fraction was then incubated at room temperature in the presence of various effectors: 500 nM okadaic acid, 2  $\mu$ g/ml human Cdc25A, 200 nM p21<sup>Cip1</sup>, 40  $\mu$ g/ml *S. pombe* p13<sup>Suc1</sup>. Reaction was stopped at different times by adding 1 mM Na-orthovanadate at 4°C. Samples were collected either for western blot analysis and for kinase assays.

### Plx1 immunoprecipitation and immunodepletion

The polyclonal anti-Plx1 antibody was prepared and purified as described by Abrieu et al. (1998). 100  $\mu$ l of the cytosolic extract or of the F40 fraction were incubated with 1  $\mu$ l of the purified polyclonal anti-Plx1 antibody for 2 hours at 4°C. 25  $\mu$ l Protein A-Sepharose were added and the extract or fraction was further incubated for 1 hour at 4°C. Immunoprecipitates were collected after 5 minutes centrifugation at 15,000 g and the immunodepleted supernatant was used for in vitro MPF amplification assays.

### Kinase assays

Samples collected during the in vitro MPF amplification assay were incubated with p13<sup>Suc1</sup>-Sepharose beads and submitted to Cdc2 kinase assay (Jessus et al., 1991). Plx1 kinase activity was measured in Plx1 immunoprecipitates as described by Karaïskou et al. (1998).

### Western blotting

Proteins were electrophoresed on a 12.5% SDS-PAGE Anderson gel (Anderson et al., 1973) and transferred to nitrocellulose filters (Schleicher and Schuell) as described by Jessus et al. (1991). The polyclonal rabbit antibody against the C terminus of *Xenopus* Cdc25 and polyclonal rabbit anti-Plkk1 antibody are kindly provided by Dr J. Maller and Dr Y. W. Qian (Qian et al., 1998b). The polyclonal sheep antibody against *Xenopus* cyclin B2 was a kind gift from Dr J. Maller (Gautier and Maller, 1991). The polyclonal rabbit anti-Plx1, used in western blotting, is a kind gift from Dr P. Descombes and Dr E. A. Nigg (Descombes and Nigg, 1998). Polyclonal rabbit antibody directed against Cdc2 phosphorylated on tyrosine was purchased at NEB (New England Biolabs). Polyclonal rabbit anti-14-3-3 $\epsilon$  antibody was purchased at Santa Cruz Biotechnology Inc. Polyclonal guinea-pig anti-

PR65 (or A subunit), anti-PR55 (or B subunit) (Bosch et al., 1995) and anti-catalytic subunit (or C subunit) of *Xenopus* PP2A were produced in the laboratory. In all cases, horseradish peroxidase-coupled antibodies were used (Jackson Immunoresearch) and immunoblots were analyzed by a chemiluminescence detection system (NEN).

### Bacterial production of recombinant proteins

Glutathione S-transferase (GST) fusion human Cdc25A (pGEX-KG-human Cdc25A provided by Dr K. Galaktionov, CSHL, USA; Galaktionov and Beach, 1991), GST-p21<sup>Cip1</sup> (pGEX-KG-p21 provided by Dr H. Zhang, CSHL, USA) were expressed using a procedure described by Taieb and Jessus (1996). *S. pombe* p13<sup>suc1</sup> protein was purified using a UnoQ chromatography column (Bio-Rad).

## RESULTS

### In vitro MPF amplification in a cytosolic extract

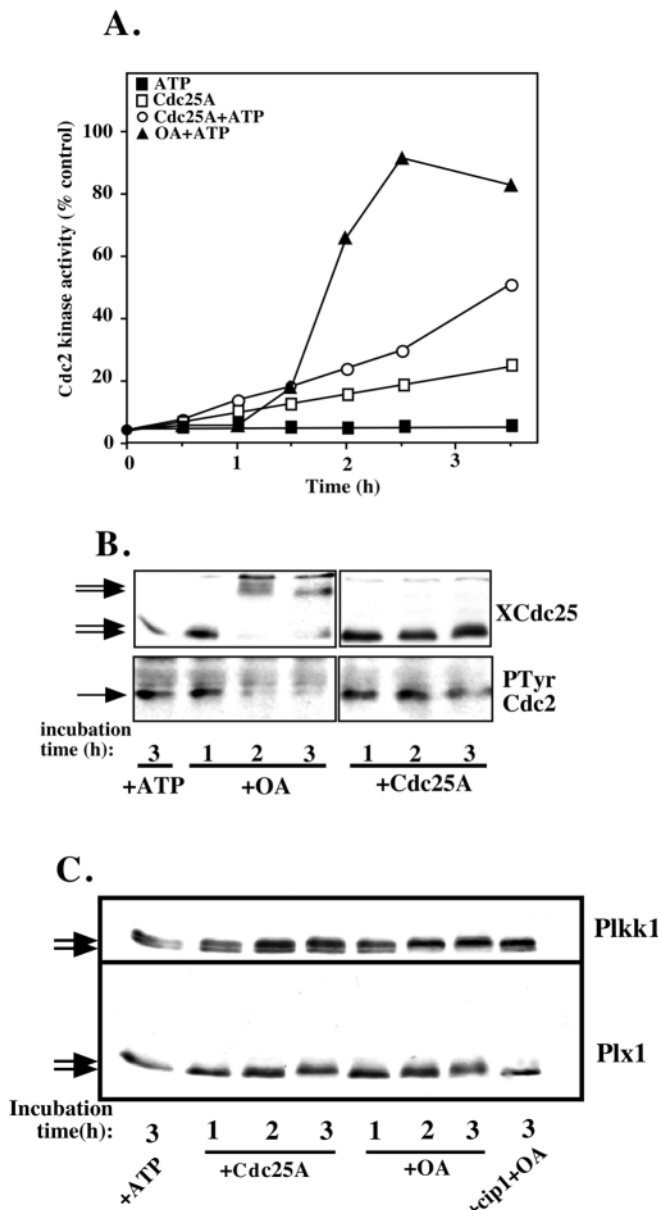
We recently described a cell-free system, derived from *Xenopus* prophase oocytes, that allows the analysis of the

molecular mechanisms involved in the Cdc2/Cdc25 amplification loop (Karaiskou et al., 1998). Briefly, *Xenopus* prophase-arrested oocytes are lysed in 4 volumes of EB buffer, and after two successive centrifugations the high-speed cytosolic fraction is obtained. This diluted 'cytosolic extract' does not contain the membrane fraction nor membrane-associated proteins and does not support protein synthesis. Incubation of this prophase extract, in either the absence or presence of ATP, does not lead to Cdc2 kinase activation.

Addition of a threshold amount of recombinant Cdc25 protein, human Cdc25A expressed in *E. coli*, leads to the direct activation of Cdc2 kinase, with a linear time-course (Fig. 1A). When ATP is added together with exogenous Cdc25, the activation of Cdc2 kinase is accelerated (Fig. 1A). The linear activation of Cdc2 kinase is correlated with a linear tyrosine dephosphorylation of Cdc2 (Fig. 1B). This direct activation of Cdc2 kinase leads to a partial phosphorylation of the endogenous Cdc25, as judged by the retardation of its electrophoretic mobility (Fig. 1B). Endogenous Cdc25 does not undergo any further mobility shift even when Cdc2 kinase reaches its maximum activation level, 5 to 6 hours after Cdc25A addition (data not shown). This process does not involve an auto-amplification loop; Cdc2 activation is linear and endogenous Cdc25 remains only partially phosphorylated.

Addition of 500 nM okadaic acid, a powerful inhibitor of type 2A phosphatase under these conditions (Karaiskou et al., 1998), induces, after a lag period (1-2 hours), an abrupt activation of Cdc2 that is ATP-dependent (Fig. 1A). This all-or-none activation of Cdc2 kinase is concomitant with the abrupt dephosphorylation of Cdc2 on tyrosine and the full phosphorylation, and therefore full activation (Izumi et al., 1992), of endogenous Cdc25 (Fig. 1B). Therefore PP2A inhibition is sufficient to initiate the Cdc2/Cdc25 positive feedback loop, occurring after a lag period.

Plx1 kinase has been shown to phosphorylate and stimulate



**Fig. 1.** Cdc2 kinase activation in a cytosolic extract. The cytosolic extract was incubated at room temperature, in the presence of various effectors. Samples were collected at indicated times and submitted to either histone H1 kinase assay (A) or western blotting (B,C). (A) The cytosolic extract was incubated in the presence of either an ATP-regenerating system (ATP), or 2  $\mu$ g/ml human Cdc25A in the absence of an ATP-regenerating system (Cdc25A), or 2  $\mu$ g/ml human Cdc25A and an ATP-regenerating system (Cdc25A+ATP), or 500 nM okadaic acid and an ATP-regenerating system (OA+ATP). The 100% histone H1 kinase activity corresponds to the activity measured after a 4 hour incubation in the presence of OA+ATP. (B) The cytosolic fraction was incubated in the presence of an ATP-regenerating system and supplemented either with buffer (+ATP), or with 500 nM okadaic acid (+OA), or with 2  $\mu$ g/ml human Cdc25A (+Cdc25A). Samples were collected at various times, as indicated, and analyzed by western blotting using an anti-*Xenopus* Cdc25 antibody (XCdc25, upper panel) or an antibody directed against the tyrosine-phosphorylated form of Cdc2 (PTyr Cdc2, lower panel). Arrows indicate the different migrating forms of each protein. (C) The cytosolic fraction was incubated in the presence of an ATP-regenerating system and supplemented with buffer (+ATP), or with 2  $\mu$ g/ml human Cdc25A (+Cdc25A), or with 500 nM okadaic acid (+OA), or with 500 nM okadaic acid and p21<sup>Cip1</sup> (+cip1+OA). Samples were collected at indicated times, and analyzed by western blotting using an anti-*Xenopus* Plk1 antibody (Plk1, upper panel) or an anti-*Xenopus* Plx1 antibody (Plx1, lower panel). Arrows on the left indicate the various migrating forms of each protein.

the activity of Cdc25 (Kumagai and Dunphy, 1996). The activation of Cdc2 kinase, in response to either exogenous Cdc25A or okadaic acid, is accompanied by the phosphorylation and activation of Plx1 (Fig. 1C). Qian et al. (1998b) have recently described the purification and cloning of a *Xenopus* Plx1 kinase kinase, termed Polo-like kinase kinase 1 or Plkk1. This Ser/Thr kinase directly phosphorylates and activates Plx1, and is itself activated by phosphorylation. Since Plx1 kinase is activated in our *in vitro* system (Fig. 1C), it was of high interest to determine whether Plkk1 is also activated under the same conditions. Plkk1 activation results from the phosphorylation of the protein, that is visualized by an electrophoretic mobility shift (Qian et al., 1998b). Therefore, Plkk1 phosphorylation state was analyzed by western blotting, with a specific anti-Plkk1 antibody. In the cytosolic extract, Plkk1 is present and migrates as a doublet (Fig. 1C). In order to induce the linear activation of Cdc2, human Cdc25A was added in the cytosolic extract. Plkk1 was phosphorylated in parallel with Plx1 phosphorylation (Fig. 1C) and activation (Karaiskou et al., 1998). This result demonstrates that Plkk1 is activated together with Plx1 and Cdc2 kinases. The addition of okadaic acid accelerates the time-course of Plkk1 and Plx1 kinases activation when compared to the addition of Cdc25A (Fig. 1C).

We have previously shown that incubation of the cytosolic extract with recombinant p21<sup>Cip1</sup>, a Cdc2 inhibitor under our conditions (Karaiskou et al., 1998; Xiong et al., 1993) prevents Cdc2 activation, phosphorylation of Cdc25 and activation of Plx1 kinase in response to okadaic acid. Similarly, Plkk1 phosphorylation is inhibited in the presence of okadaic acid and p21<sup>Cip1</sup> (Fig. 1C). This demonstrates that Plkk1 activation requires Cdc2 kinase activation and explains why Plx1 kinase is not activated in the absence of Cdc2 kinase activity under our conditions.

### Cytosolic extract fractionation removes a Cdc25 inhibitor, that does not correspond to PP2A or 14-3-3 protein

In the cytosolic extract, the linear activation of Cdc2 kinase is sufficient to activate Plx1 kinase as well as its activating kinase Plkk1 (Fig. 1C). However, the activation of both these kinases never leads to MPF auto-amplification under these *in vitro* conditions. What does prevent MPF amplification although Cdc2 and Plx1 have been turned on? And why is the inhibition of PP2A sufficient to release this block and allow the auto-amplification loop?

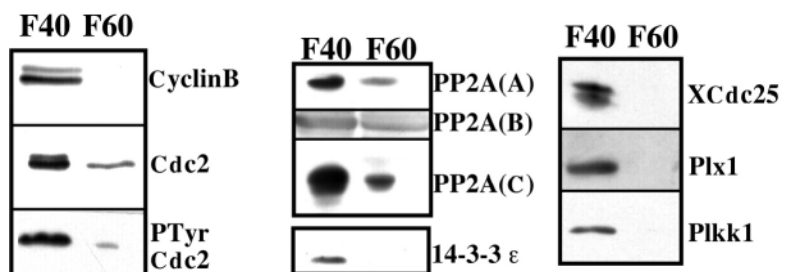
In an attempt to answer the above questions, it was important to separate the molecular partners required for the amplification loop. An ammonium sulfate fraction from a high-speed oocyte extract (0%-33%) has been reported as lacking

an inhibitor of the amplification of MPF, named INH, and identified as a trimeric form of PP2A (Cyert and Kirschner, 1988; Lee et al., 1991, 1994). Therefore, the cytosolic extract was fractionated by ammonium sulfate precipitation. The 0%-40% ammonium sulfate precipitate (or F40) and the 40%-60% ammonium sulfate precipitate (or F60) were analyzed by western blotting (Fig. 2). Cyclin B2, Plx1, Plkk1 and Cdc25 were entirely recovered in the F40, while the A (or the 65 kDa regulatory subunit), B (or the 55 kDa regulatory subunit) and C (or the catalytic subunit) subunits of PP2A were present in both fractions, F40 and F60. Cdc2 migrates as a doublet (Solomon et al., 1990). The cyclin B2-bound form of Cdc2, tyrosine phosphorylated and corresponding to pre-MPF, is represented by the upper band of the doublet and was present only in the F40. The F60 does not contain pre-MPF but only free form of Cdc2, corresponding to the lower band of the doublet (Fig. 2). Therefore, the main components involved in MPF activation, cyclin B2, Cdc2 itself, Cdc25, Plx1, Plkk1 and PP2A coprecipitate in the F40. Surprisingly, the F40 derived from the cytosolic extract, reported to be devoid of the inhibitor INH, corresponding to PP2A (Lee et al., 1994), contains the majority of the trimeric form of PP2A (Fig. 2).

Fig. 3A shows that ATP and okadaic acid addition in the F40 results in a nearly instantaneous Cdc2 kinase activation. The lag period, that precedes the okadaic acid-dependent Cdc2 activation in a cytosolic extract, is strongly shortened (compare Fig. 1A with Fig. 3A). This rapid Cdc2 kinase activation is correlated with the tyrosine dephosphorylation of Cdc2 and is accompanied by the hyperphosphorylation of Cdc25 (Fig. 3B). These results show that okadaic acid can induce the Cdc2/Cdc25 feed-back loop in the F40, with a diminished lag period; therefore, although an inhibitory mechanism responsible for the lag period has been removed from the F40, the inhibitory effect exerted by PP2A on Cdc2/Cdc25 feed-back loop is functional in this fraction.

Interestingly, incubation of the F40, without any further addition, results in a slow, linear activation of Cdc2 kinase (Fig. 3A). In the presence of a ATP-regenerating system, the rate of Cdc2 kinase activation is increased (Fig. 3A). This linear activation of Cdc2 kinase is correlated with a slight and progressive tyrosine dephosphorylation (Fig. 3B). It is also accompanied by a partial phosphorylation of endogenous Cdc25, that is ATP-dependent (Fig. 3B). These results indicate that, after ammonium sulfate fractionation, endogenous Cdc25 has acquired a basal catalytic activity. This situation is in strong contrast with the cytosolic extract where Cdc25 remains locked in an inactive state, whether ATP is present or not.

After addition of ATP or of ATP together with okadaic acid in the F40, Plkk1 and Plx1 kinases are activated in parallel with

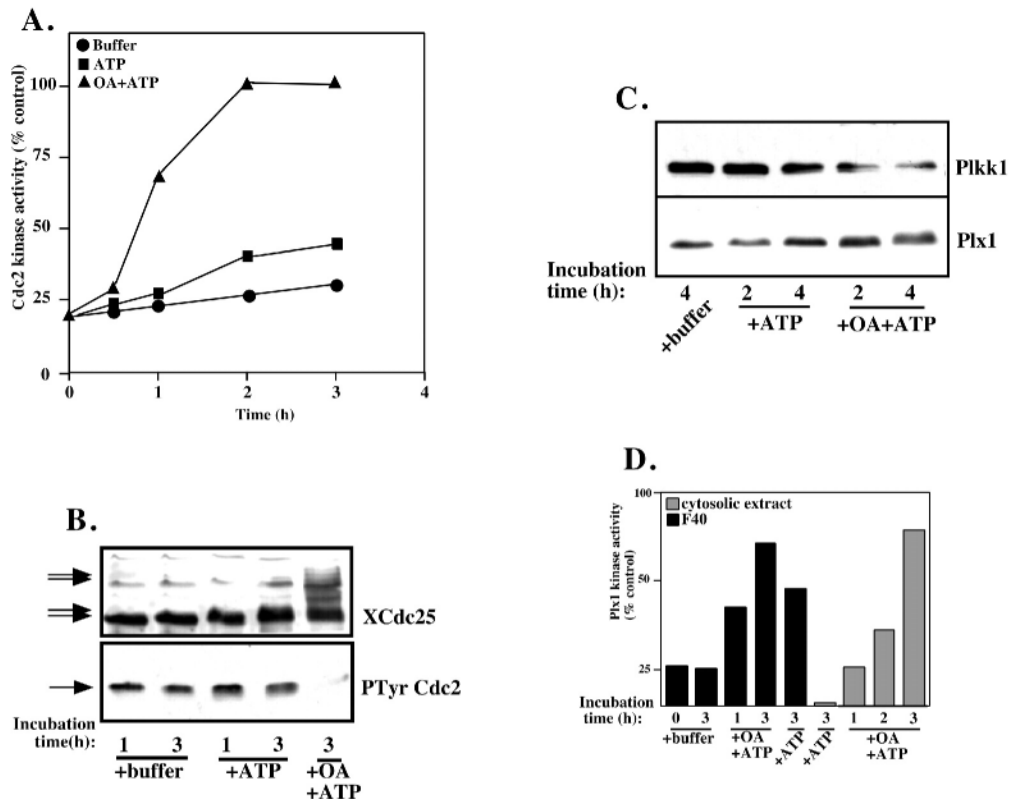


**Fig. 2.** Distribution of several proteins, potentially involved in the MPF amplification loop, after ammonium sulfate fractionation. The F40 and F60 ammonium sulfate precipitates were analyzed by western blotting. The following antibodies were used (from top to bottom): anti-cyclin B2, anti-Cdc2, anti-phosphotyrosine Cdc2, anti-A, C or B subunit of PP2A, anti-14-3-3  $\epsilon$ , anti-Cdc25, anti-Plx1 and anti-Plkk1.

**Fig. 3.** Cdc2 kinase activation in the F40. The F40 was incubated at room temperature in the presence of various effectors. Samples were collected at indicated times and submitted to histone H1 kinase activity (A), western blotting (B, C), or Plx1 kinase activity (D). (A) The F40 was incubated in the absence of effectors (Buffer), or in the presence of an ATP-regenerating system (ATP), or 500 nM okadaic acid and an ATP-regenerating system (OA+ATP). The 100% histone H1 kinase activity corresponds to the activity measured after a 4 hour incubation in the presence of OA+ATP.

(B) The F40 was incubated in the absence of effectors (+buffer), or in the presence of an ATP-regenerating system (+ATP), or 500 nM okadaic acid and an ATP-regenerating system (+OA+ATP). After 1 or 3 hours of incubation, as indicated, it was analyzed by western blotting using anti-*Xenopus* Cdc25 antibody (XCdc25, upper panel) and the anti-phosphotyrosine Cdc2 (PTyr Cdc2, lower panel).

(C) The F40 was incubated in the absence of effectors (+buffer), or in the presence of an ATP-regenerating system (+ATP), or 500 nM okadaic acid and an ATP-regenerating system (+OA+ATP). Samples were collected at various times, as indicated, and analyzed by western blotting using an anti-*Xenopus* Plk1 antibody (Plk1, upper panel) or an anti-*Xenopus* Plx1 antibody (Plx1, lower panel). (D) The cytosolic extract or the F40 were incubated in the absence of effectors (+buffer), or in the presence of an ATP-regenerating system (+ATP), or 500 nM okadaic acid and an ATP-regenerating system (+OA+ATP). Samples were collected at various times, as indicated, and Plx1 kinase activity was assayed in anti-Plx1 immunoprecipitates. The 100% Plx1 kinase activity corresponds to the activity measured after a 4 hour incubation in the presence of OA+ATP.



Cdc2 kinase, as judged by their electrophoretic mobility and Plx1 kinase assay (Fig. 3C,D) (Karaiskou et al., 1998).

In conclusion, the F40 is characterized by the possibility to support a linear activation of Cdc2 kinase without exogenous Cdc25A addition, indicating that endogenous Cdc25 has acquired a basal level of activity during the fractionation of the cytosolic extract. This basal level of Cdc25 activity accounts for the disappearance of the lag period preceding the okadaic acid-induced Cdc2 kinase amplification. However, Cdc2 amplification loop remains negatively regulated by a type 2A phosphatase, indicating that the inhibitory mechanism that has been removed by ammonium sulfate fractionation does not correspond to PP2A.

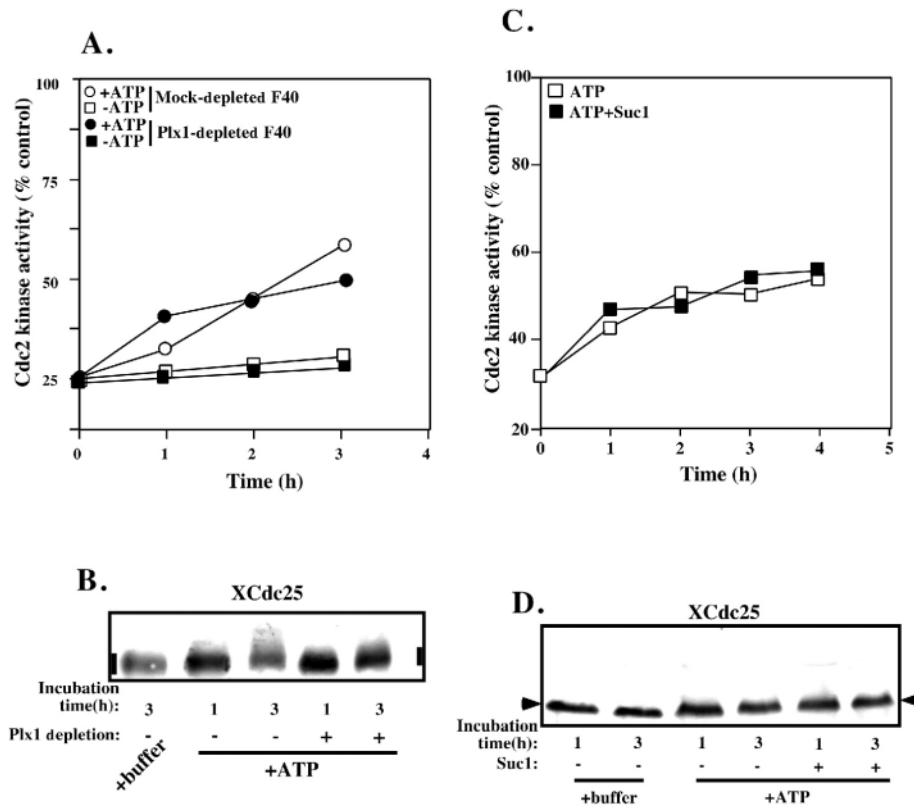
14-3-3 $\epsilon$  is present in *Xenopus* oocyte and has been shown to act as a negative regulator of mitotic Cdc25 in *Xenopus* egg extracts (Kumagai et al., 1998b). We have examined the possibility that 14-3-3 could play an inhibitory role for Cdc25, in our in vitro system. We used an antibody directed against the  $\epsilon$  isoform of the 14-3-3 family. 14-3-3 $\epsilon$  protein is present in the cytosolic extract (data not shown). It is totally recovered in the F40 and is not detectable by western blotting in the F60 fraction (Fig. 2). Therefore the inhibitory step, removed by ammonium sulfate fractionation, cannot be attributed to the removal of 14-3-3 $\epsilon$  protein, since this protein remains present in the F40.

### The linear activation of Cdc2 kinase and the partial phosphorylation of Cdc25 are independent of Plx1 kinase and Suc1/Cks

In response to ATP addition in the F40, Cdc2 and Plx1 kinases are activated and Cdc25 becomes partially phosphorylated. In order to clarify whether Plx1 kinase is required for the linear activation of Cdc2 kinase and the partial phosphorylation of Cdc25, Plx1 immunodepletion experiments were performed. The F40 was incubated either with a specific affinity-purified anti-Plx1 antibody or with a preimmune serum as control, and then treated with Protein A-Sepharose beads. Plx1 immunodepletion is complete under these conditions (see Fig. 5B). Plx1- or mock-depleted F40 was further incubated in the presence of ATP; as shown in Fig. 4A, the absence of Plx1 kinase does not modify the linear activation of Cdc2 kinase resulting from ATP addition. This demonstrates that endogenous Cdc25 does not require Plx1 kinase to linearly activate Cdc2 kinase. Interestingly, the partial phosphorylation of Cdc25, normally observed after ATP addition in the F40, does occur, even in the absence of Plx1 (Fig. 4B).

In agreement with the above results, in the Plx1-depleted cytosolic extract, addition of exogenous Cdc25A is still able to lead to the activation of Cdc2 kinase with linear kinetics and to the resulting partial phosphorylation of endogenous Cdc25, as in the control extract (data not shown), confirming that Plx1

**Fig. 4.** The linear activation of Cdc2 and the partial phosphorylation of Cdc25 occur independently of Plx1 kinase and of Suc1/ Cks protein. (A,B) The Plx1-depleted or mock-depleted F40 were incubated in the presence of various effectors. Samples were collected at indicated times and submitted to either histone H1 kinase assay (A) or western blotting with the anti-*Xenopus* Cdc25 antibody (B). (A) The mock-depleted (open symbols) or Plx1-depleted (closed symbols) F40 was incubated in the absence (–ATP) or in the presence (+ATP) of an ATP-regenerating system. (B) The mock-depleted or Plx1-depleted F40 was incubated in the absence (+buffer) or in the presence (+ATP) of an ATP-regenerating system and samples were collected at various incubation times, as indicated. (C,D) The F40 was incubated in the presence of buffer or an ATP-regenerating system, supplemented with buffer (ATP) or with 40 µg/ml p13<sup>Suc1</sup> protein (ATP+Suc1). Samples were collected at various incubation times and submitted to H1 kinase assay (C) and western blotting with the anti-XCdc25 antibody (D). Bars indicate the various migration positions of XCdc25. The 100% kinase activity corresponds to the activity measured after a 4 h incubation of the F40 in the presence of 500 nM okadaic acid and an ATP-regenerating system



kinase is not necessary for the linear activation of Cdc2 kinase, nor for the partial phosphorylation of endogenous Cdc25.

Altogether these results indicate that the partial phosphorylation of Cdc25 does not depend on Plx1 kinase activation, but is rather a direct consequence of the linear Cdc2 activation, even when Cdc2 kinase is only partially activated.

Patra and Dunphy (1996) have shown that overexpression of the *Xenopus* homologue of Suc1/Cks, Xe-p9 in *Xenopus* egg extracts delays mitosis by inhibiting the Cdc25-dependent tyrosine dephosphorylation of Cdc2, suggesting that Xe-p9 regulates the interaction between Cdc2 and Cdc25. We have studied the effect of an excess of Suc1/Cks (40 µg of Suc1/Cks per ml added towards 5 µg of estimated endogenous Xe-p9 per ml) on the ATP-dependent activation of Cdc2 in the F40. Suc1/Cks does not interfere with the linear activation of Cdc2 and does not modify the partial phosphorylation of Cdc25, that occurs after ATP addition in the F40 (Fig. 4C,D). Therefore, the ability of Cdc2 to partially phosphorylate Cdc25 is not affected by an overexpression of Suc1/Cks under our conditions.

#### The Cdc2/Cdc25 amplification loop requires Plx1 kinase and can be inhibited by an excess of Suc1/Cks

The linear activation of Cdc2 kinase and the parallel activation of Plx1 kinase are not sufficient to initiate the Cdc2/Cdc25 amplification loop. In order to determine whether Plx1 kinase is necessary for this process, Plx1-depleted F40 was tested for its ability to support the MPF amplification loop, in response to ATP and okadaic acid. Mock-depleted F40 undergoes, as expected, MPF amplification: Cdc2 kinase is abruptly activated (Fig. 5A) and Cdc25 hyperphosphorylated (Fig. 5B). In contrast, when the Plx1-depleted F40 was incubated in the presence of ATP and okadaic acid, the Cdc2/Cdc25

amplification process does not take place (Fig. 5A). Indeed, Cdc2 kinase activation occurs linearly (Fig. 5A) and Cdc25 is only partially phosphorylated (Fig. 5B). We verified that the inability of the Plx1-depleted extract to undergo MPF amplification did not result from a partial depletion in Cdc2 or Cdc25, due to an association with Plx1 protein (for Plx1 and Cdc25, see Fig. 5B, for Cdc2, data not shown). Altogether these results demonstrate that the *in vitro* okadaic acid-dependent initiation of the Cdc2/Cdc25 feed-back loop requires Plx1 kinase, even though Plx1 kinase activity is not sufficient to generate this process.

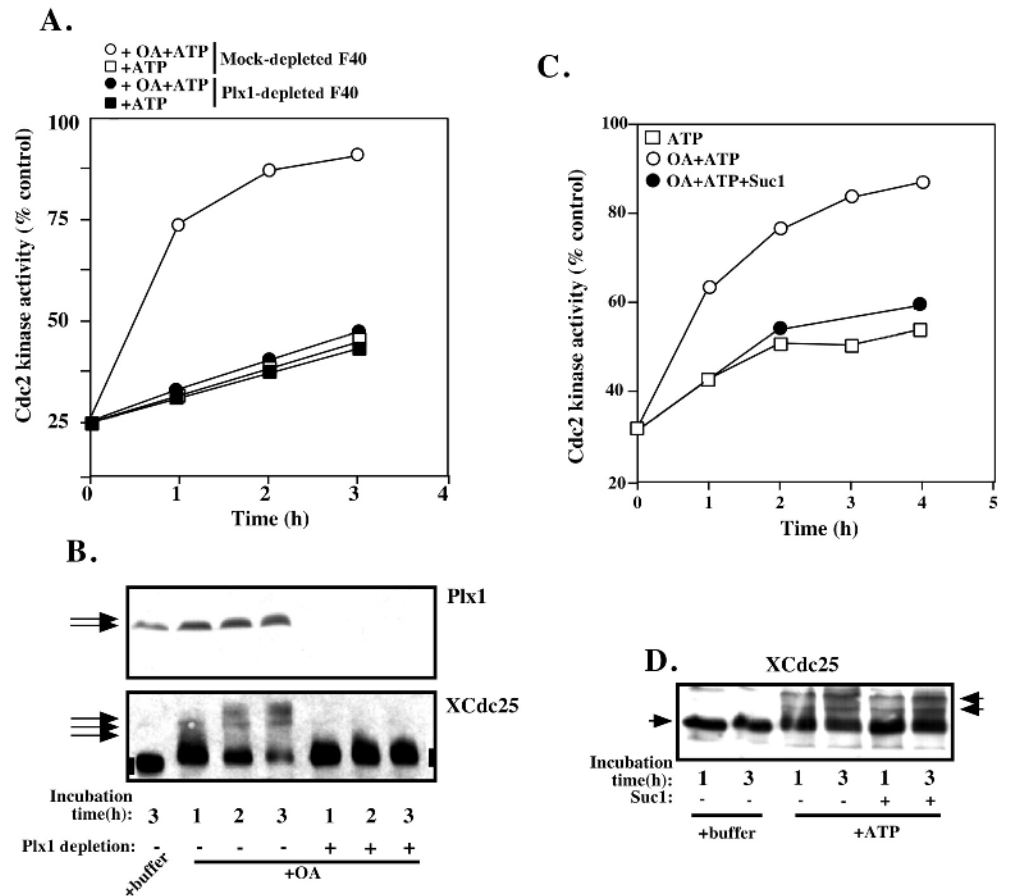
It is noteworthy that after Plx1 depletion of the F40, the ATP-dependent, linear activation of Cdc2 kinase remains functional and independent of PP2A inhibition (Fig. 5A). Furthermore, Cdc25 never undergoes its hyperphosphorylation shift. This suggests that inhibition of PP2A does not significantly affect the linear Cdc2 activation and the resulting partial phosphorylation of Cdc25, suggesting that when Cdc25 is phosphorylated by Cdc2, it represents a poor PP2A substrate.

Plx1 depletion was also performed in the cytosolic extract, in order to determine if the initiation of the Cdc2/Cdc25 feed-back loop was also dependent on Plx1 kinase activation. When okadaic acid and ATP are added in the cytosolic extract depleted in Plx1 kinase, the Cdc2/Cdc25 amplification process is prevented; Cdc2 kinase activation is inhibited and Cdc25 does not undergo any significant mobility shift (data not shown). In agreement with the results obtained in the F40, this observation confirms that Plx1 kinase is necessary for the initiation of the MPF auto-amplification.

We investigated whether Suc1/Cks could interfere with the initiation of the Cdc2 amplification loop under these conditions. When an excess of Suc1/Cks was added in the F40



**Fig. 5.** Plx1 kinase and Suc1/Cks-mediated Cdc2/Cdc25 association are necessary for the Cdc2/Cdc25 positive feed-back loop. (A,B) The Plx1-depleted or mock-depleted F40 were incubated in the presence of various effectors. Samples were collected at various times and submitted to either histone H1 kinase assay (A) or western blotting (B). The antibodies used were directed against *Xenopus* Plx1 and *Xenopus* Cdc25. (A) The mock-depleted (open symbols) or Plx1-depleted (closed symbols) F40 was incubated in the presence of an ATP-regenerating system and supplemented either with buffer (+ATP) or with 500 nM okadaic acid (+OA+ATP). (B) The mock-depleted or Plx1-depleted F40 was incubated in the absence of effectors (+buffer) or in the presence of 500 nM okadaic acid and an ATP-regenerating system (+OA). Samples were collected at various incubation times, as indicated, and immunoblotted with the anti-Plx1 antibody (upper panel) and the anti-Cdc25 antibody (lower panel). (C,D) The F40 was incubated in the presence of an ATP-regenerating system, supplemented with buffer (ATP), with 500nM okadaic acid (OA+ATP) or with okadaic acid and 40  $\mu\text{g/ml}$  p13<sup>Suc1</sup> protein (OA+ATP+Suc1). Samples were collected at various incubation times and submitted to H1 kinase assay (C) and western blotting with the anti-XCdc25 antibody (D). Bars indicate the various migrating forms of XCdc25. The 100% kinase activity corresponds to the activity measured after a 4 hour incubation of the F40 with OA+ATP.



in the presence of ATP and okadaic acid, the abrupt activation of Cdc2, that normally takes place, was inhibited (Fig. 5C). Under this condition, Cdc25 becomes hyper-phosphorylated (Fig. 5D). Therefore, although fully phosphorylated, Cdc25 is prevented to initiate the Cdc2/Cdc25 amplification loop. As expected, the linear activation of Cdc2 kinase was not prevented (Fig. 5C).

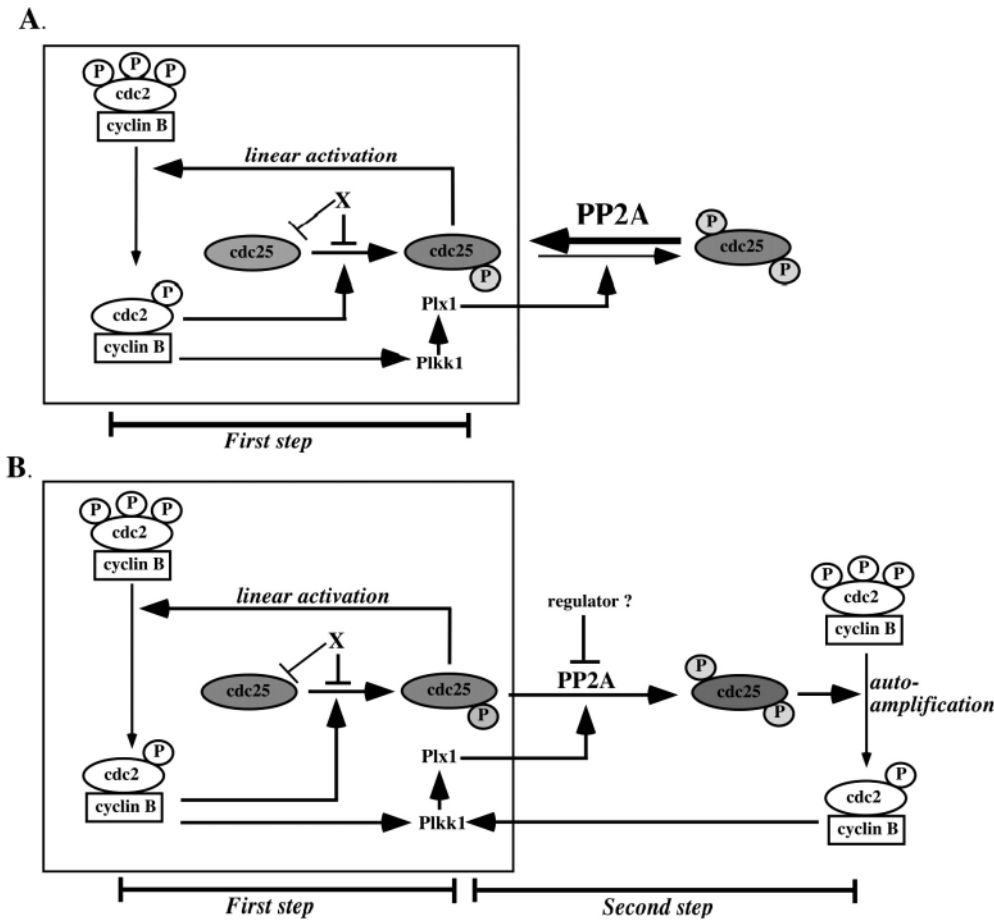
## DISCUSSION

MPF auto-amplification is an all-or-none biochemical process that triggers the conversion of a prophase I-arrested oocyte into a metaphase II-arrested oocyte. We developed *Xenopus* oocyte cell-free extracts that allow the investigation of the post-translational modifications controlling this irreversible transition. We report in this study new observations supporting the hypothesis that MPF auto-amplification depends upon a two-step mechanism (Fig. 6). In a first step, Cdc25 phosphatase activates Cdc2 kinase with linear kinetics, and active Cdc2 in turn partially phosphorylates Cdc25. This results in a slight retardation of the electrophoretic mobility of Cdc25, that is blocked by the Cdc2-inhibitor p21<sup>Cip1</sup> (Karaiskou et al., 1998), indicating that Cdc2 is responsible for this phosphorylation.

Under these basal conditions, Cdc2 kinase activation follows linear kinetics and no auto-amplification takes place. In a second and subsequent step, Plx1 kinase catalyzes the hyperphosphorylation of Cdc25, that is reflected by the high mobility shift of Cdc25. This second step occurs *in vitro* only when PP2A phosphatase is inhibited by okadaic acid. This shows that PP2A antagonizes the action of Plx1 and strongly suggests that PP2A is the physiological phosphatase that catalyzes the dephosphorylation of the Plx1-phosphorylated residues of Cdc25.

### The first step of the auto-amplification process is locked by an inhibitor in oocyte extracts

Oocyte cytosolic extracts never spontaneously generate Cdc2 kinase activity; in contrast, the 0%-40% ammonium sulfate fraction, derived from the cytosolic extract and termed F40, generates Cdc2 kinase activity with a slow linear rate, after incubation at room temperature. ATP addition in the F40 accelerates Cdc2 kinase activation and allows a partial phosphorylation of Cdc25. Therefore, ammonium sulfate precipitation removes an inhibitory activity that prevents Cdc25 from linearly activating Cdc2 kinase (Fig. 6). It was reported a decade ago that similar fractionation of cytosolic extracts removes an inhibitor of MPF amplification, INH,



**Fig. 6.** Model for the two-step mechanism of MPF auto-amplification. See text for discussion. The key features are as follows: (A) The first step of MPF auto-amplification can be induced either by a threshold level of Cdc2 kinase activity or by downregulation of the inhibitory activity X of Cdc25. The completion of the first step is independent of PP2A activity and Suc1/Cks-mediated Cdc2/Cdc25 interaction. In contrast, the second step does not occur in the absence of PP2A inhibition, although Plx1 is activated. (B) The downregulation of PP2A allows the completion of the second step and leads to the full phosphorylation and activation of Cdc25 and the auto-amplification of MPF. This step depends on Suc1/Cks-mediated Cdc2/Cdc25 association. In vivo, progesterone can lead to the activation of a threshold level of Cdc2 kinase activity by inhibiting Myt1 and/or by stimulating cyclin B synthesis.

identified as a trimeric form of PP2A (Cyert and Kirschner, 1988; Lee et al., 1991, 1994). It was therefore important to determine whether this enzyme could be the inhibitory factor preventing endogenous Cdc25 from linearly activating Cdc2 kinase. Two experimental observations argue against this possibility. First, the F40 contains the majority of the PP2A trimeric form, and second, this form remains fully active, since its inhibition by okadaic acid allows the immediate auto-amplification of Cdc2 kinase.

What is the nature of the inhibitor removed by ammonium sulfate? Previous reports have indicated that at least three candidate proteins could play this role: 14-3-3 and Pin1 proteins, known to associate with Cdc25, and Suc1/Cks that binds Cdc2 kinase. First, 14-3-3 protein was shown to segregate in the F40, arguing against a possible role of this protein as the inhibitor of the basal catalytic activity of Cdc25. Second, we have tested Suc1/Cks recombinant protein; when added to the F40, Suc1/Cks did not affect the linear Cdc2 activation. These negative results do not favor the view that the inhibitory activity results from the removal of a docking protein, which prevents the physical interaction between Cdc2 and Cdc25. An attractive candidate, that could play an inhibitory role in Cdc25 activation is the peptidyl-prolyl isomerase Pin1, a negative regulator of the G<sub>2</sub>-M transition, that has been reported to strongly interact with phosphorylated Cdc25 (Crenshaw et al., 1998; Shen et al., 1998; Yaffe et al., 1997). Indeed, *Xenopus* Pin1, that is present in the oocyte, is removed by ammonium sulfate

fractionation from the F40 and segregates in the F60 (data not shown), leaving open the possibility for an inhibitory role of Pin1. Another interesting possibility is that a phosphatase specifically dephosphorylates the Cdc2-phosphorylated residues of Cdc25 and plays the role of the inhibitor. This phosphatase must be absent from the F40, allowing Cdc2 to partially phosphorylate Cdc25, which in turn linearly activates Cdc2 kinase. It can not be a type 2A phosphatase since inhibition of PP2A in the F40, after Plx1 kinase depletion, does not facilitate Cdc25 partial phosphorylation nor modify Cdc2 kinase linear activation. These results suggest that the Cdc2-phosphorylated form of Cdc25 is a poor substrate of trimeric PP2A. This is in contrast with the generally accepted view that PP2A regulates negatively Cdc2-induced Cdc25 phosphorylation (Clarke et al., 1993; Izumi et al., 1992).

#### Plkk1 and Plx1 kinases activation depends on Cdc2 kinase and is necessary but not sufficient for the MPF auto-amplification

A surprising finding reported here indicates that Plx1 kinase and its activating kinase Plkk1 are activated in the F40, provided ATP is added. Under these conditions, only a partial Cdc2 kinase activation occurs, in the absence of auto-amplification and Cdc25 hyperphosphorylation. Furthermore, addition of a threshold level of recombinant Cdc25A into the cytosolic extract is sufficient to activate both Plx1 and Plkk1 kinases, again in the absence of auto-amplification and Cdc25 hyperphosphorylation (Karaiskou et al., 1998). In the presence of p21<sup>Cip1</sup>, Plkk1 and Plx1 kinases activation is inhibited. Therefore, the activation of these kinases appears to be Cdc2

kinase-dependent but is not sufficient itself to trigger the auto-amplification loop (Fig. 6).

### Plx1 and PP2A antagonistically regulate both Cdc25 hyperphosphorylation and MPF auto-amplification

Under our *in vitro* conditions, PP2A inhibition is absolutely required for the abrupt dephosphorylation of Cdc2 and the hyperphosphorylation of Cdc25; in the absence of Plx1, this event is totally prevented. Conversely, even fully activated, Plx1 is not able to lead to Cdc25 hyperphosphorylation in the absence of okadaic acid. The most simple interpretation is that PP2A dephosphorylates the Plx1-phosphorylated residues of Cdc25. Altogether these results indicate that the main regulators of the Cdc2/Cdc25 auto-amplification are Plx1 kinase and its opposed phosphatase, PP2A (Fig. 6).

### During the MPF auto-amplification, Cdc25 dephosphorylates Cdc2 in a Suc1/Cks-dependent manner

Suc1/Cks proteins have been proposed to act as docking molecules for Cdc2 regulators and substrates, as Cdc25 and components of the mitotic ubiquitin ligase APC (anaphase promoting complex) (Bourne et al., 1996; Patra and Dunphy, 1998). Xe-p9, the *Xenopus* homologue of Suc1/Cks proteins is required for the dephosphorylation of Cdc2 by Cdc25 in *Xenopus* egg extracts (Patra and Dunphy, 1996). Its overexpression, probably leading to the disruption of this tight interaction, results in inhibition of Cdc2 dephosphorylation even in the presence of fully phosphorylated and active Cdc25 (Patra and Dunphy, 1996). We show here that overexpression of Suc1/Cks in the F40 does not interfere with the ATP-dependent linear Cdc2 activation nor with the resulting Cdc25 partial phosphorylation. In contrast, overexpression of Suc1/Cks inhibits the okadaic acid-induced Cdc2 kinase all-or-none activation, whereas the linear Cdc2 activation and Plx1 activation take place and Cdc25 is hyper-phosphorylated. In conclusion, high concentrations of Suc1/Cks protein inhibit the initiation of the Cdc2/Cdc25 amplification loop, although Cdc25 has been fully phosphorylated and activated. We suggest that partially phosphorylated Cdc25 acts independently of Suc1 for Cdc2 dephosphorylation and activation whereas the interaction between hyper-phosphorylated Cdc25 and Cdc2 is Suc1/Cks-dependent.

### How is the MPF auto-amplification process initiated?

A major question is to know whether a threshold level of Cdc2 kinase, corresponding to the basal activation observed in the F40, is required for the subsequent establishment of the positive feed-back loop. In other words, is the first partial phosphorylation of Cdc25 by Cdc2 kinase a necessary step that allows Plx1 kinase to further phosphorylate Cdc25 on new residues, leading to a full activation? When oocyte extracts are incubated in the absence of Cdc2 kinase activity, with an excess of p21<sup>Cip1</sup>, inhibition of PP2A by okadaic acid does not activate neither Plk1 nor Plx1, clearly arguing that activation of both kinases lays downstream Cdc2 kinase. On the other hand, when Plx1 kinase is removed by immunodepletion, Cdc2 kinase can be activated linearly although the feed-back loop is blocked. This indicates that an active Plx1 is not necessary for the generation of basal Cdc2 kinase activity. It may then be concluded that both Cdc2 and Plx1 kinases are necessary for

MPF auto-amplification and strongly suggest that Cdc2-induced phosphorylation of Cdc25 is a prerequisite for Cdc25 full activation and phosphorylation by Plx1 kinase.

### The *in vivo* relevance of the two-step mechanism of MPF amplification

Our model proposes that the first step of the MPF auto-amplification mechanism is triggered by a threshold level of Cdc2 kinase activity. In the oocyte, such a threshold could be provided by the downregulation of the Myt1 kinase and an increased level of cyclins, two events possibly induced by progesterone, as it has been already proposed (Kobayashi et al., 1991; Nebreda et al., 1995; Palmer et al., 1998). According to this model, progesterone could also negatively regulate the inhibition that locks Cdc25 under an inactive form in prophase. Concerning the second step, a frustrating question is to understand how PP2A activity could be regulated. It is clear from our experiments that neither Cdc2 nor Plx1 and MAP kinases (Karaiskou et al., 1998) are sufficient to down regulate PP2A in the oocyte cytosolic extract. An interesting possibility could be that a PP2A regulator is associated with oocyte organelles or membranes and has been removed during the preparation of the oocyte cytosolic extract. Further investigation will be necessary to understand the physiological down-regulation of PP2A.

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