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Multiple Roles for Protein Phosphatase 1 in Regulating the *Xenopus* Early Embryonic Cell Cycle

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Using cytostatic factor metaphase II-arrested extracts as a model system, we show that protein phosphatase 1 is regulated during early embryonic cell cycles in *Xenopus*. Phosphatase 1 activity peaks during interphase and decreases shortly before the onset of mitosis. A second peak of activity appears in mitosis at about the same time that cdc2 becomes active. If extracts are inhibited in S-phase with aphidicolin, then phosphatase 1 activity remains high. The activity of phosphatase 1 appears to determine the timing of exit from S-phase and entry into M-phase; inhibition of phosphatase 1 by the specific inhibitor, inhibitor 2 (Inh-2), causes premature entry into mitosis, whereas exogenously added phosphatase 1 lengthens the interphase period. Analysis of DNA synthesis in extracts treated with Inh-2, but lacking the A- and B-type cyclins, shows that phosphatase 1 is also required for the process of DNA replication. These data indicate that phosphatase 1 is a component of the signaling pathway that ensures that M-phase is not initiated until DNA synthesis is complete.

INTRODUCTION

The onset of mitosis in eukaryotic cells is determined by the activity of the cdc2 protein kinase (Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1989; Lee and Nurse, 1988). Although the level of cdc2 polypeptide is constant throughout the cell cycle (Simanis and Nurse, 1986; Draetta and Beach, 1988; Draetta *et al.*, 1989), cdc2 is active only after forming a complex with a cyclin partner (Booher *et al.*, 1989; Moreno *et al.*, 1989; Gautier *et al.*, 1990; Solomon *et al.*, 1990). The cyclins are a class of proteins first identified in marine invertebrates by their appearance before meiosis, followed by their rapid destruction at the metaphase/anaphase transition (Evans *et al.*, 1983; Swenson *et al.*, 1986; Standardt *et al.*, 1987). Recently, a great deal has been learned about the regulation of cdc2/cyclin complexes in the control of mitosis. The levels of two cyclins, the A- and B-type, accumulate during late S-phase and G_2 (Minshull *et al.*, 1990; Walker and Maller, 1991), although A-type cyclins form active complexes earlier than B-type cyclins. B-type cyclins complex with cdc2 shortly after synthesis, but the complex remains inactive due to phosphorylation of the cdc2 moiety on threonine 14 and tyrosine 15 (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989). The wee1/mik1 protein kinase is thought to be responsible for this phosphorylation (Russell and Nurse, 1987; Lundgren *et al.*, 1991; Parker *et al.*, 1991). At the end of G_2, cdc2/cyclin B becomes rapidly activated by dephosphorylation of tyrosine 15 by the cdc25 protein-tyrosine phosphatase (Russell and Nurse, 1986; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Lee *et al.*, 1992), and the activated kinase is sufficient for the initiation of mitosis. At the metaphase/anaphase boundary, the cyclin B moiety is rapidly degraded by the ubiquitin pathway, resulting in the inactivation of cdc2 protein kinase (Glotzer *et al.*, 1991).

Despite the well-documented role of protein kinases in mitosis, little is known regarding the role serine/threonine protein phosphatases play in this process. In eukaryotic cells, protein phosphorylation is catalyzed by a large number of specific kinases, yet only four general classes of protein serine/threonine phosphatase appear to be largely responsible for dephosphorylation of serine/threonine residues (for reviews, see Cohen, 1989; Shenolikar and Nairn, 1990). These phosphatases may be identified in a number of ways (Ingebritsen and Cohen, 1983). Protein phosphatase 1 prefers the β-subunit of phosphorylase kinase and is specifically inhibited

by the protein inhibitors, inhibitor 1 (Inh-1) and inhibitor 2 (Inh-2). Phosphatase 2A, on the other hand, prefers the α-subunit of phosphorylase kinase and is inhibited by nanomolar concentrations of okadaic acid (OA) (Cohen, 1989). PrP-1 is also inhibited by OA but at micromolar concentrations (Cohen, 1989). PrP-2B, also called calcineurin, is a Ca^{++}/calmodulin dependent enzyme, whereas PrP-2C is dependent on Mg for activity. Although these four classes of protein phosphatases act on a wide range of substrates and have important roles in many cellular processes, some specialized phosphatases do not fit in these categories (Andres and Maller, 1989).

Protein serine/threonine phosphatases have been identified as having a number of roles in the cell cycle. Felix *et al.* (1990) demonstrated that inhibition of PrP-2A by OA in high-speed supernatants of activated *Xenopus* eggs resulted in the activation of cdc2 H1 kinase activity, suggesting a role for this phosphatase before mitosis. OA also has been shown to activate maturation promoting factor activity when injected into starfish and *Xenopus* oocytes (Goris *et al.*, 1989; Picard *et al.*, 1989, 1991). This effect appears to be due to specific inhibition of phosphatase 2A. INH, an activity which is capable of preventing spontaneous activation of inactive cdc2/cyclin complexes in oocyte extracts (Cyert and Kirschner, 1988), has been purified partially and found to contain a phosphatase 2A catalytic subunit (Lee *et al.*, 1991). Finally, a role for PrP-2A upstream of mitosis has been proposed in fission yeast (Kinoshita *et al.*, 1990). In addition, a mutation in the gene coding for one of the regulatory subunits of PrP-2A leads to defective cytokinesis in budding yeast (Healy *et al.*, 1991).

A number of groups have shown that phosphatase 1 (PrP-1) is required for the completion of mitosis, as judged by the arrest at midmitosis of PrP-1 mutants (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990). Booher and Beach (1989) also have suggested a role for phosphatase 1 before mitosis; in a wee1$^-$/cdc25ts double mutant at the restrictive temperature, the overexpression of PrP-1 caused a G_2 arrest and prevented the onset of mitosis. In addition, the levels of Inh-2 have been shown to oscillate in a cell cycle-dependent manner (Brautigan *et al.*, 1990). This implies that the activity of PrP-1 is regulated in the cell cycle. Despite these implications of roles for phosphatases in the regulation of the cell cycle, little cell cycle-dependent change in activity has been shown when these phosphatases have been directly assayed (Kinoshita *et al.*, 1990; Ruediger *et al.*, 1991), limiting our understanding of the roles these important regulatory molecules play during the cell cycle.

The nature of the signals upstream of wee1 and cdc25 that regulate the activity of cdc2 at the G_2/M boundary remain largely unknown. To identify other potential components of the feedback pathway, we and others have used *Xenopus* egg extracts as a model system. These extracts can carry out complete cycles of mitosis and DNA synthesis in vitro. Dasso and Newport (1990) demonstrated that in extracts prepared from activated *Xenopus* eggs, the presence of unreplicated DNA prevented mitosis, presumably due to the activation of feedback controls that delay the cell cycle in interphase until the completion of DNA synthesis. On activation of feedback controls, extracts continue to accumulate cdc2/cyclin B in a tyrosine phosphorylated state, and cdc25 remains inactive (Kumagai and Dunphy, 1991; Izumi, Walker, and Maller, unpublished data). Agents able to bypass feedback controls will ultimately activate the cyclin/cdc2 complexes by tyrosine dephosphorylation, and the extracts will enter mitosis prematurely. The cdc25 protein already has been shown to bypass such a block (Kumagai and Dunphy, 1991). We recently have demonstrated that cyclin A/cdc2 is required for these feedback controls in *Xenopus* egg extracts, because in the absence of cyclin A, extracts arrested with high concentrations of DNA or aphidicolin enter mitosis prematurely without completion of DNA synthesis (Walker and Maller, 1991). Here we show that OA also is able to overcome a DNA/aphidicolin block to initiation of mitosis. We have identified PrP-1 as being active both before mitosis as well as at mitosis. The timing of mitosis in egg extracts can be controlled by altering the levels of PrP-1 in the extract. These data demonstrate a role for protein PrP-1 in the feedback control mechanism regulating the dependence of mitosis on completion of DNA synthesis.

MATERIALS AND METHODS

Materials

The catalytic subunit of protein phosphatase 1 was prepared from rabbit skeletal muscle as described by Tung *et al.* (1984). Recombinant inhibitor 2 was produced by a procedure similar to that described by Pickering *et al.* (1991). OA was purchased from Upstate Biotechnology, Lake Placid, NY. Sperm chromatin was isolated by the method described by Lohka and Maller (1985). All other reagents were of the highest quality available.

Preparation of Extracts

Metaphase II-arrested (cytostatic factor [CSF]) extracts were prepared from freshly laid eggs as described previously, omitting leupeptin from all buffers (Murray and Kirschner, 1989a). These extracts are normally arrested at meiosis II with high levels of cyclin B/*cdc*2 H1 kinase activity due to the activity of CSF. The extracts can be induced to exit metaphase and enter S-phase by the addition of 0.4 mM calcium to cause CSF release. Control (untreated or buffer treated) extracts were incubated at 23°C with sperm chromatin to form ~750 nuclei/μl and 0.4 μM $CaCl_2$ to induce exit from metaphase arrest. An interphase extract is defined as an extract that is in S-phase with a low level of *cdc*2 H1 kinase activity. All additions were made as described in the text. To arrest extracts with DNA and aphidicolin, sperm chromatin to form 2000 nuclei/μl and 50 μg/ml aphidicolin were added to the extracts at the same time as the $CaCl_2$. Unless otherwise indicated, extracts were incubated for 80 min to bring them into interphase (low H1 kinase activity) before further manipulation. (For a complete description of the events occurring in DNA/aphidicolin-blocked extracts, see Dasso and Newport, 1990). At this time, the

extracts typically have synthesized enough cyclin to induce mitosis after release from the aphidicolin block. At various times throughout extract cycles, aliquots were removed and assayed for H1 kinase and H1 phosphatase activity (see below). The H1 kinase activity in the extracts was assayed in a final volume of 20 μl, which included 1 μl of extract and 19 μl of a reaction mixture containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5, 10 mM $MgCl_2$, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), 0.2 mM ATP (1.5 cpm/fmol), 0.5 mg/ml histone H1. After a 10-min incubation at 23°C, the assays were stopped by the addition of 20 μl 50% acetic acid, and an aliquot of the assay was spotted onto a 2-cm square of p81 paper. The papers were washed three times for 2 min each in 75 mM phosphoric acid, dried, and the incorporation of label was determined by scintillation counting. All assays were corrected for a blank assay of extract brought into interphase in the presence of protein synthesis inhibitors. The inclusion of 2 μM OA in the assay mixture had no effect on the outcome of the assays, indicating that the assay selectively determined the level of H1 kinase activity in the extracts. All extracts were routinely inspected for nuclear formation and nuclear envelope breakdown (NEBD) at various times throughout the time course of the experiment by both phase contrast and fluorescence microscopy using the DNA stain DAPI. Cyclin A and B immune complex H1 kinase assays were performed as described previously (Walker and Maller, 1991). All immunoprecipitates were washed extensively by the method described by Gautier *et al.* (1989) before assay for H1 kinase activity.

Unless otherwise stated, the results shown are representative of three experiments that gave similar results.

Preparation of ^{32}P-H1

^{32}P-H1 was prepared by phosphorylation of H1 with cyclin A/cdc2 in the following manner. High activity cyclin A/cdc2 was prepared by incubating bovine protein A–cyclin A fusion protein (PACA) (Bandara *et al.*, 1991) at a final concentration of 150 nM in 100 μl of interphase CSF extract for 60 min at 23°C. Under these conditions, the H1 kinase activity of the extract was activated approximately threefold above normal mitotic levels. The active PACA/cdc2 complex was purified by immunoprecipitation with rabbit IgG-Sepharose, followed by washing as described previously for immunoprecipitation (Gautier *et al.*, 1989). The purified complex was then incubated with 5 mg H1 in 50 mM HEPES, pH 7.2, 200 μM ^{32}P-ATP (50 000 cpm/pmol), 10 mM $MgCl_2$, 2 mM EGTA, and 2 mM DTT in a final volume of 250 μl for 2 h at 23°C. The PACA/cdc2 beads were removed by centrifugation at 10 000 × *g* for 5 min. The beads were washed with 250 μl of TE (10 mM tris(hydroxymethyl)aminomethane, pH 7.5, 2 mM EDTA), the supernatants combined, and cold ATP added to a final concentration of 2 mM. The supernatant was dialyzed against TE for 36 h at 20°C with six changes of buffer. By this protocol, ≥0.2 mol ^{32}P was incorporated per mol H1, and <1% of the ^{32}P remained soluble after precipitation with 30% trichloroacetic acid (TCA).

Assay of H1 Phosphatase in Extracts

Typically, 2 μl of extract was incubated with 20 μM ^{32}P-H1 in a final volume of 20 μl TE for 3 min at 23°C. Under these conditions, the release of ^{32}Pi was linear for ≥8 min. Assays were terminated by the addition of 30 μl of 30% TCA, followed by incubation at 4°C for 15 min. Because extracts have a high concentration of protein, no carrier was added. When purified phosphatases were assayed, 10 μl of 0.2 mg/ml sheep IgG was added to the tubes after the TCA step. Control assays were performed exactly as above, except that 100 mM paranitrophenylphosphate, 100 mM NaF, 5 mM EDTA, and 600 μM $NaVO_4$ were included. The counts released under these conditions were attributable to mechanisms other than dephosphorylation, such as nonspecific degradation or proteolysis of the H1 (~20% of the total released counts in the case of crude extracts). The samples were centrifuged at 10 000 × *g* for 5 min, and a 25-μl aliquot of the supernatant was removed and counted. To determine the activity of PrP-1, aliquots were incubated either without or with 100 units/μl of Inh-2 for 10 min before the addition of H1. The difference between counts released in the absence and presence of Inh-2 was attributable to PrP-1. One unit of Inh-2 is defined as the amount of Inh-2 required to inhibit 0.004 U PrP-1 by 50%. One unit of PrP-1 is defined as the amount required to release 1 nmol Pi/min using phosphorylase *a* at 2 mg/ml as a substrate.

Determination of DNA Synthesis

The extent of DNA synthesis was determined by the incorporation of [α-^{32}P]dATP into sperm chromatin as described previously (Blow and Laskey, 1986; Walker and Maller, 1991). Extracts were treated either with or without cycloheximide for 10 min before CSF release. On $CaCl_2$ addition to cause CSF release, 10 ng/μl sperm chromatin and 100 μCi/ml [α-^{32}P]dATP were added. Each sample was then treated with or without 100 units/μl Inh-2 10 min later. Aliquots of the reaction were removed and assayed either for H1 kinase activity or for incorporation of [α-^{32}P]dATP into DNA.

RESULTS

OA Causes Premature Mitosis in a DNA/Aphidicolin-Blocked Extract

DNA/aphidicolin-blocked CSF extracts serve as an ideal model to investigate the mechanisms controlling exit from S-phase and entry into M-phase in early *Xenopus* embryos. In these extracts, DNA synthesis is blocked, and, as a consequence, the extracts arrest in interphase with high levels of inactive cyclin/cdc2 complexes (Dasso and Newport, 1990) due to feedback controls that prevent mitosis from occurring in the presence of unreplicated DNA. To enter mitosis, therefore, the signals that maintain cyclin/cdc2 in an inactive state must be overcome. It is likely that these signals are also involved in the normal transition from S-phase to M-phase in early embryos. It previously has been demonstrated that addition of OA, a potent inhibitor of protein phosphatases 1 and 2A, is able to induce cdc2 H1 kinase activation in high-speed supernatants from *Xenopus* interphase extracts made without added DNA (Felix *et al.*, 1990). This was proposed to be due to the inhibition of a type 2A phosphatase. To investigate the potential role of protein phosphatases in coordinating the S → M transition in *Xenopus* embryonic cycles, the effect of OA in DNA/aphidicolin-arrested extracts was therefore examined. OA was added to a final concentration of 1 μM to a DNA/aphidicolin-treated extract 80 min after release from metaphase II-arrest (CSF release) had been induced by the addition of 0.4 mM $CaCl_2$. As shown in Figure 1A, after a short lag of ~20 min, a large increase in H1 kinase occurred with rapid kinetics. This increase was accompanied by NEBD, which confirmed that the extract had entered mitosis (Figure 1B). No increase in H1 kinase or NEBD occurred in the control extract (Figure 1). The profiles of cyclin A and cyclin B/cdc2 H1 kinase are shown in Figure 1C. Before the addition of OA, the activity of cyclin A/cdc2 H1 kinase was elevated as expected (Walker and Maller,

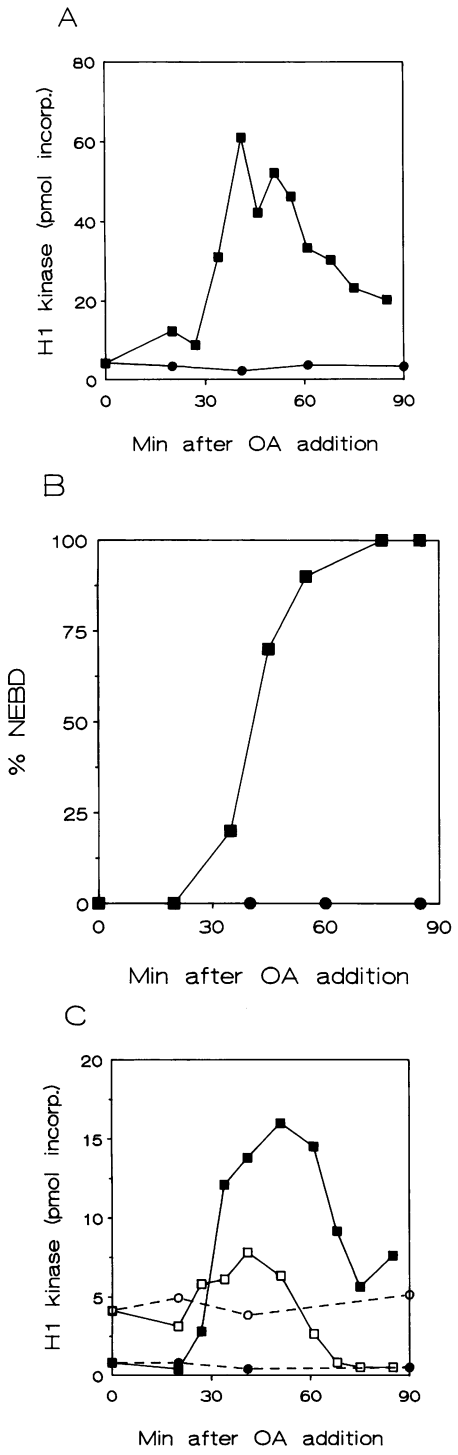

Figure 1. Okadaic acid releases a DNA/aphidicolin-blocked interphase extract. Metaphase II-arrested extracts were brought into interphase in the presence of DNA and aphidicolin as described in MATERIALS AND METHODS. At 80 min after metaphase II release, the extracts were treated either with (□, ■) or without (○, ●) 1 μM OA. At various times, aliquots were removed and assayed for total H1 kinase (A), NEBD (B), or for cyclin A/cdc2- (□, ○) or cyclin B/cdc2- (■, ●) immunoprecipitated H1 kinase activity (C) as described previously (Walker and Maller, 1991).

1991) but did not change throughout the time course of the control extract. In the OA-treated extract, however, cyclin A/cdc2 H1 kinase increased further after a 20-min lag and then began to decline after 45 min. Cyclin B/cdc2 H1 kinase activity was almost undetectable before addition of OA but followed a profile of activation similar to that of cyclin A in the OA-treated extract. In the absence of OA, cyclin B H1 kinase activity remained at basal levels.

Regulation of Total H1 Phosphatase in Cycling Extracts

It seemed likely that the effect of OA was due to the inhibition of a protein phosphatase. To investigate the regulation of the putative phosphatase, the rate of total H1 dephosphorylation was followed in both control and DNA/aphidicolin-treated extracts. The profiles of H1 kinase and H1 phosphatase in a control extract are shown in Figure 2A. Shortly after release from metaphase II arrest, H1 phosphatase activity rapidly increased about fourfold. This activity stayed high throughout interphase and then declined in concert with the activation of H1 kinase as the extract entered mitosis. The activation of H1 phosphatase after release from metaphase II arrest was also rapid in the DNA/aphidicolin-treated extract shown in Figure 2B. In this case, however, the extract remained in interphase, and the phosphatase activity remained high, whereas the H1 kinase activity remained low.

The total H1 phosphatase profile could reflect the activity of a combination of several different phosphatases. It is unlikely that either type 2B or 2C phosphatases are involved, because the conditions of the assay include EDTA, which would inhibit the activity of both of these enzymes. It is relatively easy to distinguish a type 1- from a type 2- phosphatase, because type 1 activity is specifically inhibited by either of two inhibitory proteins termed Inh-1 and Inh-2 (Cohen, 1989). Inh-1 requires phosphorylation by the cyclic AMP-dependent protein kinase to inhibit PrP-1 and is readily dephosphorylated in crude extracts, whereas Inh-2 is active in its dephospho-form. For this reason, Inh-2 was chosen for further experiments. Figure 3A shows the effect of addition of Inh-2 and OA on the total H1 phosphatase activity in both metaphase II-arrested (CSF) and interphase (40 min after release from metaphase II arrest) extracts. In the metaphase II-arrested extract, the H1 phosphatase activity was relatively low, and the preincubation of the extract with Inh-2 caused little change in the activity. Incubation of these extracts with OA caused an increase in the H1 phosphatase activity. The reason for this intriguing observation is unknown. As expected, the total H1 phosphatase activity in the interphase extract was higher than in the metaphase II-arrested extract (see Figure 2). Incubation of the interphase extract with 100 units/μl Inh-2 before the phosphatase assay caused a

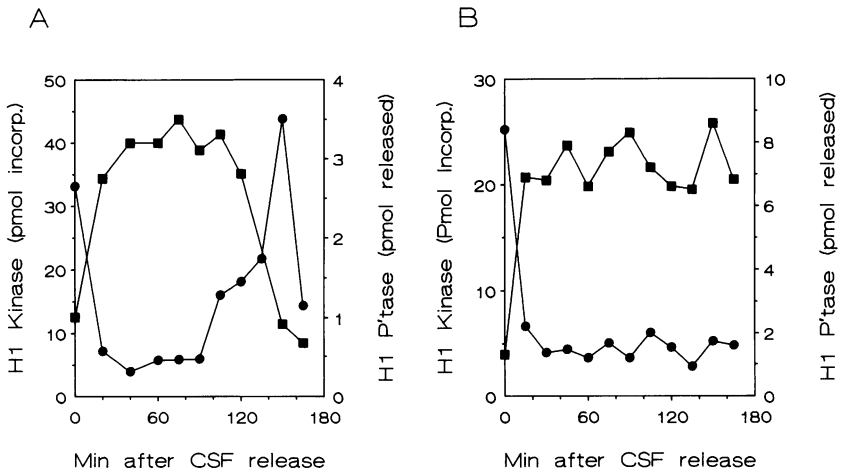

Figure 2. Oscillation of H1 phosphatase in the cell cycle. A shows a control extract and B a DNA/aphidicolin-blocked extract, following release of metaphase II arrest with 0.4 mM $CaCl_2$. Metaphase II-arrested extracts were prepared as described either in the presence of 750 nuclei/μl (A) or 2000 nuclei/μl plus 50 μg/ml aphidicolin (B). At various times, aliquots were removed and assayed either for H1 kinase (●) or for H1 phosphatase (■).

30% decline in phosphatase activity. A 42% decrease in phosphatase activity was also observed in extracts incubated with 1.5 μM OA before assay, whereas only a slight (5%) decline was observed if the concentration of OA was 0.2 μM, at which Prp-2A would be expected to be preferentially affected (Cohen, 1989; Felix *et al.*, 1990). A dose response to Inh-2 is shown in Figure 3B. Maximal inhibition by Inh-2 occurred at 150 units/μl, and 50% maximal inhibition occurred at 50 units/μl. The amount of Inh-2 required is in the same range as that used by Foulkes and Maller (1982) for the inhibition of PrP-1 in *Xenopus* oocytes.

Based on these results, the specific regulation of PrP-1 in the cell cycle was investigated. Aliquots were taken at various time points from a cycling extract and incubated either with or without 100 units/μl Inh-2 for 10 min before assaying H1 phosphatase. The profile of PrP-1 in a cell cycle after release from metaphase II arrest is shown in Figure 4A. PrP-1 activity remained low for ~20 min after CSF release and then increased to a level approximately threefold over basal during S-phase. Shortly before the activation of cdc2 H1 kinase activity, the PrP-1 activity declined. A second highly reproducible (repeated in 5 separate experiments) peak of PrP-1 activity was seen when the extract reached maximal H1 kinase activity and entered mitosis as determined by NEBD. As was the case for total H1 phosphatase activity (Figure 2B), PrP-1 was activated and then remained high when extracts were blocked in S-phase by treatment with DNA and aphidicolin (Figure 4B).

Effect of Inh-2 and PrP-1 on the Cell Cycle

To investigate the role of PrP-1 in regulating early embryonic cycles, extracts were treated with either Inh-2 or PrP-1 shortly after release from metaphase II arrest, and the effect on the subsequent cycle was studied. The extracts used in these experiments contained 500 nuclei/μl, an amount insufficient to delay the timing of the entry into mitosis. Figure 5 shows that the addition of 80 units/μl Inh-2 to extracts 20 min after release from metaphase II arrest resulted in early entry into mitosis as determined by H1 kinase activity (Figure 5A) and NEBD compared with a control (buffer-treated) extract. NEBD occurred ~40 min earlier in the Inh-2-treated extract than in the control. In addition, H1 kinase activity increased gradually over a 60-min time course, whereas the activation of H1 kinase in the control occurred with abrupt kinetics as expected. It should also be noted that chromatin decondensation did not occur in nuclei from Inh-2-treated extracts, although the formation of nuclear envelopes was normal as determined by phase contrast microscopy. Inh-2 was also able to overcome a DNA/aphidicolin block, as shown in Figure 5B. A metaphase II-arrested extract was released into interphase in the presence of DNA and aphidicolin for 70 min. Thirty minutes later, the extract was treated with either buffer, 100 units/μl Inh-2, or 1 μM OA. H1 kinase activity of the control extract remained low throughout the experiment, and no NEBD was observed. As expected, the addition of OA to the extract caused the activation of H1 kinase and NEBD after a lag period. Addition of Inh-2 also resulted in both an increase in *cdc2* H1 kinase to the same level as in the OA-treated extract and NEBD. The lag between the addition of Inh-2 and the activation of H1 kinase was ~15 min shorter than with OA (30 compared with 45 min). Although the difference between the lag for Inh-2 and OA was variable in different experiments, Inh-2 consistently activated H1 kinase more rapidly than OA.

The converse experiment relative to the addition of Inh-2 investigated the effect of excess PrP-1 on the cell cycle. Twentyfive minutes after CSF release, purified catalytic subunit of PrP-1 was added to a final concentration sufficient to dephosphorylate 6 pmol of phospho-H1 $\cdot$ $min^{-1} \cdot \mu l^{-1}$. This amount resulted in an approximate threefold increase over the basal level of PrP-1 in the extract. As shown in Figure 6, the control extract reached maximum cdc2 H1 kinase activity 90 min after

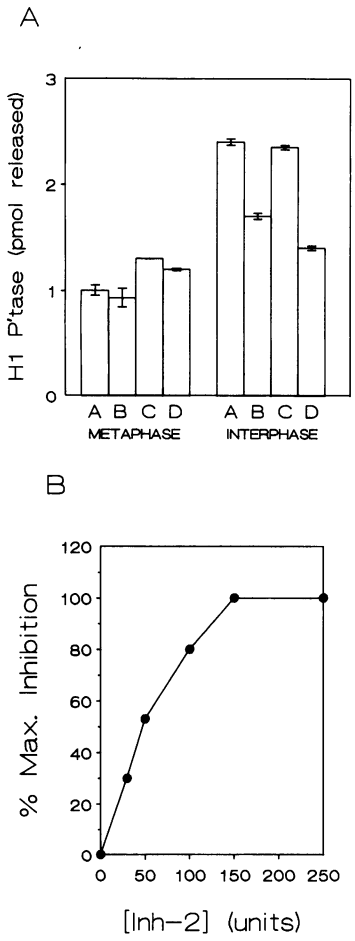

Figure 3. Characterization of the cell cycle-regulated H1-phosphatase. (A) effect of Inh-2 and OA on the cell cycle-regulated H1-phosphatase. Aliquots of either metaphase II-arrested or interphase (40 min after $CaCl_2$ addition) extracts were incubated with phosphatase inhibitors for 10 min and then assayed for H1 phosphatase activity. A, control; B, plus 100 units/μl Inh-2; C, plus 200 nM OA; D, plus 1500 nM OA. (B) dose response of the effect of Inh-2 in an interphase extract. Aliquots from an interphase extract were incubated with varying concentrations of Inh-2 for 10 min and then assayed for H1 phosphatase.

CSF release, and 100% NEBD occurred by 100 min (not shown). The extract treated with PrP-1, on the other hand, remained in interphase for ≥140 min as determined by H1 kinase activity and nuclear morphology. H1 kinase activity just began to increase by 160 min, the limit of this experiment, and no NEBD occurred. It was possible that the delay in activation of H1 kinase and NEBD in the extract was due to the fact that the exogenous PrP-1 was dephosphorylating cdc2 substrates, thereby preventing entry into mitosis. However, the addition of the same concentration of PrP-1 to a metaphase II-arrested extract had no detectable effect on the H1 kinase activity in the extract even after 30 min of exposure to the phosphatase, and the extract remained in mitosis as determined by nuclear morphology. This result suggests that PrP-1 is not simply dephosphorylating cdc2 substrates, although it is difficult to exclude the possibility that PrP-1 can specifically dephosphorylate cdc2 substrates in the extract at a higher rate than H1.

Effect of Inh-2 on DNA Synthesis in Extracts

The data suggest that PrP-1 has some role in the regulation of DNA synthesis, either in ensuring the progression of DNA replication itself or as a signal of the state of DNA synthesis (i.e., it signals that S-phase is not complete), or both. To test these hypotheses, DNA synthesis was determined in both control and cycloheximide-treated extracts in the presence and absence

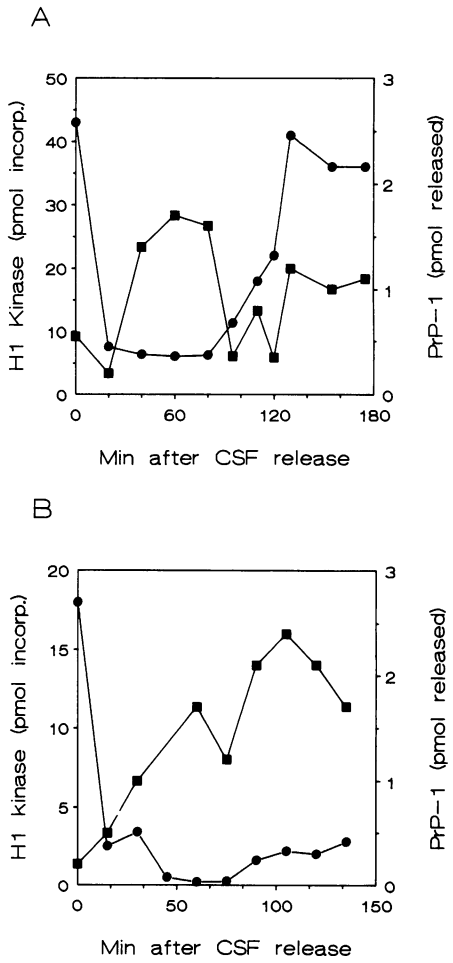

Figure 4. Oscillation of PrP-1 in the cell cycle. A shows a control extract and B a DNA/aphidicolin-blocked extract. Metaphase II-arrested extracts were prepared as described in MATERIALS AND METHODS, either in the presence of 750 nuclei/μl (A) or with 2000 nuclei/μl plus 50 μg/ml aphidicolin (B). At various times after $CaCl_2$ addition, aliquots were removed and assayed for either H1 kinase (●) or for PrP-1 (■) using phospho-H1 as a substrate.

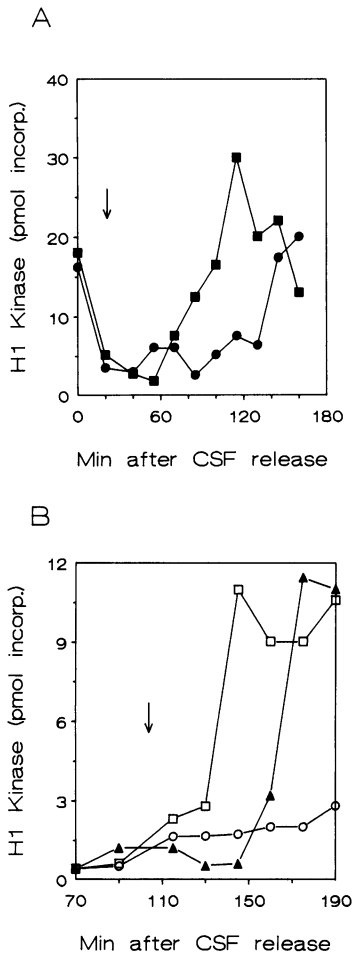

Figure 5. Effect of inhibitor 2 on the cell cycle. Metaphase II-arrested extracts were prepared as described in MATERIALS AND METHODS, either in the presence of 750 nuclei/μl (A) or in the presence of 2000 nuclei/μl plus 50 μg/ml aphidicolin (B). At various times after the addition of $CaCl_2$ to induce cycling, aliquots were removed and assayed for H1 kinase activity. (A) 20 min after the addition of $CaCl_2$, the extracts were treated either with (■) or without (●) 80 units/μl Inh-2. (B) extracts were brought into interphase for 70 min before removal of the first aliquot. Thirty minutes later, the extracts were treated either with buffer (○), 80 units/μl Inh-2 (□), or 1 μM OA (▲). The arrows indicate the time at which Inh-2 and OA were added.

of 100 units/μl Inh-2. Despite the fact that neither cyclin A nor B are present after release from metaphase II arrest in cycloheximide-treated extracts, there is no effect on the amount of DNA synthesis (Blow and Laskey, 1986; Walker and Maller, 1991). If PrP-1 were required for DNA synthesis itself, then the addition of Inh-2 to both the control and the cycloheximide-treated extracts should result in the inhibition of this process. On the other hand, if the inhibition of PrP-1 were to serve only as a signal for the activation of cdc2 H1 kinase, then DNA synthesis should be inhibited in the control extract, as a result of entry into mitosis, but remain unaffected in cycloheximide-treated extracts that cannot enter mitosis because of the lack of cyclin synthesis. The results of this experiment are shown in Figure 7. Figure 7A shows a time course of DNA synthesis either with or without Inh-2 in an untreated extract. The addition of Inh-2 to the extract resulted in ~80% inhibition of DNA synthesis relative to the untreated control, and the extracts entered mitosis prematurely as judged by both H1 kinase activity and NEBD. The effect of Inh-2 on extracts treated with cycloheximide is shown in Figure 7B. In the absence of new cyclin synthesis, Inh-2 also inhibited DNA synthesis. In these extracts, the lack of cyclin synthesis was confirmed by the absence of mitosis determined by nuclear morphology and basal levels of H1 kinase activity up to 3 h after calcium addition.

DISCUSSION

We have investigated the potential roles of protein phosphatases in the early embryonic cell cycle. OA, a potent inhibitor of both PrP-1 and -2A, was found to bypass a DNA/aphidicolin block and to cause premature mitosis. This suggests a role for phosphatases in controlling the transition from S-phase to M-phase in the early embryonic cell cycle. It should be pointed out, however, that OA has numerous effects on *Xenopus* egg extracts depending on the time of addition to the extract. OA has been observed to cause the activation of H1 kinase in high-speed supernatants of extracts prepared from interphase eggs (Felix *et al.*, 1990). The addition of OA to metaphase II-arrested extracts, on the other hand, results in release from metaphase II and entry into interphase (Lorca *et al.*, 1991). In addition, the interphase period after OA addition at metaphase II-arrest

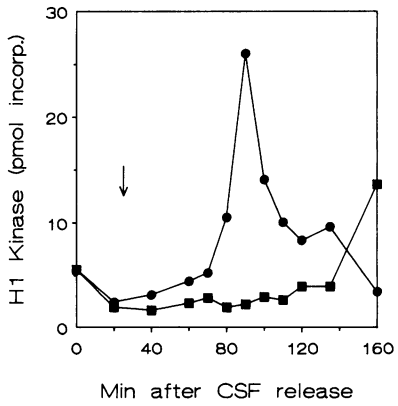

Figure 6. Protein phosphatase 1 delays entry into mitosis. Metaphase II-arrested extracts were prepared in the presence of 750 nuclei/μl as described in MATERIALS AND METHODS. At various times, aliquots were removed and assayed for H1 kinase activity. Twenty-five minutes after the addition of 0.4 mM $CaCl_2$ to induce cycling, the extracts were treated either with (■) or without (●) the purified catalytic subunit of PrP-1 to a final concentration of 6 pmol · min^{-1} · μl^{-1}. This elevated concentration of PrP-1 remained evident after addition to the extract. The arrow indicates the time of addition of PrP-1 to the extract.

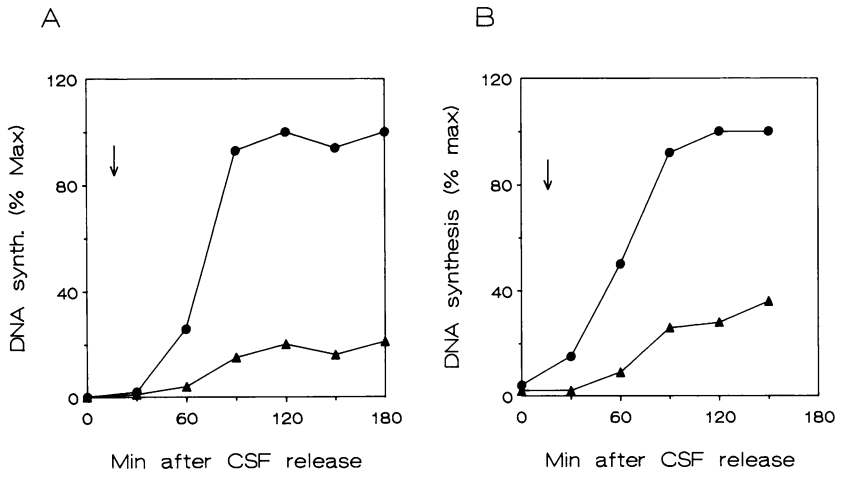

Figure 7. Treatment of extracts with Inh-2 inhibits DNA synthesis. Metaphase II-arrested extracts were induced to cycle by $CaCl_2$ addition and assayed for DNA synthesis by incorporation of [α-^{32}P]dATP into sperm chromatin as described in MATERIALS AND METHODS. At various times, aliquots were removed and assayed for H1 kinase activity and DNA synthesis. Extracts were treated either without (A) or with (B) 100 μg/ml cycloheximide for 10 min before the addition of calcium to the extracts. Twenty minutes after CSF release, extracts were treated either without (●) or with (▲) 100 units/μl Inh-2. The arrows indicate the time of addition of Inh-2 to the extracts.

is prolonged because of the inhibition of cyclin B synthesis (Walker and Maller, unpublished observations). The wide range of effects of OA raises questions about the use of OA to assign effects to specific phosphatases in crude systems. At best, OA may be used to imply a role for phosphorylation/dephosphorylation in controlling a step in a pathway of metabolic regulation.

To more specifically identify a role for protein phosphatases before mitosis, we directly investigated the regulation of phosphatase activity using phospho-H1 as a substrate. H1 phosphorylated by cdc2 was chosen as a substrate for the following reasons. H1 previously has been shown to be a substrate for both the type 1 and 2A phosphatases (Lee *et al.*, 1991). In addition, because H1 is phosphorylated at cdc2 consensus sites, it would be reasonable to expect that any phosphatase responsible for dephosphorylating mitotic substrates would be able to dephosphorylate phospho-H1. Importantly, the total H1 phosphatase activity changes during the cell cycle, increasing shortly after release from metaphase II arrest and falling in concert with the onset of mitosis. Furthermore, we demonstrate that an Inh-2-sensitive protein phosphatase activity, most likely a PrP-1 type, accounts for at least part of this regulated phosphatase activity (Figure 4). The Inh-2-sensitive phosphatase activity first becomes active as the extracts begin DNA synthesis and declines to basal levels shortly before the onset of mitosis. A second peak of activity was consistently observed to coincide with the peak of *cdc*2 H1 kinase activity in mitosis. The importance of this phosphatase in the control of mitosis was demonstrated by the ability to manipulate the timing of the initiation of mitosis in these extracts by artificially altering the levels of this enzyme. On inhibition of the phosphatase with the specific inhibitor, Inh-2 extracts prematurely activate *cdc*2 H1 kinase and enters mitosis early (Figure 5). When added to a DNA/aphidicolin-blocked extract, Inh-2 induces an increase in H1 kinase activity and NEBD, consistent with entry into mitosis. Conversely, extracts could be induced to delay in interphase by the addition of exogenous PrP-1 (Figure 6), suggesting that the Inh-2-regulated phosphatase is indeed PrP-1. These data provide evidence that PrP-1 is involved in the control of the cell cycle, specifically in the feedback pathway that delays mitosis until the completion of DNA synthesis.

In addition, inhibition of this phosphatase has a direct effect on DNA synthesis. Even in the absence of new cyclin synthesis, treatment of the extracts with Inh-2 results in a loss of DNA synthesis (Figure 7B). Because no new cdc2/cyclin active complexes are formed, it is highly unlikely that the loss of DNA synthesis is a result of entry into mitosis. This suggests that PrP-1 is also required for the onset of DNA synthesis in *Xenopus* early cell cycles. A mutation in PrP-1 has been shown to cause excessive chromatin condensation in *Drosophila* (Axton *et al.*, 1990), and we also observe that chromatin remains condensed in Inh-2-treated extracts even though nuclear envelopes form normally. From these observations, it appears likely that the loss of DNA synthesis in Inh-2-treated extracts is due to the inability to decondense the chromatin during entry into interphase. The effect of PrP-1 on DNA synthesis is probably distinct from the regulation of the onset of mitosis, because the premature activation of cdc2/cyclin induced by Inh-2 occurs in extracts that have not been delayed by high concentrations of DNA (Figure 5A). The inhibition of PrP-1 by Inh-2 also accelerates the activation of cdc2 H1 kinase activity even in the total absence of DNA (not shown). Such broad effects in the control of the timing of the early embryonic cell cycle strongly suggest that PrP-1 plays a pivotal role in the regulation of S-phase in *Xenopus* embryonic cell cycles. One attractive hypothesis would be that PrP-1 is regulated by the mitotic clock that determines the timing of S- and M-phases in a normal cell cycle (Murray and Kirschner, 1989b). Feedback mechanisms, such as the one triggered by incomplete DNA synthesis, which disrupt the normal cell cy-

cle, would maintain PrP-1 in an active state to prevent the premature onset of mitosis.

Evidence from fission yeast also supports a role for PrP-1 during S-phase. Booher and Beach (1989) reported that a gene, termed bws1+, caused a G_2 arrest in a wee1$^-$/cdc25ts double mutant at the restrictive temperature. This double mutant normally expresses a wee phenotype at the restrictive temperature. The bws1+ gene product is 82% identical to the catalytic subunit of mammalian PrP-1. This is in keeping with our own observations that an excess of PrP-1 significantly delays the progression of the cell cycle, whereas the removal of PrP-1 significantly speeds up the cell cycle. These genetic data imply a role for PrP-1 in controlling the regulation of the onset of mitosis. The exact point at which PrP-1 exerts its action remains unclear, but one likely explanation of this data is that the overexpression of PrP-1 sends a signal that DNA synthesis is still ongoing. Although it is possible that PrP-1 is involved in the regulation of the cdc25 and wee1 activities, the absence of both wee1 and cdc25 in the double mutants studied by Booher and Beach (1989) implies that the activation of cdc2 is regulated by additional controls other than tyrosine dephosphorylation and that these controls involve PrP-1. Evidence that supports the regulation of mitosis by mechanisms other than tyrosine 15 phosphorylation/dephosphorylation recently has been demonstrated in budding yeast (Amon *et al.*, 1992; Sorger and Murray, 1992). One good candidate for a further regulation point is the phosphorylation of T161; to become active, cdc2 must be phosphorylated at this site (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Solomon *et al.*, 1991). An intriguing hypothesis is that regulation of both the tyrosine phosphorylation (by the wee1 and cdc25 gene products) and the threonine 161 phosphorylation (by the as yet unknown kinase[s] and/or phosphatase[s]) are controlled upstream by the activity of PrP-1. If PrP-1 is indeed a component of the cell cycle clock, then this is a reasonable possibility.

During interphase, cyclin A/cdc2 is active and is also probably nuclear (Pines and Hunter, 1991). Cyclin A/cdc2 also has been shown to have a role in controlling entry into mitosis in *Xenopus* egg extracts (Walker and Maller, 1991). It is possible that the cyclin A activity present during interphase interacts with PrP-1 to mediate the dependence of mitosis on completion of DNA synthesis. At first inspection, there are similarities between the two activities. The ablation or inhibition of either activity results in exit from DNA synthesis and premature entry into mitosis (Walker and Maller, 1991; this work). One major difference between the regulation of cdc2 kinase activation by PrP-1 and cyclin A is in the timing of entry into mitosis as a result of loss of these activities. Loss of cyclin A results in premature mitosis only when the cycle has been delayed due to incomplete DNA synthesis, whereas loss of PrP-1 activity results in premature mitosis even in normally progressing cycles. Although both proteins may ultimately act on the same pathway of activation of cyclin B/cdc2, this observation does not preclude the possibility that cyclin A/cdc2 and PrP-1 act along two different feedback pathways, each with a distinct role in regulation of the S → M transition. The nature of the relationship between the actions of cyclin A/cdc2 and PrP-1 merits investigation.

In apparent disagreement with our results, Felix *et al.* (1990) previously reported that PrP-1 does not have a role in OA-induced mitosis. There are significant differences between the two experimental approaches, however, that may account for the discrepancy. Our extracts from metaphase II-arrested cells carry out functional cycles of DNA synthesis and mitosis in the presence of exogenously added DNA. Felix *et al.* performed their experiments with frozen high-speed supernatants from activated eggs, which contained no DNA and could not carry out protein synthesis. If PrP-1 has a role in coordinating the transition from S-phase to M-phase as we have suggested, then it is likely that in partially fractionated extracts in the absence of DNA, the feedback mechanism involving PrP-1, which would normally regulate this step, is not in place.

In addition to activity during S-phase, we also observed PrP-1 to be active at mitosis (Figure 4A). A requirement for PrP-1 in the completion of mitosis has been well documented. Mutants of PrP-1 in fission yeast (Ohkura *et al.*, 1989), *Aspergillus nidulans* (Doonan and Morris, 1989) and *Drosophila* (Axton *et al.*, 1990), cause arrest of the cell cycle in midmitosis. A number of roles of PrP-1 at this time have been suggested. These include involvement in chromatin packaging and spindle dynamics (Axton *et al.*, 1990), the dephosphorylation of mitotic substrates (Doonan and Morris, 1989; Axton, *et al.*, 1990), and sister chromatid disjoining (Ohkura *et al.*, 1989; Axton *et al.*, 1990). Our observations provide the first biochemical evidence that PrP-1 activity is increased in mitosis and confirm a role for this phosphatase at this time in the cell cycle. Interestingly, PrP-1 activity does not appear to be elevated in metaphase II-arrested extracts (meiotic M-phase-arrested), even though the activity of *cdc2* kinase is high at this time. Metaphase II-arrested extracts often spontaneously exit mitosis, and the low levels of PrP-1 activity may reflect the early stages of such spontaneous release. Alternatively, the low levels of PrP-1 in metaphase II-arrested extracts may reflect a difference between meiotic and mitotic metaphases. Differences in the effects of PrP-1 inhibiting antibodies also have been observed in systems that have different cell cycle arrest points (Picard *et al.*, 1989). Further work needs to be done to investigate the role of PrP-1 at meiosis II in *Xenopus*.

It is clear from this work that there are significant changes in PrP-1 activity during the embryonic cell cycle. However, the activities of both PrP-1 using phosphorylase as a substrate (Kinoshita *et al.*, 1990) and PrP-

2A using either phosphorylase a (Kinoshita *et al.*, 1990) or myosin light chain (Ruediger *et al.*, 1991) as substrates have been reported to change little during the cell cycle. One possible explanation for these apparent discrepancies is that the kinetics with respect to specific substrates (such as phospho-H1) change within the cycle. The kinetics of PrP-1G, the PrP-1 activity involved in the regulation of glycogen metabolism, have been shown to change for specific substrates in response to hormones (for review, see Cohen, 1989). In rabbit skeletal muscle, PrP-1G is a complex of the PrP-1 catalytic subunit and the G subunit, a 160 000-KDa protein (Tang *et al.*, 1991) that interacts with glycogen. The G subunit may be phosphorylated at two sites, site 1 and 2, by the cyclic AMP-dependent protein kinase resulting in the dissociation of the C subunit from the G subunit. The free C subunit is five to eightfold less effective than PrP-1G in the dephosphorylation of glycogen synthase. In response to insulin, however, the G subunit becomes phosphorylated at site 1 only, resulting in a 2.8-fold increase in the glycogen synthase phosphatase activity of PrP-1G and tight binding to glycogen (Dent *et al.*, 1990). It is possible that a similar mechanism of regulation exists in the early cell cycles of *Xenopus*, where PrP-1 might associate and dissociate with a specific targeting subunit in a cell cycle-dependent manner. The likelihood of this possibility is increased by the finding that the G-subunit kinase specific for site 1 is a homolog of S6 kinase II, which is cell cycle regulated in *Xenopus* oocytes and eggs (Lavoinne *et al.*, 1991). Another likely explanation is that in the simple cycles of early *Xenopus* embryos, only the phosphatases required for progression through repetitive S- and M-phases are present or active, and so their activities are not masked by other phosphatase activities participating in other cellular processes.

The data presented here demonstrate multiple roles for PrP-1 in the cell cycle. Not only is it involved in mitosis as has been demonstrated previously (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990), but it is also active during interphase and is involved both in the process of DNA replication and as part of the feedback system that controls the transition from DNA synthesis to mitosis in early *Xenopus* cell cycles. A model for the action of PrP-1 in the activation of cyclin B/cdc2 is shown in Figure 8. The ability of PrP-1 to block the activation of cdc2, which is normally catalyzed by cdc25 (Russell and Nurse, 1986; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Lee *et al.*, 1992), supports the possibility that cdc25 itself is regulated by phosphorylation. The fact that PrP-1 is integrally involved at several steps in the cell cycle demonstrates the importance of this enzyme in controlling the cell cycle. Because the activity of PrP-1 is easily measured using cdc2-phosphorylated H1, cell free extracts from *Xenopus* eggs will continue to serve as an excellent model to elucidate the mechanism of action and upstream regulation of this phosphatase in the cell cycle.

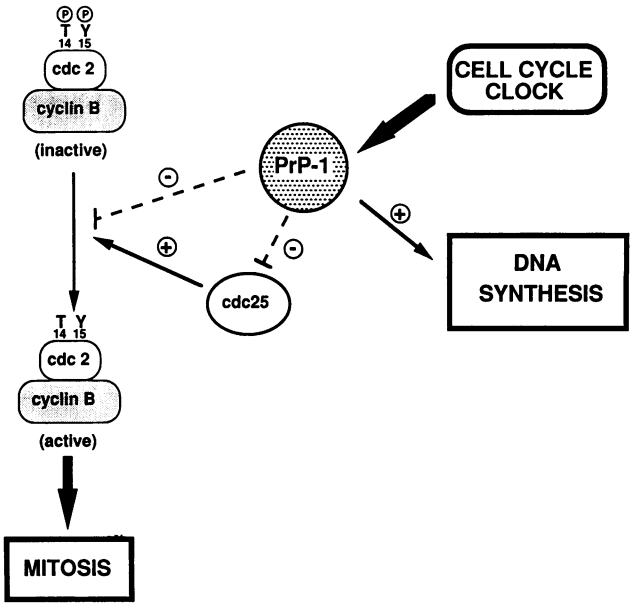

Figure 8. Model for the role of PrP-1 in the activation of cyclin B/cdc2. We propose that PrP-1 plays a central role in the regulation of the activation of cdc2. Active PrP-1 is required to maintain cyclin B/cdc2 in an inactive tyrosine-phosphorylated state. It is likely that one target of PrP-1 is the cdc25 phosphatase that dephosphorylates tyrosine 15. In addition, PrP-1 is required for DNA synthesis. PrP-1 is proposed to be regulated by the hypothetical "Cell Cycle Clock" that determines the timing of S- and M-phases in a normal cell cycle (Murray and Kirschner, 1989b).

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REFERENCES

Amon, A., Surana, U., Muroff, I., and Nasmyth, K. (1992). Regulation of $p34^{CDC28}$ tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. Nature *355*, 368–371.

Andres, J.L., and Maller, J.L. (1989). Purification and characterization of a novel protein phosphatase highly specific for ribosomal protein S6. J. Biol. Chem. *264*, 151–156.

Axton, J.M., Dombradi, V., Cohen, P.T.W., and Glover, D.M. (1990). One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. Cell *63*, 33–46.

Bandara, L.R., Adamczewski, J.P., Hunt, T., and La Thangue, N.B. (1991). Cyclin A and the retinoblastoma gene product complex with a common transcription factor. Nature *352*, 249–251.

Blow, J.J., and Laskey, R.A. (1986). Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. Cell *47*, 577–587.

Booher, R., Alfa, C.E., Hyams, J.S., and Beach, D.H. (1989). The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activation and nuclear localization. Cell *58*, 485–497.

Booher, R., and Beach, D. (1989). Involvement of a type 1 protein phosphatase encoded by *bws1+* in fission yeast mitotic control. Cell *57*, 1009–1016.

Brautigan, D.L., Sunwoo, J., Labbé, J.C., Fernandez, A., and Lamb, N.J.C. (1990). Cell cycle oscillation of phosphatase inhibitor-2 in rat fibroblasts coincident with $p34^{cdc2}$ restriction. Nature *344*, 74–78.

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. *58*, 453–508.

Cyert, M.S., and Kirschner, M.W. (1988). Regulation of MPF activity in vitro. Cell *53*, 185–195.

Dasso, M., and Newport, J.W. (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in *Xenopus*. Cell *61*, 811–823.

Dent, P., Lavoinne, A., Nakielny, S., Caudwell, F. B., Watt, P., and Cohen, P. (1990). The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature *348*, 302–308.

Doonan, J.H., and Morris, N.R. (1989). The *bimG* gene of *Aspergillus nidulans*, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. Cell *57*, 987–996.

Draetta, G., and Beach, D. (1988). Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. Cell *54*, 17–26.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell *56*, 829–838.

Ducommun, B., Brambilla, P., Felix, M.-A., Franza, B.R., Jr., Karsenti, E., and Draetta, G. (1991). cdc2 phosphorylation is required for its interaction with cyclin. EMBO J. *10*, 3311–3319.

Dunphy, W.G., Brizuela, L., Beach, D., and Newport, J. (1988). The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell *54*, 423–431.

Dunphy, W.G., and Kumagai, A. (1991). The cdc25 protein contains an intrinsic phosphatase activity. Cell *67*, 189–196.

Dunphy, W.G., and Newport, J.W. (1989). Fission yeast p13 blocks mitotic activation and tyrosine dephosphorylation of the *Xenopus* cdc2 protein kinase. Cell *58*, 181–191.

Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D., and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell *33*, 389–396.

Felix, M.A., Cohen, P., and Karsenti, E. (1990). cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cell cycle: evidence from the effects of okadaic acid. EMBO J. *9*, 675–683.

Foulkes, J.G., and Maller, J.L. (1982). In vivo actions of protein phosphatase inhibitor-2 in *Xenopus* oocytes. FEBS Lett. *150*, 155–160.

Gautier, J., Matsukawa, T., Nurse, P., and Maller, J.L. (1989). Dephosphorylation and activation of *Xenopus* $p34^{cdc2}$ protein kinase during the cell cycle. Nature *339*, 626–629.

Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J.L. (1990). Cyclin is a component of MPF from *Xenopus*. Cell *60*, 487–494.

Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988). Purified maturation promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene cdc2+. Cell *54*, 433–439.

Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F., and Kirschner, M.W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell *67*, 197–208.

Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature *349*, 132–138.

Goris, J., Hermann, J., Hendrix, P., Ozon, R., and Merlevede, W. (1989). Okadaic acid, a specific protein phosphatase inhibitor, induces maturation and MPF formation in *Xenopus laevis* oocytes. FEBS Lett. *245*, 91–94.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S., and Nurse, P. (1991). Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* $p34^{cdc2}$ function. EMBO J. *11*, 3297–3309.

Gould, K.L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature *342*, 39–45.

Healy, A.N., Zolnierowicz, S., Stapelton, A.E., Goebel, M.G., DePaoli-Roach, A.A., and Pringle, J.R. (1991). CDC55 a *Saccharomyces cerevisiae* gene involved in cellular morphogenesis: identification, characterization and homology to the B subunit of mammalian type 2A protein phosphatase. Mol. Cell. Biol. *11*, 5767–5780.

Ingebritsen, T.S., and Cohen, P. (1983). The protein phosphatases involved in cellular regulation. 1. Classification and substrate specificities. Eur. J. Biochem. *132*, 263–274.

Kinoshita, N., Ohkura, H., and Yanagida, M. (1990). Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. Cell *63*, 405–415.

Kumagai, A., and Dunphy, W.G. (1991). The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell free system. Cell *64*, 903–914.

Lavionne, A., Erikson, E., Maller, J.L., Price, D.J., and Cohen, P. (1991). Purification and characterisation of the insulin-stimulated protein kinase from rabbit skeletal muscle: close similarity to S6 kinase II. Eur. J. Biochem. *199*, 723–728.

Lee, M., and Nurse, P. (1988). Cell cycle control genes in fission yeast and mammalian cells. Trends Genet. *4*, 287–290.

Lee, M.S., Ogg, S., Xu, M., Parker, L.L., Donoghue, D.J., Maller, J.L., and Piwnica-Worms, H. (1992). cdc25+ encodes a protein phosphatase that dephosphorylates $p34^{cdc2}$. Mol. Biol. Cell *3*, 73–84.

Lee, T.H., Solomon, M.J., Mumby, M.C., and Kirschner, M.W. (1991). INH, a negative regulator of MPF, is a form of protein phosphatase 2A. Cell *64*, 415–424.

Lohka, M.J., and Maller, J.L. (1985). Induction of nuclear envelope breakdown, chromosome condensation and spindle formation in cell-free extracts. J. Cell Biol. *101*, 518–523.

Lorca, T., Fesquet, D., Zindy, F., Le Bouffant, F., Cerruti, M., Brechot, C., Devauchelle, G., and Dorée, M. (1991). An okadaic acid sensitive phosphatase negatively controls the cyclin degradation pathway in amphibian eggs. Mol. Cell. Biol. *11*, 1171–1175.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M., and Beach, D. (1991). mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell *64*, 1111–1122.

Minshull, J., Golsteyn, R., Hill, C., and Hunt, T. (1990). The A- and B-type cyclin-associated cdc2-kinases in *Xenopus* turn on and off at different times in the cell cycle. EMBO J. *9*, 2865–2875.

Moreno, S., Hayles, J., and Nurse, P. (1989). Regulation of $p34^{cdc2}$ protein kinase during mitosis. Cell *58*, 361–372.

Morla, A., Draetta, G., Beach, D., and Wang, J.Y.J. (1989). Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. Cell *58*, 193–203.

Murray, A.W., and Kirschner, M. (1989a). Cyclin synthesis drives the early embryonic cell cycle. Nature *339*, 275–280.

Murray, A.W., and Kirschner, M. (1989b). Dominoes and clocks: the union of two views of cell cycle regulation. Science *246*, 614–621.

Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T., and Yanagida, M. (1989). The fission yeast *dis2+* gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. Cell *57*, 997–1007.

Parker, L., Atherton-Fessler, S., Lee, M.S., Ogg, S., Falk, J.L., Swenson, K.I., and Piwnica-Worms, H. (1991). Cyclin promotes the tyrosine phosphorylation of $p34^{cdc2}$ in a $wee1^{+}$ dependent manner. EMBO J. *10*, 1255–1263.

Picard, A., Capony, J.P., Brautigan, D.L., and Dorée, M. (1989). Involvement of protein phosphatases 1 and 2A in the control of M phase-promoting factor in starfish. J. Cell Biol. *109*, 3347–3354.

Picard, A., Labbé, J.C., Barakat, H., Cavadore, J.C., and Dorée, M. (1991). Okadaic acid mimics a nuclear component required for cyclin B-cdc2 kinase microinjection to drive starfish oocytes into M phase. J. Cell Biol. *115*, 337–344.

Pickering, W.D., Kudlicki, W., Kramer, G., Hardesty, B., Vandenheede, J.R., Merlevede, W., Park, I., and DePaoli-Roach, A.A. (1991). Fluorescence studies on the interaction of inhibitor 2 and okadaic acid with the catalytic subunit of type 1 protein phosphatases. Biochemistry *30*, 10280–10287.

Pines, J., and Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle dependent nuclear transport. J. Cell Biol. *115*, 1–17.

Ruediger, R., Van Hood, J.E., Mumby, M., and Walter, G. (1991). Constant expression and activity of protein phosphatase 2A in synchronized cells. Mol. Cell. Biol. *11*, 4282–4285.

Russell, P., and Nurse, P. (1986). cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell *45*, 145–153.

Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by *wee1+*, a gene encoding a protein kinase homolog. Cell *49*, 559–567.

Shenolikar, S., and Nairn, A.C. (1990). Protein phosphatases: recent progress. Adv. Second Messenger Phosphoprotein Res. *23*, 1–121.

Simanis, V., and Nurse, P. (1986). The cell cycle control gene $cdc2^{+}$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. Cell *45*, 261–268.

Solomon, M., Glotzer, M., Lee, T.H., Philippe, M., and Kirschner, M.W. (1990). Cyclin activation of $p34^{cdc2}$. Cell *63*, 1013–1024.

Solomon, M.J., Lee, T., and Kirschner, M.W. (1991). The role of phosphorylation in $p34^{cdc2}$ activation: identification of an activating kinase. Mol. Biol. Cell *3*, 13–27.

Sorger, P.K., and Murray, A.W. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of $p34^{CDC28}$. Nature *355*, 365–368.

Standardt, N., Minshull, J., Pines, J., and Hunt, T. (1987). Cyclin synthesis, modification and destruction during meiotic maturation of the starfish oocyte. Dev. Biol. *124*, 248–258.

Swenson, K., Farrell, K.M., and Ruderman, J.V. (1986). The clam embryo protein cyclin A induces entry into M-phase and resumption of meiosis in *Xenopus* oocytes. Cell *47*, 861–870.

Tang, P.M., Bondor, J.A., Swiderek, K., and DePaoli-Roach, A.A. (1991). Molecular cloning of and expression of the regulatory (R_{GL}) subunit of the glycogen-associated protein phosphatase. J. Biol. Chem. *266*, 15782–15789.

Tung, H.Y.L., Resnik, T.J., Hemmings, B.A., Shenolikar, S., and Cohen, P. (1984). The catalytic subunits of protein phosphatase-1 and protein phosphatase 2A are distinct gene products. Eur. J. Biochem. *138*, 635–641.

Walker, D.H., and Maller, J.L. (1991). Role for cyclin A in the dependence of mitosis on the completion of DNA replication. Nature *354*, 314–317.