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Dephosphorylation of cdc25-C by a Type-2A Protein Phosphatase: Specific Regulation During the Cell Cycle in *Xenopus* Egg Extracts

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We have examined the roles of type-1 (PP-1) and type-2A (PP-2A) protein-serine/threonine phosphatases in the mechanism of activation of $p34^{cdc2}$/cyclin B protein kinase in Xenopus egg extracts. $p34^{cdc2}$/cyclin B is prematurely activated in the extracts by inhibition of PP-2A by okadaic acid but not by specific inhibition of PP-1 by inhibitor-2. Activation of the kinase can be blocked by addition of the purified catalytic subunit of PP-2A at a twofold excess over the activity in the extract. The catalytic subunit of PP-1 can also block kinase activation, but very high levels of activity are required. Activation of $p34^{cdc2}$/cyclin B protein kinase requires dephosphorylation of $p34^{cdc2}$ on Tyr15. This reaction is catalysed by cdc25-C phosphatase that is itself activated by phosphorylation. We show that, in interphase extracts, inhibition of PP-2A by okadaic acid completely blocks cdc25-C dephosphorylation, whereas inhibition of PP-1 by specific inhibitors has no effect. This indicates that a type-2A protein phosphatase negatively regulates $p34^{cdc2}$/cyclin B protein kinase activation primarily by maintaining cdc25-C phosphatase in a dephosphorylated, low activity state. In extracts containing active $p34^{cdc2}$/cyclin B protein kinase, dephosphorylation of cdc25-C is inhibited, whereas the activity of PP-2A (and PP-1) towards other substrates is unaffected. We propose that this specific inhibition of cdc25-C dephosphorylation is part of a positive feedback loop that also involves direct phosphorylation and activation of cdc25-C by $p34^{cdc2}$/cyclin B. Dephosphorylation of cdc25-C is also inhibited when cyclin A-dependent protein kinase is active, and this may explain the potentiation of $p34^{cdc2}$/cyclin B protein kinase activation by cyclin A. In extracts supplemented with nuclei, the block on $p34^{cdc2}$/cyclin B activation by unreplicated DNA is abolished when PP-2A is inhibited or when stably phosphorylated cdc25-C is added, but not when PP-1 is specifically inhibited. This suggests that unreplicated DNA inhibits $p34^{cdc2}$/cyclin B activation by maintaining cdc25-C in a low activity, dephosphorylated state, probably by keeping the activity of a type-2A protein phosphatase towards cdc25-C at a high level.

INTRODUCTION

Progression through the cell cycle is regulated by the activity of protein-serine/threonine kinases, which have catalytic subunits belonging to the cdc2 family (Pines and Hunter, 1991). They are known as cyclin-dependent protein kinases because their activity is governed by association with cyclin subunits that are synthesised and degraded during specific phases of the cell cycle (Hunt, 1991). The major protein kinase involved in the induction of mitosis is a one-to-one complex between $p34^{cdc2}$ and cyclin B (reviewed in Nurse, 1990). As cyclin B is synthesised, beginning in S-phase and proceeding during G2-phase, it associates with $p34^{cdc2}$ and induces rapid phosphorylation at three sites (reviewed in Clarke and Karsenti, 1991). Phosphorylation at one site (Thr 161 in vertebrate $p34^{cdc2}$) is required for subsequent kinase activity and appears to stabilize the interaction with cyclin (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Krek and Nigg, 1992; Lorca *et al.*, 1992; Solomon *et al.*, 1992).

However, the complex is not immediately active but is suppressed by phosphorylation at two other sites (Thr 14 and Tyr 15) close to the ATP binding site of the kinase (Gould and Nurse, 1989; Krek and Nigg, 1991a,b; Norbury *et al.*, 1991; Solomon *et al.*, 1992). Thus an inactive "pro-kinase" form of $p34^{cdc2}$/cyclin B accumulates during G2-phase. Final activation of $p34^{cdc2}$/cyclin B and entry into mitosis occurs when the inhibitory sites on $p34^{cdc2}$ are dephosphorylated. So it seems that the transition from G2 to M-phase is gated by a molecular switch involving a change in the relative kinase and phosphatase activities that regulate the phosphorylation of $p34^{cdc2}$ on Thr14 and Tyr15. In somatic cell cycles, this post-translational control allows the activation of $p34^{cdc2}$/cyclin B to be coupled to checkpoint signals that maintain cells in G2 (Dasso and Newport, 1990; Enoch and Nurse, 1991). In early embryonic cell cycles without such controls, it may determine the intrinsic timing of $p34^{cdc2}$/cyclin B protein kinase activation and hence the period of the cell cycle.

To understand how the switch works, it is necessary to elucidate the nature of the protein kinases and protein phosphatases that constitute it and how their activity is regulated. Tyrosine kinase activity towards $p34^{cdc2}$ is probably catalysed by the wee1 and mik1 protein kinases in the fission yeast *Schizosaccaromyces pombe* (Russell and Nurse, 1987b; Lundgren *et al.*, 1991; Parker *et al.*, 1992) and their homologues in vertebrates (Igarashi *et al.*, 1991; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993). Genetic and biochemical evidence suggests that this tyrosine kinase activity is negatively regulated by serine/threonine phosphorylation, being more active during interphase than mitosis (Russell and Nurse, 1987a; Smythe and Newport, 1992). The kinase that phosphorylates Thr14 may be distinct from the Tyr15 kinase (Honda *et al.*, 1992; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993), but it has not yet been characterized. Dephosphorylation of Tyr15 and possibly also Thr14 on $p34^{cdc2}$ is catalysed by the cdc25 phosphatase (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991c; Strausfeld *et al.*, 1991; Lee *et al.*, 1992). In human cells, three types denoted cdc25-A, -B, and -C have been identified (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991). In Xenopus, the cdc25 species that have been found are closest to the C type, although there are several very similar isoforms (Millar *et al.*, 1991b; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). Constant amounts of cdc25-C are present throughout the cell cycle in both human somatic cells and Xenopus early embryos (Millar *et al.*, 1991a; Izumi *et al.*, 1992; Jessus and Beach, 1992; Kumagai and Dunphy, 1992). Its activity is regulated post-translationally, being increased by serine/threonine phosphorylation during mitosis (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993). Phosphorylation of cdc25-C is dependent on $p34^{cdc2}$/cyclin B itself, creating a positive feedback loop that accelerates the activation of $p34^{cdc2}$/cyclin B (Hoffmann *et al.*, 1993). The phosphatase that dephosphorylates serine/threonine residues on cdc25-C has not been characterized yet.

Evidence for a role of protein-serine/threonine phosphatases in the timing of mitosis has come from genetic and biochemical experiments. Protein phosphatase-1 (PP-1) has been shown to be required for exit from mitosis in Drosophila, Aspergillus, and fission yeast (Doonan and Morris, 1989; Ohkura *et al.*, 1989; Axton *et al.*, 1990). PP-1 might act downstream of $p34^{cdc2}$ to reverse mitotic phosphorylations and may also be involved in the inactivation of $p34^{cdc2}$/cyclin B. An additional role for PP-1 during G-2 has been inferred from experiments using fission yeast in which over-expression of the catalytic subunit of PP-1 delays entry into mitosis (Booher and Beach, 1989; Yanagida *et al.*, 1992). However, this catalytic subunit is normally complexed with regulatory subunits that direct it to specific subcellular localizations and modulate its activity towards particular substrates (Cohen, 1989). Therefore its overexpression may lead to nonspecific dephosphorylation of proteins. In a cold-sensitive mutant of a type-1 phosphatase (dis2-11) at the restrictive temperature, $p34^{cdc2}$ protein kinase activity is high and cells arrest in a defective mitotic state consistant with premature entry into mitosis (Kinoshita *et al.*, 1991). PP-1 may be involved in the suppression of $p34^{cdc2}$ protein kinase during G2, although this could be an indirect effect by acting on processes that provide checkpoint regulation of entry into mitosis. In Xenopus, injection of specific inhibitors of PP-1 into oocytes slows progesterone-induced maturation (Huchon *et al.*, 1981; Foulkes and Maller, 1982) but not maturation-promoting factor (MPF) auto-amplification (Huchon *et al.*, 1981). This suggests that PP-1 is required for the reinitiation of meiosis but not during maturation itself. Apparently conflicting results have been reported on the role of PP-1 in the regulation of $p34^{cdc2}$ protein kinase in egg extracts. Félix *et al.* (1990) have shown that complete inhibition of PP-1 by inhibitor-1 (I-1) and inhibitor-2 (I-2) proteins has no effect on the kinetics of activation of $p34^{cdc2}$ bound to endogenous cyclins, whereas Walker *et al.* (1992) found that I-2 could advance activation of this kinase when it is dependent on cyclin synthesis and is coupled to DNA replication.

Protein phosphatase-2A (PP-2A) has been shown to play a negative role in the entry into mitosis. In *S. pombe*, loss of function of one PP-2A gene results in premature mitosis. Genetic evidence also indicates an interaction with cdc25 (Kinoshita *et al.*, 1990; Yanagida *et al.*, 1992). In Xenopus, inhibition of PP-2A induces oocyte maturation (Goris *et al.*, 1989) and results in premature activation of $p34^{cdc2}$ protein kinase activity in egg extracts (Félix *et al.*, 1990). Moreover, an inhibitor of MPF activation, called INH, has been purified and found to

contain the catalytic subunit of PP-2A, the activity of which is required for INH function (Lee *et al.*, 1991).

In the work reported in this paper, we have examined the possible roles of PP-1 and PP-2A in the timing of tyrosine dephosphorylation and activation of the $p34^{cdc2}$/cyclin B protein kinase. We have used Xenopus egg extracts as a model system in which the basic cell-cycle mechanism is fully functional but relieved from checkpoint controls that occur in somatic cell cycles (Murray, 1992). However, these can be reimposed, for instance by adding nuclei to couple mitosis to the completion of S-phase (Dasso and Newport, 1990). We have used extracts without endogenous cyclins and that do not synthesise them. The kinase activity of the endogenous $p34^{cdc2}$ protein is induced by the addition of exogenously produced cyclins. This approach avoids the complication of potential effects of phosphatase activity on protein synthesis that may occur in "cycling" extracts in which the cell cycle is driven by the synthesis of endogenous cyclins. It also allows the activation of a defined cyclin-dependent protein kinase to be examined. We find that a type-2A protein phosphatase negatively regulates $p34^{cdc2}$/cyclin B protein kinase activation. This phosphatase acts at the stage of tyrosine dephosphorylation of $p34^{cdc2}$ and may be mediated through dephosphorylation and inhibition of cdc25-C. Moreover, we show that the type-2A protein phosphatase that dephosphorylates and inhibits cdc25-C is specifically repressed during mitosis. Finally, we provide evidence supporting the idea that the negative feedback control exerted by unreplicated DNA over activation of $p34^{cdc2}$/cyclin B protein kinase involves the type-2A protein phosphatase/cdc25-C pathway.

MATERIALS AND METHODS

Materials

Escherichia coli BL21 (DE3) carrying the Arbacia cyclin BΔ90 expression vector (T7Δ90, a modified T7 vector pET3b) was a gift of M. Glotzer and M. Kirschner (University of California, San Francisco). Cyclin BΔ90 was expressed and purified as described in Glotzer *et al.* (1991). Xenopus cyclin B1, a clone provided by J. Minshull and T. Hunt, was expressed as a maltose-binding protein fusion using a pMAL vector in *E. coli* TB1 (New England Biolabs, Beverly, MA). The protein was mostly soluble and was purified by affinity chromatography on amylose resin (Félix *et al.*, 1993; in preparation). Human cdc25-C cDNA (a gift of P. Russell) was inserted into a modified pGEX-2T vector which was used to transform *E. coli* DH5a cells (Hoffmann *et al.*, 1993). Expression of the 82kDa glutathione-S-transferase (GST) cdc25-C fusion protein (Smith and Johnson, 1988) was induced by isopropyl β-D-thiogalactoside (IPTG). The fusion protein was purified on glutathione sepharose beads and eluted by 10 mM glutathione in 50 mM tris(hydroxymethyl)aminomethane (Tris) HCl (pH 8.0), 50 mM NaCl, and 1 mM dithiothreitol (DTT).

Catalytic subunits of protein phosphatase-1 and protein phosphatase-2A purified from rabbit skeletal muscle (Tung *et al.*, 1984) and ^{32}P-labeled glycogen phosphorylase *a*, phosphorylase kinase, and histone H1 (the latter phosphorylated using a purified mammalian $p34^{cdc2}$ protein kinase; Langan *et al.*, 1989; Sola *et al.*, 1991) were donated by P. Cohen (University of Dundee). Recombinant inhibitor-2 (Pickering *et al.*, 1991) was donated by A.A. DePaoli-Roach (Indiana University). Okadaic acid was also a gift from P. Cohen or purchased from Gibco (Grand Island, NY). Microcystin-LR was purchased from Calbiochem (La Jolla, CA). Na vanadate, aphidicolin, and γ-S-ATP were from Sigma (St. Louis, MO). Other chemicals were from Merck (Rahway, NJ).

Preparation of Interphasic Extracts of Xenopus Eggs

Extracts of Xenopus eggs arrested in interphase were prepared by a modification of a previous method (Félix *et al.*, 1989). Eggs were dejellied with 2% (mass/vol) cysteine-HCl (pH 7.8), then washed extensively with modified Ringer MMR/4 (25 mM NaCl, 0.4 mM KCl, 0.25 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], and 25 μM EDTA, pH 7.2). Eggs were activated by an electric shock (Karsenti *et al.*, 1984), incubated at 20°C in MMR/4 plus 100 μg/ml cycloheximide for 90 min, then transferred to centrifuge tubes filled with ice-cold acetate buffer (100 mM K acetate, 2.5 mM Mg acetate, 60 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 5 μg/ml cytochalasin D, and 1 mM dithiothreitol, pH 7.2). Subsequent steps were performed at 4°C. Excess buffer was removed and the eggs crushed by centrifugation at 10 000 × *g* for 10 mins. The cytoplasmic material between the upper yellow lipid layer and the lower yolk was collected and an ATP-regenerating system (final concentrations of 10 mM creatine phosphate, 80 μg/ml creatine kinase, 1 mM ATP) added. In some experiments, this cytoplasmic fraction was used ("10 000 × *g* extracts"). To prepare extracts without heavy membranes ("100 000 × *g* extracts"), the cytoplasmic fraction was further centrifuged at 100 000 × *g*, the supernatant collected, aliquoted, frozen, and stored in liquid nitrogen. These extracts contained ~40 mg protein/ml.

Incubation of Cyclins in Egg Extracts and Histone H1 Kinase Assays

Cyclins diluted to 10 μM in buffer A (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 10 μg/ml leupeptin) were added in 1/10 vol to interphasic extracts devoid of endogenous cyclin to give a final concentration of 1 μM. When phosphatase inhibitors were used, they were also added in 1/10 vol and the extract preincubated for 15 min before cyclin addition. Incubations were carried out at 23°C, and samples for kinase assays, typically 1 μl, were removed into 20 vols of Extraction Buffer (80 mM β-glycerophosphate [pH 7.3], 15 mM $MgCl_2$, 20 mM EGTA, 1 mM dithiothreitol, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride [PMSF] plus 2 mM Na vanadate. Histone H1 kinase assays were performed essentially as in Félix *et al.*, 1989 with the use of exogenous histone H1 (type IIIS from calf thymus, Sigma).

Isolation of kinase molecules from Xenopus egg extracts using $p13^{suc1}$-Sepharose beads, assay of associated histone H1 kinase and detection of kinase molecules or phosphotyrosine by Western blotting was carried out as described in Clarke *et al.* (1992). A mouse monoclonal antibody against the PSTAIRE sequence of $p34^{cdc2}$ or a specific antibody against phosphotyrosine were used (gifts of M. Yamashita and G. Peaucellier, respectively).

Incubation of cdc25-C in Extracts

Human cdc25-C diluted in buffer A was added to extracts together with cyclins and phosphatase inhibitors in 1/2 vol to give a final concentration of 0.02 μg/μl, except in the experiment shown in Figure 8, where cdc25-C was added in an equal volume to give a concentration of 0.035 μg/ml. Incubations were carried out at 23°C and samples containing ~0.02 μg of cdc25-C were removed into gel sample buffer [80 mM Tris/HCl (pH 6.8), 5% (mass/vol) sodium dodecyl sulfate (SDS), 50 mM Na fluoride, 5 mM EDTA, 4 mM Na vanadate, 10% (by vol) 2-mercaptoethanol]. Detection by western blotting was carried out as described in Clarke *et al.* (1992) except for the following modifications. SDS-gel electrophoresis of samples was carried out using

10% acrylamide gels with prestained molecular mass marker proteins (Biorad, Richmond, VA; low molecular mass range). Electrophoresis was continued until the 32.5 kDa marker was almost at the bottom of the gel. After transfer to nitrocellulose membranes and blocking of the membrane (Clarke *et al.*, 1992), detection was carried out by using anti-cdc25-C C-terminal peptide serum IH37 (1/1000 dilution), followed by protein A-horseradish peroxidase complex (Amersham, Arlington Height, IL; 1/2000) and developed using a chemiluminescent method (Amersham ECL) according to the manufacturers instructions.

Protein Phosphatase-1 and Protein Phosphatase-2A Assays

Activities of PP-1 and PP-2A were assayed by using glycogen phosphorylase *a* and phosphorylase kinase as substrates with the use of established methods (Cohen *et al.*, 1988). The inhibitory activities of inhibitor-2, okadaic acid, and microcystin-LR were standardized using the catalytic subunits of protein phosphatase-1 and -2A, with phosphorylase *a* as substrate. This was particularly important for okadaic acid because we have found that some other commercial supplies are much less potent. By using catalytic subunits at <0.1 U/ml, inhibitor-2 completely blocked PP-1 activity at 0.1 μM but did not affect PP-2A activity. Okadaic acid (5 nM) completely inhibited PP-2A but did not affect PP-1, whereas 1 μM okadaic acid blocked both PP-1 and PP-2A. Microcystin-LR inhibited PP-1 and PP-2A with similar potency; 10 nM completely blocked both. These potencies conform to previous characterizations (Cohen *et al.*, 1989; MacKintosh *et al.*, 1990; Cohen, 1991).

Unless otherwise stated, assays of phosphatase activity in egg extracts were carried out at 1/100 dilution in phosphatase buffer (50 mM Tris/HCl [pH 7.0 at 20°C], 2 mM EGTA, 2 mM EDTA, 0.1% (by vol), 2-mercaptoethanol, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM benzamidine, and 0.5 mM PMSF) and by using phosphorylase *a* as substrate. Incubation was for 10 min at 23°C, during which time the reaction rate was linear. PP-1 activity was taken as that inhibited by 2 μM inhibitor-2 or that inhibited by 5 μM okadaic acid but not 5 nM okadaic acid. PP-2A activity was taken as that inhibited by 5 nM okadaic acid, or inhibited by 5 μM okadaic acid but not 2 μM inhibitor-2. In both cases, assessment by either measure agreed within 10%. PP-1c and PP-2Ac were also assayed using phosphorylase *a*. 1 unit (U) of phosphatase activity catalyses the release of 1 nmol of phosphate/min.

PP-1 and PP-2A activities in concentrated egg extracts (diluted by addition of 1/2 vol of buffer as in experiments examining the dephosphorylation of cdc25-C) were assessed by the dephosphorylation of phosphorylase kinase β and α subunits, respectively. In this case very much higher concentrations of inhibitors were required to completely block phosphatase activity. Inhibitor-2 blocked β-subunit dephosphorylation (PP-1) by only 10% at 0.25 μM, by ~50% at 2.5 μM and by >90% at 10 μM. There was no effect of inhibitor-2 on α-subunit dephosphorylation (PP-2A). Okadaic acid cannot be used to inhibit PP-2A completely specifically in such concentrated extracts, but at 0.2 μM, α-subunit dephosphorylation (PP-2A) was inhibited by ~30% and β-subunit dephosphorylation (PP-1) was not affected. At 0.4 μM okadaic acid, α-subunit dephosphorylation (PP-2A) was inhibited by ~80% and β-subunit dephosphorylation (PP-1) was inhibited by <10%. At 1.0 μM okadaic acid, α-subunit dephosphorylation (PP-2A) was inhibited by >90% and β-subunit dephosphorylation (PP-1) was inhibited by ~80%. At 2 μM okadaic acid or 2 μM microcystin-LR, both α- and β-subunit dephosphorylation was inhibited by >90% (see also Félix *et al.*, 1990).

Phosphorylation of cdc25-C by $p34^{cdc2}$/Cyclin B

Human GST-cdc25-C was phosphorylated by an immunoprecipitate of cdc2/cyclin B from mitotic Hela cell extracts in the presence of 10 mM $MgCl_2$ and 1 mM ATP for 30 min at 23°C (Hoffmann *et al.*, 1993). The phosphorylated protein was recovered as described in Smith and Johnson (1988) on glutathione Sepharose beads and either used directly in phosphatase assays or eluted by 10 mM glutathione in 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT. Thiophosphorylated cdc25-C was prepared in the same way except that 10 mM γ-S-ATP was used instead of ATP.

Activation of $p34^{cdc2}$/cyclin B by cdc25-C

To prepare the substrate for cdc25-C, $p34^{cdc2}$/cyclin B was immunoprecipitated using anti-cyclin B antibodies from Hela cells that were arrested in S-phase by treatment with 10 mM hydroxyurea for 14 h. Under these conditions, $p34^{cdc2}$ complexed with cyclin B was phosphorylated on tyrosine and was inactive (Pagano *et al.*, 1992; Hoffmann, unpublished observations). The immunoprecipitate was incubated with cdc25-C for 15 min at 30°C in Tris/HCl (pH 8.0), 1 mM DTT, and the activity of the kinase measured by phosphorylation of histone H1 as described in Hoffmann *et al.* (1993).

Formation of Nuclei in Egg Extracts

Demembranated Xenopus sperm nuclei were prepared as described by Philpott *et al.* (1991) and stored at −70°C. Concentration was determined by counting with a hemocytometer. Sperm nuclei were added in 1/10 final volume to 10 000 × *g* extracts and incubation carried out at room temperature. When used, aphidicolin was added at the start of the incubation from a stock solution of 10 mg/ml in dimethyl sulfoxide (DMSO) to give a final concentration of 50 μg/ml. Aliquots were removed at 10-min intervals and diluted with an equal volume of Hoechst buffer [15 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) (pH 7.4), 200 mM sucrose, 7 mM $MgCl_2$, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 3.7% formaldehyde, 20 μg/ml Hoechst 33258 DNA dye]. Chromatin decondensation was examined by fluorescence microscopy and nuclear membrane formation by phase contrast. Typically, complete decondensation and nuclear envelope formation was observed after ~60 min. Some extracts failed to support the formation of complete nuclei and were discarded.

RESULTS

Activation of $p34^{cdc2}$/Cyclin B Protein Kinase is Negatively Regulated by a Type-2A Protein Phosphatase

In a previous study, Félix *et al.* (1990) found that a type-2A protein phosphatase was responsible for the suppression of activation of histone H1 kinase in 100 000 × *g* egg extracts containing subthreshold levels of cyclin molecules. This conclusion was based on the use of protein phosphatase inhibitors: okadaic acid induced $p34^{cdc2}$ protein kinase activation at a concentration that blocked PP-2A but only partially inhibited PP-1, whereas complete inhibition of PP-1, using I-1 and I-2, did not activate $p34^{cdc2}$ protein kinase. Here, we have performed similar experiments but using extracts devoid of endogenous cyclins, $p34^{cdc2}$ protein kinase being activated by the addition of bacterially produced cyclin B. Both 10 000 × *g* and 100 000 × *g* extracts were used, although we obtained very similar results with either type of extract. Initially a truncated B-type cyclin from sea urchin (*Arbacia*), denoted cyclin BΔ90 was used, since the mechanism of activation of $p34^{cdc2}$ by this cyclin has been previously characterized in detail. Cyclin BΔ90 lacks the "destruction box" sequence required for degradation and is stable in mitotic extracts (Glotzer *et*

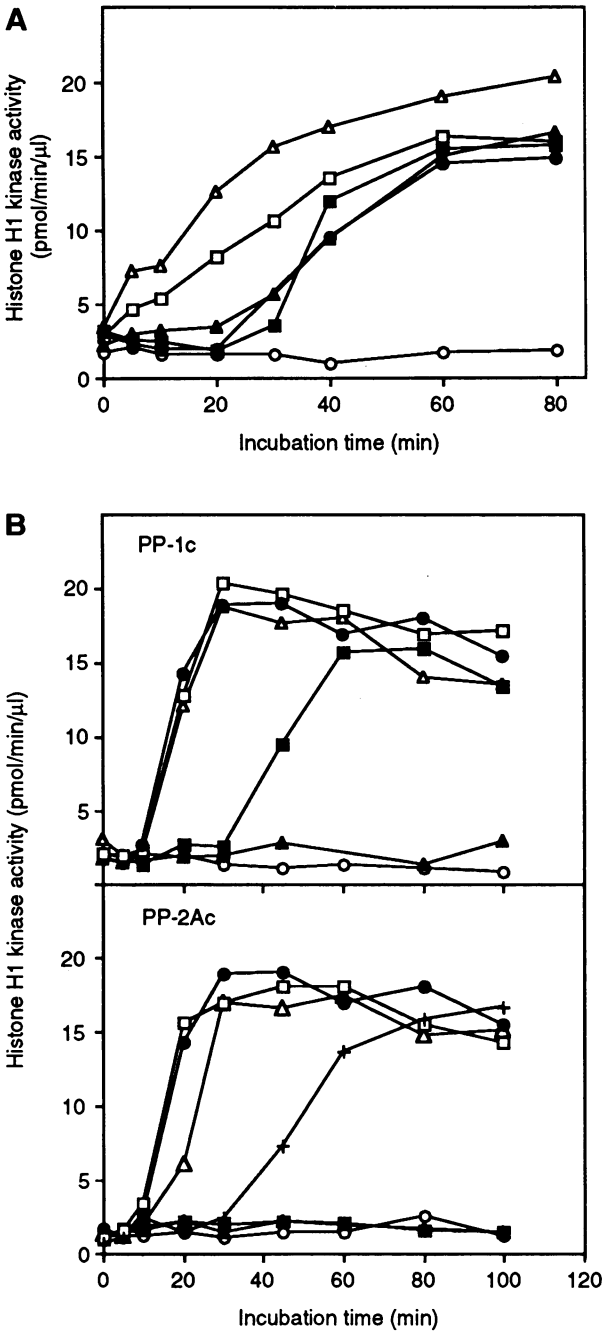

Figure 1. Regulation of the activation of $p34^{cdc2}$/cyclin BΔ90. (A) Premature activation of $p34^{cdc2}$/cyclin BΔ90 by okadaic acid but not inhibitor-2. Cyclin BΔ90 was added at 1 μM to an interphasic 10 000 × *g* extract. Incubations carried out in the absence of protein phosphatase inhibitors (●), with addition of 0.4 μM okadaic acid (□), 2 μM okadaic acid (△), 2 μM inhibitor-2 (■), or 10 μM inhibitor-2 (▲). An incubation without cyclin BΔ90 (○) is also shown. Very similar results were obtained by using 100 000 × *g* extracts. (B) Effect of the addition of purified catalytic subunits of protein phosphatase-1 (PP-1c, top) and protein phosphatase-2A (PP-2Ac, bottom) on $p34^{cdc2}$/cyclin BΔ90 activation. Phosphatases were added to 100 000 × *g* extracts containing 1 μM cyclin BΔ90. Control incubations in the absence of added phosphatases were carried out with (●) or without (○) cyclin BΔ90. PP-2Ac activities added to the extracts were 50 (▲), 37.5 (■), 25 (crosses), 12.5 (△) and 5 (□) U/ml. PP-1c activities added were 1400 (▲), 700 (■), 350 (△), 175 (□) U/ml. Activities of PP-1 and PP-2A in the extracts were 9.9 and 17.9 U/ml, assayed at 1/100 dilution.

al., 1991). Activation of $p34^{cdc2}$ by this cyclin normally occurs after a lag period during which $p34^{cdc2}$ is inhibited by tyrosine phosphorylation and requires a threshold concentration of cyclin to be exceeded (Solomon *et al.*, 1990; Clarke *et al.*, 1992; Figure 1A). Similar to previous observations, okadaic acid at concentrations that inhibited PP-2A caused a premature activation of $p34^{cdc2}$/cyclin BΔ90 (Figure 1A). In contrast, inhibitor-2, which specifically inhibits PP-1, had little or no effect on $p34^{cdc2}$/cyclin BΔ90 activation at concentrations that completely blocked PP-1 activity in the extract.

We also examined the effect on $p34^{cdc2}$/cyclin B activation of increasing the activity of PP-1 or PP-2A in the extracts through addition of purified phosphatase catalytic subunits (denoted PP-1c and PP-2Ac, respectively). Addition of PP-2Ac to extracts in a twofold or greater excess, over the activity of the native endogenous enzyme, completely blocked the activation of $p34^{cdc2}$/cyclin BΔ90. Addition of an activity, 1.4-fold greater than that already present in the extract, lengthened the lag period, although normal levels of kinase activity were eventually achieved (Figure 1B). At lower activities, PP-2Ac had no effect on $p34^{cdc2}$/cyclin BΔ90 activation. PP-1c could produce a very similar effect on the kinetics of kinase activation, although much higher activities were required: addition of a 140-fold excess of PP-1 activity over that present in the extract completely blocked $p34^{cdc2}$/cyclin BΔ90 activation. A 70-fold excess produced a delay in activation, but lower amounts did not affect kinase activation (Figure 1B). The catalytic subunits were active in the extracts, as judged by assay after their addition.

Taken together, these results suggest that a type 2A protein phosphatase suppresses $p34^{cdc2}$/cyclin B protein kinase activation in egg extracts, although over expression of a type 1 catalytic subunit can also suppress kinase activation, probably by nonspecific phosphatase activity.

Activation of $p34^{cdc2}$/Cyclin B Protein Kinase is Suppressed by a Type-2A Protein Phosphatase at the Stage of Tyrosine Dephosphorylation

We have produced full length Xenopus cyclins in a soluble form in *E. coli* as fusion proteins with the maltose-binding protein. We have previously shown that addition of Xenopus cyclin A to egg extracts resulted in the activation of $p34^{cdc2}$ without any lag or threshold requirement, consistent with the low level of tyrosine phosphorylation observed on $p34^{cdc2}$ when it is associated with cyclin A (Clarke *et al.*, 1992). Xenopus cyclin B1 added to interphasic egg extracts also interacted with $p34^{cdc2}$ but efficiently induced tyrosine phosphorylation

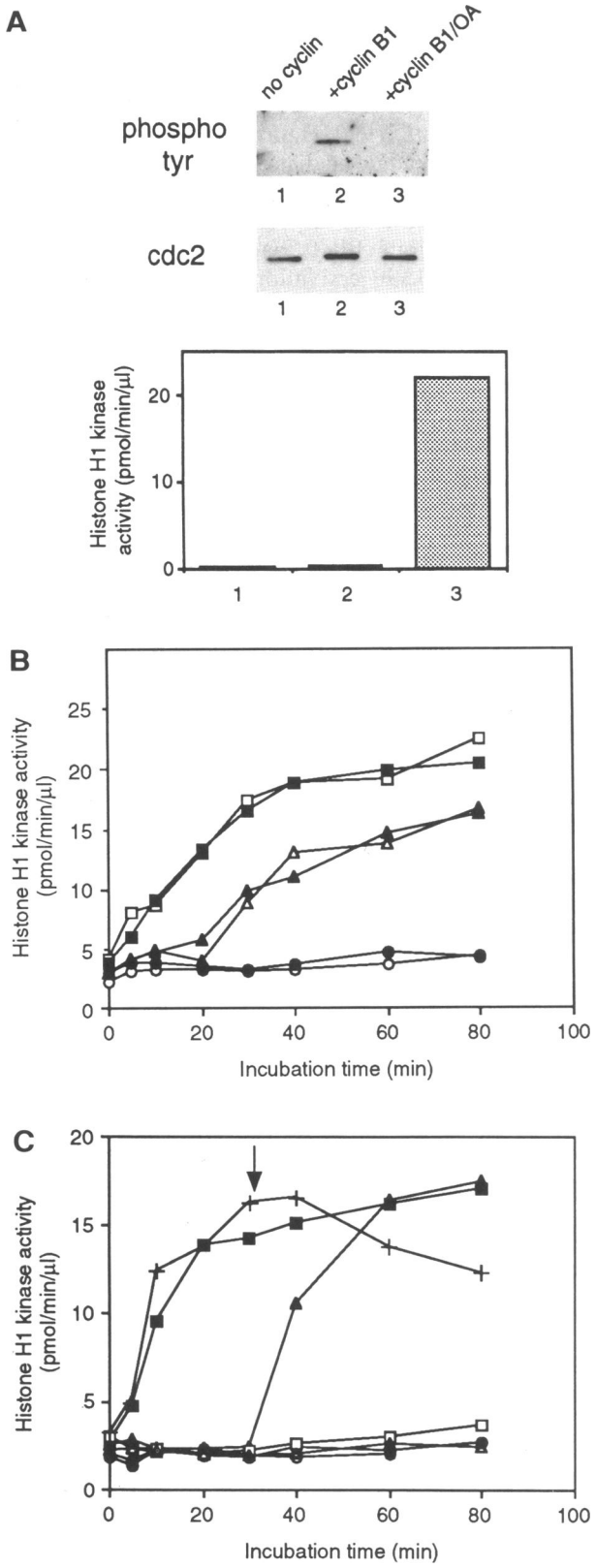

Figure 2. Activation and tyrosine dephosphorylation of $p34^{cdc2}$ complexed to Xenopus cyclin B1 in egg extracts treated with okadaic acid. (A) Detection of phosphotyrosine (top) and $p34^{cdc2}$ (middle) by antibodies after partial purification on $p13^{suc1}$ beads and western blotting. $p34^{cdc2}$ was detected by using an anti-PSTAIRE sequence antibody; on longer exposure, the less abundant $p33^{cdk2}$ was also apparent. Kinase activity on the beads was also measured (bottom). Incubations were carried out for 60 min without cyclin (lane 1), with cyclin B1 (lane 2), or cyclin B1 plus 1 μM okadaic acid (lane 3). (B) Kinase activity during incubation of extracts after addition of cyclin B1. Incubations contained no added phosphatase inhibitors (○), 10 μM inhibitor-2 (●), 0.2 μM okadaic acid (△), 0.2 μM okadaic acid plus 10 μM inhibitor-2 (▲), 1 μM okadaic acid (□), and 1 μM okadaic acid plus 10 μM inhibitor-2 (■). Other incubations containing 0.1 μM or less okadaic acid, with or without 10 μM inhibitor-2, or without cyclin B1 also did not show any kinase activation and are omitted for clarity. (C) Effect of vanadate ions on the activation of $p34^{cdc2}$/cyclin B1 in response to okadaic acid. 0.4 μM okadaic acid was added to extracts immediately before (○, ●) or 30 min after (△, ▲) addition of cyclin B1, with (open symbols) or without (closed symbols) preincubation of the extract for 30 min with 4 mM Na vanadate. Vanadate was also added 30 min after addition of cyclin B1 and okadaic acid (crosses). Cyclin B1 alone was added to one incubation (○).

and a decrease in mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2A). However, spontaneous activation of the kinase activity of $p34^{cdc2}$ did not occur even upon prolonged incubation, unlike $p34^{cdc2}$ bound to sea urchin cyclin BΔ90 (Figure 2 and Hoffmann *et al.*, 1993). We do not yet understand the precise reasons for these differences, but they are probably due to differences in the affinities of the tyrosine kinase and cdc25 phosphatase for $p34^{cdc2}$ when complexed to different cyclins. The Xenopus cyclin B1 is functional because it activates the kinase efficiently in extracts supplemented with active cdc25-C (Hoffmann *et al.*, 1993) and in extracts containing low levels of mitotic cyclins (Clarke, unpublished observations). Its behavior is not affected by the maltose binding protein fused to the cyclin B1 molecule because we have obtained similar results after removal of this protein from cyclin B1 by specific proteolytic cleavage. Nevertheless, this cyclin provides a useful tool to investigate the final stage of tyrosine dephosphorylation and activation of $p34^{cdc2}$ in egg extracts.

We investigated the effect of protein phosphatase inhibitors on the activation of the $p34^{cdc2}$/cyclin B1 complex. In these undiluted extracts, much higher concentrations of inhibitors are required to inhibit protein phosphatases than in dilute solutions (see METHODS). In extracts to which 1 μM okadaic acid had been added, cyclin B1 produced a rapid activation of $p34^{cdc2}$ protein kinase without any detectable lag (Figure 2B). Almost no tyrosine phosphate was detected in $p34^{cdc2}$, in contrast to the phosphorylation induced by cyclin B1 in the absence of okadaic acid (Figure 2A). Reducing the concentration of okadaic acid in the extracts to 0.2 μM resulted in a lag before kinase activation (Figure 2B). At this concentration of okadaic acid, type-2A phosphatase activity is partially inhibited, but type-1 activity is not affected (see METHODS). At 0.1 μM or less okadaic

acid, no kinase activation was observed. Preincubation of the extracts with inhibitor-2 did not elicit any activation of $p34^{cdc2}$/cyclin B1, even at concentrations sufficiently high to completely block PP-1 activity (≤37 μM was tested with no effect on kinase activation). When inhibitor-2 was added in addition to okadaic acid, no alteration of the kinetics of activation occurred. These results indicate that the tyrosine dephosphorylation and activation of $p34^{cdc2}$/cyclin B1 is suppressed by the activity of protein phosphatase that is partially inhibited at 0.2 μM okadaic acid, more potently inhibited at 1 μM but is not sensitive to inhibitor-2. This is consistent with it being a type-2A protein phosphatase. They also suggest that type-1 protein phosphatase activity blocked by inhibitor-2 does not affect this stage of $p34^{cdc2}$/cyclin B1 activation.

To determine at which stage in the activation of $p34^{cdc2}$/cyclin B the type-2A protein phosphatase was acting, we did the following experiments. First, cyclin B1 was incubated in an extract for 30 min to ensure that binding to $p34^{cdc2}$ was complete (Figure 2C). After this time, an inactive $p34^{cdc2}$/cyclin B1 complex was formed in which $p34^{cdc2}$ was phosphorylated on Tyr15 and probably also on Thr14 and Thr161 (Figure 2B, see also Solomon *et al.*, 1992). Addition of okadaic acid after incubation for 30 min resulted in immediate activation of the kinase (Figure 2C), showing that okadaic acid was affecting a step subsequent to the binding of cyclin and phosphorylation of $p34^{cdc2}$. We then examined the effect of adding 2 mM vanadate ions (sufficient to inhibit cdc25 in egg extracts; Clarke *et al.*, 1992). Incubation of the extracts with vanadate, before addition of cyclin B1, completely blocked the activation normally observed after okadaic addition. This was true both when okadaic acid was added immediately before or 30 min after cyclin B1 (Figure 2C). These results indicate that a type-2A protein phosphatase inhibits the activation of $p34^{cdc2}$/cyclin B1 at the stage of tyrosine dephosphorylation by cdc25.

Dephosphorylation of cdc25-C in Egg Extracts Requires the Activity of a Type 2A Protein Phosphatase

It has been shown recently that cdc25-C is activated by phosphorylation at the onset of mitosis and that this phosphorylation is required for $p34^{cdc2}$/cyclin B protein kinase activation. However, phosphorylated cdc25-C is rapidly dephosphorylated in interphasic egg extracts, this event being associated with an apparent decrease in molecular mass of ~15 kDa. Dephosphorylation can be prevented by addition of microcystin or okadaic acid to the extracts (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993). This suggested that suppression of $p34^{cdc2}$/cyclin B activation in interphasic extracts could be due to dephosphorylation and inactivation of cdc25-C. In vitro, purified catalytic subunits of both phosphatase-1 and phosphatase-2A can dephosphorylate cdc25-C (Izumi *et al.*, 1992; Clarke, unpublished observations), although in the absence of regulatory subunits this could be due to nonspecific activity. We therefore examined more closely the nature of the native phosphatases responsible for cdc25-C dephosphorylation in extracts. When phosphorylated cdc25-C (Figure 3A, lane 6) was incubated in an interphasic extract for 30 mins, the protein was dephosphorylated and shifted down on SDS-PAGE to the position of the unphosphorylated protein (Figure 3A, lane 1). In the presence of 0.4 or 2 μM okadaic acid, this down-shift was completely blocked (Figure 3A; 2 μM microcystin-LR was as effective). In contrast, inhibition of PP-1 by inhibitor-2 protein failed to block cdc25-C dephosphorylation even at 10 μM inhibitor-2 (Figure 3A). When several time points were examined between 0 and 30 min of incubation, no decrease in the rate of dephosphorylation in the presence of inhibitor-2 over interphasic extracts was observed. Identical results were obtained using 10 000 × *g* extracts (Figure 3) or 100 000

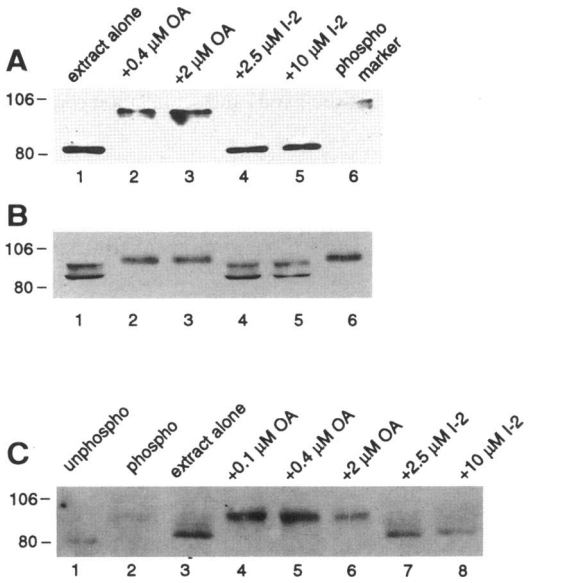

Figure 3. Effect of okadaic acid and inhibitor-2 on the dephosphorylation of cdc25-C in concentrated interphasic extracts of Xenopus eggs (A), of exponentially growing Hela cells (B), or interphasic Xenopus egg extracts diluted 1/10 in buffer containing EDTA (C). Human cdc25-C, phosphorylated in extracts of mitotic Hela cells, was added to extracts at 0.4 μM final concentration. After 30-min incubation at 23°C, gel sample buffer was added and the phosphorylation state of cdc25-C was examined by SDS-PAGE and western blotting (see METHODS). In (A) and (B), incubations were performed without further additions (lane 1), the addition of 0.4 μM (lane 2) or 2 μM (lane 3) okadaic acid, or 2.5 μM (lane 4) or 10 μM inhibitor-2 (lane 5). Phosphorylated cdc25-C was also incubated in the absence of extracts (lane 6). In (C) lanes show unphosphorylated cdc25-C (1), phosphorylated cdc25-C incubated without extract (2), cdc25-C incubated with 1/10 diluted egg extract alone (3), plus 0.1 μM, (lane 4), 0.4 μM (lane 5) or 2 μM (lane 6) okadaic acid, or 2.5 μM (lane 7) or 10 μM inhibitor-2 (lane 8).

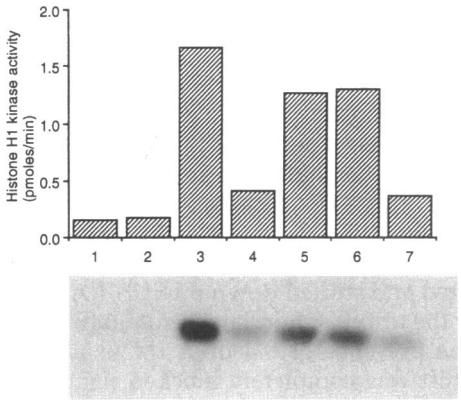

Figure 4. Activation of cdc25-C by direct phosphorylation by $p34^{cdc2}$/cyclin B, and inactivation in egg extracts. Tyrosine phosphorylated and inactivated $p34^{cdc2}$/cyclin B was immunoprecipitated from Hela cells blocked in early S-phase by hydroxyurea treatment. Histone H1 kinase activity was assayed without further treatment (column 1) or after incubation with unphosphorylated cdc25-C (2) or cdc25-C phosphorylated by active $p34^{cdc2}$/cyclin B immunoprecipitated from mitotic Hela cells (3). In conditions 4–7, the reactivated cdc25-C was further incubated for 30 min with 1/10 dilution of Xenopus egg extract without phosphatase inhibitors (4), plus 0.4 μM (5) or 5 μM okadaic acid (6), or plus 5 μM inhibitor-2 (7) before reisolation of cdc25-C and assay of ability to activate tyrosine phosphorylated $p34^{cdc2}$/cyclin B. In the bottom panel, the autoradiograph after separation of histones on SDS-PAGE is shown; the top diagram shows the kinase activities quantified by excision of the bands and scintillation counting.

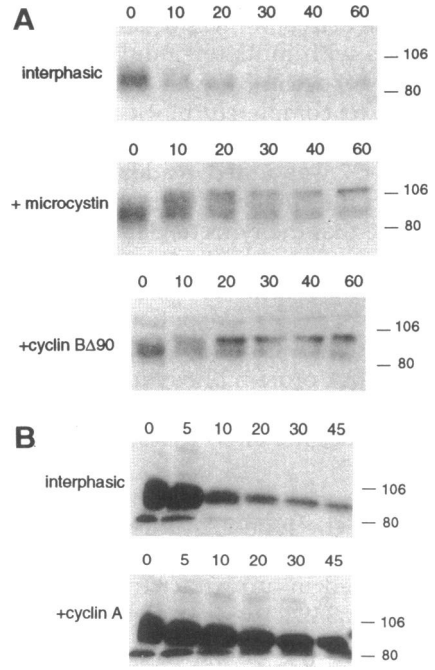

Figure 5. Rate of dephosphorylation of cdc25-C in Xenopus egg extracts. ^{32}P-labeled phosphorylated cdc25-C was prepared by phosphorylation with active $p34^{cdc2}$/cyclin B immunoprecipitates. After purification, labeled cdc25-C was incubated in Xenopus egg extracts under various conditions. At the times indicated (min) aliquots were removed into gel sample buffer and analysed by SDS-PAGE and autoradiography. In (A) incubations were carried out without further additions (interphasic, top), plus 5 μM microcystin-LR (middle), plus 1 μM cyclin BΔ90 (bottom). In a separate experiment (B), incubations were carried out without further additions or plus 1 μM cyclin A.

$\times$ *g* extracts. Inhibition of the dephosphorylation of cdc25-C was not simply due to the activation of an opposing kinase activity, because very similar results were obtained with the use of extracts that were diluted in a buffer containing EDTA to inhibit such activity. In this case 0.1 μM okadaic acid was sufficient to completely block cdc25-C dephosphorylation (Figure 3B). Because we used a human cdc25-C protein in these experiments, we verified that this result was also valid in an extract of exponentially growing Hela cells (Figure 3C). In this extract, the down-shift of cdc25-C was also very clear although not as extensive as in the interphasic Xenopus egg extract. Nevertheless, the down-shift was completely blocked by 0.4 or 2 μM okadaic acid, whereas inhibitor-2 had no effect. These results demonstrate that the phosphatase activity that dephosphorylates cdc25-C in interphase is entirely due to an okadaic acid-sensitive but inhibitor-2-insensitive activity, that is a type-2A protein-serine/threonine phosphatase.

Regulation of cdc25-C Activity by $p34^{cdc2}$/Cyclin B and a Type-2A Protein Phosphatase

To confirm the effect of phosphorylation/dephosphorylation on the activity of cdc25-C used in these experiments, unphosphorylated cdc25-C was first incubated with active $p34^{cdc2}$/cyclin B that had been immunoprecipitated from mitotic cells. Active $p34^{cdc2}$/cyclin B was then removed by centrifugation and cdc25-C was bound through its GST tag to glutathione beads. We then assayed cdc25-C activity by using tyrosine phosphorylated and inactive $p34^{cdc2}$/cyclin B as a substrate. Phosphorylated cdc25-C produced a dramatic increase (>10-fold) in the activity of $p34^{cdc2}$/cyclin B (Figure 4), whereas unphosphorylated cdc25-C produced little or no activation. The activation of cdc25-C by phosphorylation was reversed by dephosphorylation in interphasic egg extracts. This inactivation of cdc25-C was inhibited by 0.4 or 5 μM okadaic acid but not by 5 μM I-2, indicating that it was mostly due to dephosphorylation by a type-2A protein phosphatase.

Dephosphorylation of cdc25-C in Egg Extracts is Specifically Inhibited by Either $p34^{cdc2}$/Cyclin A or $p34^{cdc2}$/Cyclin B Protein Kinases

We also examined whether the activity of the type-2A protein phosphatase that dephosphorylated cdc25-C was regulated during the cell cycle. ^{32}P-labeled cdc25-C was prepared by phosphorylation using $p34^{cdc2}$/cyclin B (Figure 5). Upon incubation in interphasic extracts there was a rapid loss of ~90% of the radioactivity from cdc25-C within 10 min (Figure 5A). There was no

loss of the protein as judged by detection with antibodies on western blots. In the presence of 1 μM microcystin, the radioactive label was stable, and there was also an increase in the overall level of phosphorylation indicated by an up-shift of the protein on SDS-PAGE. Very similar results were obtained by using 0.4 μM okadaic acid. In extracts arrested in mitosis using cyclin BΔ90 but without the addition of phosphatase inhibitors, there was also an overall increase in phosphorylation of cdc25-C. In addition, and most interestingly, loss of radioactive label from cdc25-C was almost completely prevented. Activation of $p34^{cdc2}$ by addition of cyclin A to extracts also inhibited the dephosphorylation of cdc25-C (Figure 5B). Inhibition of phosphatase activity towards cdc25-C might have coincided with a general inhibition of phosphatase activity in the extracts when $p34^{cdc2}$ protein kinase was activated. To examine this point we assayed protein phosphatase activity in extracts using other phosphatase substrates (Figure 6). Assays were also carried out after addition of inhibitor-2 and okadaic acid to assess the contribution of type-1 and type-2A protein phosphatases (see METHODS). Activation of $p34^{cdc2}$ protein kinase by cyclin A or cyclin BΔ90 did not affect phosphatase activity towards glycogen phosphorylase *a* assayed in 100-fold diluted (Figure 6A) or in concentrated extracts (Figure 6B). Similarly, there was no effect of $p34^{cdc2}$/cyclin BΔ90 protein kinase activation on phosphatase activity towards histone H1 (phosphorylated by $p34^{cdc2}$ protein kinase) assayed in 100-fold (Figure 6C) or 10-fold (Figure 6D) diluted extracts. In addition, no significant effect of kinase activation on phosphatase activity towards phosphorylase *a* or histone H1 was observed when PP-1 or PP-2A were selectively inhibited using I-2 and okadaic acid.

Inhibition of $p34^{cdc2}$/Cyclin B1 Activation by Unreplicated DNA Involves a Type-2A Protein Phosphatase

Although we did not find a role for a type-1 protein phosphatase in the control of the final stage of $p34^{cdc2}$/cyclin B activation, we also wished to investigate whether it could have a role "upstream", such as in the negative control exerted on tyrosine dephosphorylation and activation of $p34^{cdc2}$/cyclin B by unreplicated DNA in somatic cell cycles. We therefore examined the effect of nuclei on the activation of $p34^{cdc2}$/cyclin B protein kinase in our extracts. Incubation of demembranated Xenopus sperm chromatin in 10 000 × *g* extracts resulted in the formation of intact nuclei. When nuclei were added at a concentration of 1000/μl, activation of the $p34^{cdc2}$/cyclin BΔ90 protein kinase was delayed by ~30 min, and when DNA replication was also inhibited by aphidicolin, kinase activation was completely blocked (Figure 7A). This block was released by okadaic acid treatment, which produced a rapid activation of $p34^{cdc2}$/cyclin BΔ90 without any lag (Figure 7B), similar to that observed in the absence of unreplicated DNA. Microcystin had a very similar effect to okadaic acid (see Figure 8). Inhibition of type-1 phosphatase activity by inhibitor-2 did not induce $p34^{cdc2}$/cyclin BΔ90 activation, even when very high concentrations of inhibitor-2 were used (Figure 7B). These results indicate that a type-2A protein phosphatase is involved in the negative control

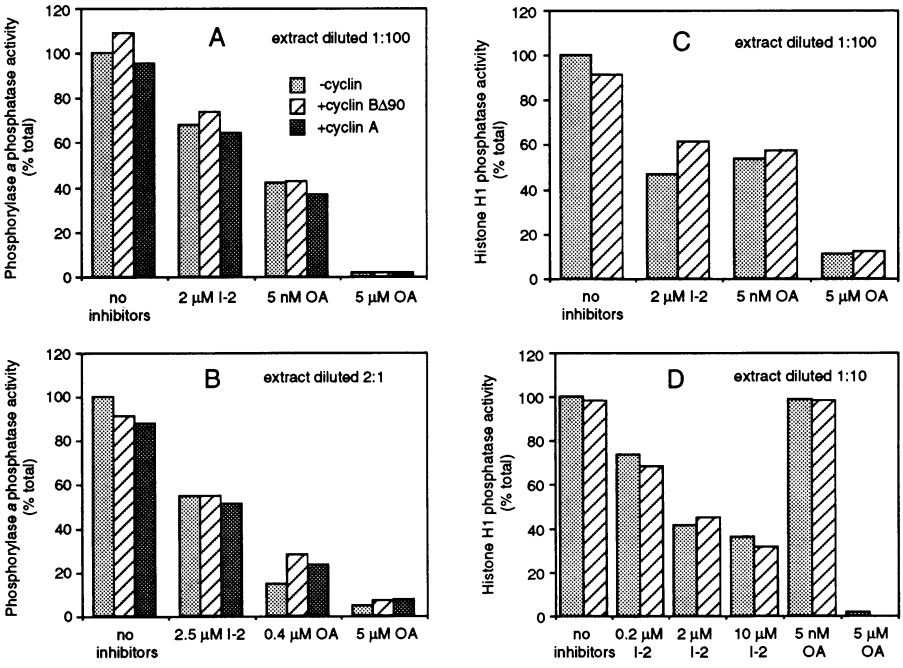

Figure 6. Effect of activation of $p34^{cdc2}$ by cyclin A or cyclin BΔ90 on phosphorylase a phosphatase (A and B) or histone H1 phosphatase (C and D) activity in egg extracts. Extracts (100 000 × *g*) were incubated for 60 min with the addition of cyclins or buffer. Samples were removed for assay of histone H1 kinase activity, which was 2.3 (no cyclin), 21.3 (plus cyclin BΔ90), and 24.7 (plus cyclin A) pmol · min^{-1} · μl^{-1} in (A) and (B), and 1.9 (no cyclin) and 19.8 (plus cyclin BΔ90) in (C) and (D). Phosphatase activity was assayed at 23°C in 1:100 (A and C), 1:10 (D), or 2:1 final dilutions of extracts. The substrate concentrations used were 3.3 μM phosphorylase *a* (A and B), 2 μM histone H1 (C), or 10 μM histone H1 (D). Activities are expressed as percentage of total activity in extracts without cyclins, which were 35.4 (A), 2.7 (B), 16.3 (C), and 26.05 U/ml (D).

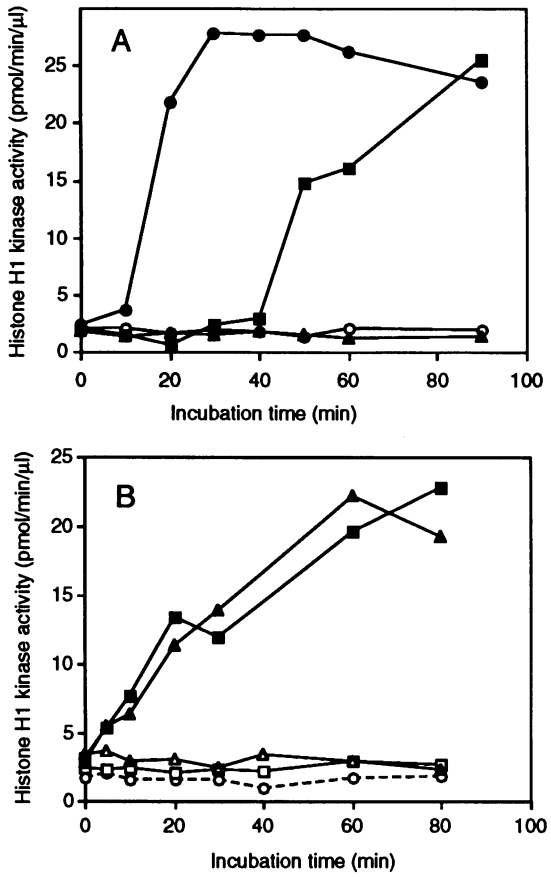

Figure 7. Effect of okadaic acid and inhibitor-2 on the block imposed on $p34^{cdc2}$/cyclin B activation by unreplicated DNA. (A) Demembranated Xenopus sperm nuclei (1000/µl) were incubated at 23°C in interphasic Xenopus egg extracts prepared as 10 000 × *g* supernatants with (▲) or without (■) 50 µg/ml aphidicolin. Incubations were also carried out without chromatin (○, ●). After 30 min, 1 µM cyclin BΔ90 was added (closed symbols) except to a control incubation (○). At the times shown, aliquots were removed for histone H1 kinase assay. In a similar experiment (B), after incubation of chromatin in extracts plus aphidicolin for 30 min, 0.4 µM (▲) or 2 µM (■) okadaic acid, or 2.5 µM (△) or 10 µM (□) inhibitor-2 were added to the extracts and incubation continued for a further 15 min before addition of cyclin BΔ90. A control incubation without phosphatase inhibitors (○) is also shown.

of $p34^{cdc2}$/cyclin B protein kinase activation by unreplicated DNA.

Stable Thiophosphorylation of cdc25-C Overcomes the Block on $p34^{cdc2}$/Cyclin B Activation by Unreplicated DNA

We also studied the effect of human cdc25-C in different phosphorylation states on the block of $p34^{cdc2}$/cyclin BΔ90 activation by unreplicated DNA. Addition of 0.4 µM unphosphorylated cdc25-C (a concentration similar to that estimated for the endogenous cdc25-C; Clarke, unpublished observations) to extracts containing cyclin BΔ90 and nuclei in the presence of aphidicolin did not overcome the block on $p34^{cdc2}$/cyclin BΔ90 activation (Figure 8). Conventionally phosphorylated cdc25-C added at the same concentration also did not activate the kinase. However, thiophosphorylated cdc25-C successfully released $p34^{cdc2}$/cyclin BΔ90 activation from suppression by unreplicated DNA. We have also obtained very similar results when examining the ability of cdc25-C to induce oocyte maturation or activation of $p34^{cdc2}$ complexed to Xenopus cyclin B1 in the absence of nuclei (Hoffmann *et al.*, 1993). Because thiophosphorylation is less easily reversed by the action of phosphatases (Eckstein, 1985), thiophosphorylated cdc25-C probably remains activated in the extracts, whereas the conventionally phosphorylated form is inactivated by dephosphorylation. This suggests that unreplicated DNA may inhibit the activation of $p34^{cdc2}$/cyclin BΔ90 by maintenance of the activity of the type-2A protein phosphatase that dephosphorylates cdc25-C at a high level and so preventing cdc25-C activation.

DISCUSSION

In this paper we show that tyrosine dephosphorylation and activation of $p34^{cdc2}$/cyclin B can be triggered by an inhibition of type-2A protein phosphatase activity with the use of the inhibitors okadaic acid or microcystin, but not by specific inhibition of type-1 protein phosphatase activity with the use of inhibitor-2. We have also found that a type-2A protein phosphatase acts at the final stage of kinase activation and requires

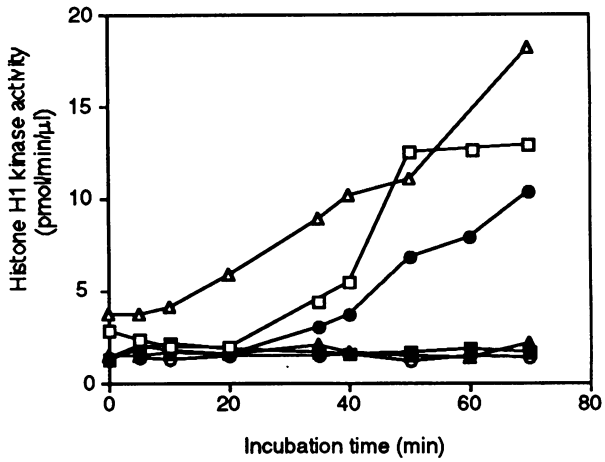

Figure 8. Effect of cdc25-C on the block imposed on $p34^{cdc2}$/cyclin B activation by unreplicated DNA. Xenopus sperm chromatin plus aphidicolin was incubated in an egg extract as in Figure 7. After 30 min, 1 µM cyclin BΔ90 was added together with unphosphorylated (▲), conventionally phosphorylated (■), or thiophosphorylated (●) cdc25-C at a final concentration of 0.4 µM. Other incubations were carried out without chromatin (□) or with chromatin plus 10 µM microcystin-LR (△). The rate of kinase activation here is less than in Figure 7 because of a greater dilution of the extract (see METHODS).

the activity of cdc25-C. We have previously found that cdc25-C is activated by phosphorylation on entry into mitosis. We therefore examined the phosphatase responsible for maintaining cdc25-C in its dephosphorylated, low activity state in interphase, and found that this was a type-2A protein phosphatase by the criteria of selective inhibition of its activity. Inhibition of cdc25-C dephosphorylation coincides with conditions that activate $p34^{cdc2}$/cyclin B. These results strongly suggest that cdc25-C is the major site of action of the type-2A protein phosphatase that inhibits $p34^{cdc2}$/cyclin B activation in egg extracts.

Other possible sites of action of type-2A protein phosphatase activity include the threonine phosphorylation site on $p34^{cdc2}$ itself, which is required for kinase activity (Thr161/167). In vitro, the purified catalytic subunit of mammalian PP-2A (or PP-1), can inactivate $p34^{cdc2}$/cyclin B, as can a native form of PP-2A from Xenopus oocytes called INH (Gould *et al.*, 1991; Lee *et al.*, 1991). This is probably due to dephosphorylation of Thr161/167. However, in egg extracts, phosphorylation at this site cannot be induced by the inhibition of PP-2A in the absence of cyclin. Moreover, in the presence of cyclin, but presumably with PP-2A still active, this site is very rapidly phosphorylated to a high stoichiometry in the inactive prokinase complex formed before the final activation step when Tyr15 and Thr14 are dephosphorylated (Ducommun *et al.*, 1991; Solomon *et al.*, 1992). It therefore seems unlikely that a type-2A protein phosphatase could normally suppress activation of $p34^{cdc2}$/cyclin B by acting at Thr161/167. In fact, it would be of interest to examine the stability of phosphorylation at this site when $p34^{cdc2}$ is bound to cyclin B; cyclin may effectively block dephosphorylation. At the metaphase-anaphase transition, Thr161/167 seems to be dephosphorylated after cyclin removal (Lorca *et al.*, 1992). A type-2A protein phosphatase might also maintain the activity of the tyrosine kinase that phosphorylates $p34^{cdc2}$, which is thought to be inhibited by increased phosphorylation (Russell and Nurse, 1987a; Smythe and Newport, 1992).

We have also found that in the mitotic state induced by $p34^{cdc2}$/cyclin BΔ90, dephosphorylation of cdc25-C catalysed by a type-2A protein phosphatase is specifically inhibited; phosphatase activity towards other substrates, including histone H1 phosphorylated by $p34^{cdc2}$, was not affected. Recently, Kumagai and Dunphy (1992) have reported that dephosphorylation of a cdc25 species is also inhibited in extracts arrested in meiotic M-phase. Our results showing that phosphorylated cdc25-C added to interphase extracts does not cause activation of $p34^{cdc2}$/cyclin B or relieve suppression by unreplicated DNA unless cdc25-C is stable to dephosphorylation suggest that inhibition of cdc25-C dephosphorylation may be required to enter the mitotic state. It seems that specific regulation of a type-2A protein phosphatase is indeed part of the mechanism of $p34^{cdc2}$/cyclin B activation. So far, little has been discovered about the control of type-2A protein phosphatases in any system. In cells, the catalytic subunit is complexed to subunits that suppress the activity towards most substrates (Cohen, 1989). However, the form of PP-2A in which the catalytic subunit is associated with A (65 kDa) and B (55 kDa) subunits, called $PP\text{-}2A_1$, is more active than the catalytic subunit and other native forms towards sites phosphorylated by $p34^{cdc2}$ (Sola *et al.*, 1991; Agostinis *et al.*, 1992). This may be relevant to the regulation of cdc25-C, because its phosphorylation is carried out by $p34^{cdc2}$/cyclin B. Another possibility is that specificity towards cdc25-C could be the property of a particular catalytic subunit. Different isoforms of PP-2A and a closely related catalytic subunit called PP-X have been identified by cDNA cloning (Cohen, 1991). Such a specific catalytic subunit could be regulated directly. At present, we classify the phosphatase that dephosphorylates cdc25-C as type-2A because of its sensitivity to okadaic acid and microcystin and insensitivity to inhibitor-2. If it does indeed have a PP-2A catalytic subunit, this may account for the role of PP-2A identified genetically in the entry into mitosis. However, its precise nature and means of regulation will require purification of the native form and identification of the constituent subunits. Purification will also allow the relationship between this phosphatase and INH to be examined.

We also examined the possible roles of PP-1 and PP-2A in the negative control exerted by unreplicated DNA on $p34^{cdc2}$/cyclin B activation in extracts. It should be kept in mind that although such experiments may inform about mechanisms involved later in development, this is emphatically not the normal situation in the early embryonic cell cycles. We found that inhibition of PP-2A but not PP-1 overcomes suppression of $p34^{cdc2}$/cyclin BΔ90 activation by unreplicated DNA. Suppression of $p34^{cdc2}$/cyclin BΔ90 activation could also be overcome by addition of thiophosphorylated cdc25-C to extracts, indicating that dephosphorylation of cdc25-C by a type-2A protein phosphatase may be part of the coupling mechanism. Our results concerning the lack of role for a type-1 protein phosphatase that is sensitive to I-2 at the final stage of $p34^{cdc2}$/cyclin B activation are at variance with the conclusions of a recent report by Walker *et al.* (1992). These authors found that in extracts supplemented with nuclei and driven into mitosis by the synthesis of endogenous cyclins, inhibition of type-1 protein phosphatase using I-2, advanced activation of histone H1 kinase activity and progression through the cell cycle. However, in these experiments, the activation of $p34^{cdc2}$ was dependent on the synthesis of cyclins and was coupled to DNA replication, so it is possible that inhibition of PP-1 influenced kinase activation indirectly through the rate of cyclin synthesis or DNA replication, which indeed the authors found were affected. Our experiments show that inhibition of PP-1 does not affect the dephosphorylation of cdc25-C or

the tyrosine dephosphorylation and activation of p34^{cdc2}/cyclin B directly, and that kinase activation can be coupled to unreplicated DNA even in the absence of PP-1 activity. They do not exclude, however, the possibility that PP-1 could be involved elsewhere in the control of p34^{cdc2}/cyclin B activation, for instance in cyclin synthesis or degradation. It also remains a possibility that a form of PP-1 that is insensitive to I-2, or other protein-serine/threonine phosphatases could be involved in the timing of mitosis.

Our knowledge of what triggers entry into mitosis is becoming more clear, although it is still not complete. It seems that p34^{cdc2}/cyclin B can be considered as the central component in a mitotic oscillator that consists of an interacting network of protein kinases and protein phosphatases (Figure 9). On passing from interphase (or G2) to mitosis the phosphorylation state and activity of each of the components changes. In embryonic cell cycles, where the oscillator is not subject to checkpoint controls, this switch might be triggered merely by the accumulation of cyclin B. If a small fraction of active p34^{cdc2} complex is formed either due to a low level of cdc25 activity or because it escapes inhibitory phosphorylation, the activation of the majority of p34^{cdc2}/cyclin B could be triggered once a threshold of kinase activity has been achieved. p34^{cdc2}/cyclin B would activate cdc25-C by direct phosphorylation and by inhibition of cdc25-C dephosphorylation by a type-2A phosphatase, producing a positive feedback. In Figure 9 we show the control of this type-2A phosphatase by direct phosphorylation, although this remains speculative at present. In addition, the tyrosine kinase that phosphorylates p34^{cdc2} may be negatively controlled by p34^{cdc2}/cyclin B. This process could account for the spontaneous activation of p34^{cdc2} in egg extracts by sea urchin cyclin B after a defined lag period (Solomon *et al.*, 1990; Clarke *et al.*, 1992) and the ability of exogenous cyclin RNA translated in the extracts to drive the cell cycle (Murray *et al.*, 1989).

However, we find that p34^{cdc2} complexed with Xenopus cyclin B1 does not spontaneously activate in extracts in which protein synthesis has been inhibited by cycloheximide. A similar result has also been reported for a starfish cyclin B (Devault *et al.*, 1992). These findings suggest that some B-type cyclins may not be able to drive the embryonic cell cycle on their own. One possibility is that, in vivo, synthesis of another protein is also required for p34^{cdc2}/cyclin B protein kinase activation. This suggestion is supported by the ability of Xenopus cyclin B1 to activate p34^{cdc2} in extracts prepared without cycloheximide and containing low levels of mitotic cyclins (Clarke, unpublished observations). During the early embryonic cell cycle, cyclin A is synthesized before cyclin B and forms an active kinase complex that accumulates gradually as cyclin A is synthesized (Minshull *et al.*, 1990). This seems to be due to inefficient inhibitory tyrosine phosphorylation of p34^{cdc2} bound to

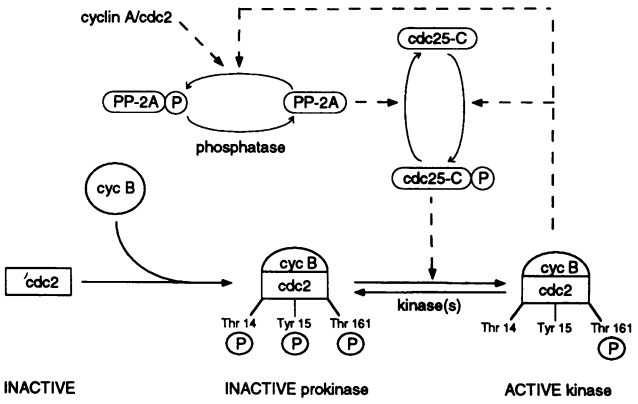

Figure 9. Model for the positive feedback activation of p34^{cdc2}/cyclin B at the G2-M phase transition. There is an accumulation of a threshold amount of active p34^{cdc2}/cyclin B which phosphorylates cdc25-C directly. In addition, a type-2A protein phosphatase activity towards cdc25-C is specifically inhibited, either by direct or indirect phosphorylation. This results in the generation of phosphorylated and activated cdc25-C, which catalyses the dephosphorylation of Thr14 and Tyr15 on p34^{cdc2} and complete kinase activation. Cyclin A-dependent protein kinase activity potentiates the activation of p34^{cdc2}/cyclin B by inhibiting the dephosphorylation of cdc25-C. The identity of the phosphatase proposed to reverse the inactivation of the type-2A protein phosphatase by cyclin-dependent protein kinases is unknown.

cyclin A, so that generation of kinase activity is not dependent on cdc25 activity (Clarke *et al.*, 1992; Devault *et al.*, 1992). When cyclin A is added to extracts containing inactive p34^{cdc2}/cyclin B prokinase, the activation of p34^{cdc2}/cyclin B is potentiated (Buendia *et al.*, 1991; Devault *et al.*, 1992). In oocytes, early embryos and somatic cells, cyclin A has been found to promote entry into M-phase (Swenson *et al.*, 1986; Pines and Hunt, 1987; Lehner and O'Farrell, 1989, 1990; Murray and Kirschner, 1989; Pagano *et al.*, 1992). In contrast, in egg extracts supplemented with nuclei, Walker and Maller (1991) have found that ablation of cyclin A releases p34^{cdc2}/cyclin B from suppression by unreplicated DNA and advances entry into mitosis. However, we (this paper) and others (Kumagai and Dunphy, 1991) have found that p34^{cdc2}/cyclin B can be coupled to DNA replication in cycloheximide-treated extracts that apparently have no cyclin A present. Further work is required to clarify this issue. A positive role for cyclin A in the entry into mitosis might be explained if p34^{cdc2}/cyclin A phosphorylated cdc25-C, like p34^{cdc2}/cyclin B. However, this does not seem to be the case (Hoffmann *et al.*, 1993). Our results have also suggested that inhibition of cdc25-C dephosphorylation is involved in the activation of p34^{cdc2}/cyclin B. It may be of particular interest therefore that cyclin A can induce the inactivation of PP-2A activity towards cdc25-C. In this way, cyclin A could potentiate phosphorylation and activation of cdc25-C, and hence the activation of p34^{cdc2}/cyclin B.

In somatic cell cycles, it is clear that p34^{cdc2}/cyclin B activation is not simply driven by cyclin B synthesis but

also requires release from checkpoint restrictions that monitor the completion of events such as DNA replication. The mechanism of such signals is largely unknown, but they may suppress the switch to the mitotic form of the cell cycle oscillator by impinging on the activity of the kinases or phosphatases involved. Protein phosphatase-2A may be an important component of these regulatory pathways, maintaining cdc25-C in a low activity dephosphorylated state until entry into mitosis is permitted.

In summary, we have found that a type-2A protein phosphatase plays an important role in the activation of $p34^{cdc2}$/cyclin B protein kinase. Its main effect is to suppress the phosphorylation and activation of cdc25-C phosphatase that acts on $p34^{cdc2}$/cyclin B. This may be part of the coupling between the completion of DNA replication and entry into mitosis in somatic cell cycles. The activity of this type-2A protein·phosphatase towards cdc25-C is not just constitutive, but is specifically decreased by cyclin A- or cyclin B-dependent protein kinase activity, allowing cdc25-C phosphorylation and $p34^{cdc2}$/cyclin B activation. Thus PP-2A or a PP-2A like protein phosphatase plays an active role in the regulation of entry into mitosis.

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REFERENCES

Agostinis, P., Derua, R., Sarno, S., Goris, J., and Merlevede, W. (1992). Specificity of the polycation-stimulated (type-2A) and ATP, Mg-dependent (type-1) protein phosphatases towards substrates phosphorylated by $p34^{cdc2}$ kinase. Eur. J. Biochem. *205*, 241–248.

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

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.

Buendia, B., Clarke, P.R., Félix, M.A., Karsenti, E., Leiss, D., and Verde, F. (1991). Regulation of protein kinases associated with cyclin A and cyclin B and their effects on microtubule dynamics and nucleation in Xenopus egg extracts. Cold Spring Harbor Symp. Quant. Biol. *56*, 523–532.

Clarke, P.R., and Karsenti, E. (1991). Regulation of $p34^{cdc2}$ protein kinase: new insights into protein phosphorylation and the cell cycle. J. Cell Sci. *100*, 409–414.

Clarke, P.R., Leiss, D., Pagano, M., and Karsenti, E. (1992). Cyclin A- and cyclin B-dependent protein kinases are regulated by different mechanisms in *Xenopus* egg extracts. EMBO J. *11*, 1751–1761.

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

Cohen, P. (1991). Classification of Protein-serine/threonine phosphatases: identification and quantitation in cell extracts. Methods Enzymol. *201*, 389–398.

Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Strålfors, P., and Tung, H.Y.L. (1988). Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. Methods Enzymol. *159*, 390–408.

Cohen, P., Klumpp, S., and Schelling, D.L. (1989). An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. FEBS Lett. *250*, 596–600.

Cohen, P.T.W. (1991). Cloning of protein-serine/threonine phosphatases. Methods Enzymol. *201*, 398–408.

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.

Devault, A., Fesquet, D., Cavadore, J.-C., Garrigues, A.M., Labbé, J.C., Lorca, T., Picard, A., Philippe, M., and Dorée, M. (1992). Cyclin A potentiates maturation-promoting factor activation in the early Xenopus embryo via inhibition of the tyrosine kinase that phosphorylates cdc2. J. Cell Biol. *118*, 1109–1120.

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.

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

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

Eckstein, F. (1985). Nucleoside phosphorothioates. Ann. Rev. Biochem. *54*, 367–402.

Enoch, T., and Nurse, P. (1991). Coupling M phase and S phase: controls maintaining the dependence of mitosis on chromosome replication. Cell *65*, 921–923.

Félix, M.-A., Clarke, P.R., Coleman, J., Verde, F., and Karsenti, E. (1993). Frog egg extracts as a system to study mitosis. In: The Cell Cycle: A Practical Approach, ed. P. Fantes, Oxford, Washington D.C.: IRL Press, (in press).

Félix, 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.

Félix, M.A., Pines, J., Hunt, T., and Karsenti, E. (1989). A post-ribosomal supernatant from activated Xenopus eggs that displays post-translationally regulated oscillation of its cdc2+ mitotic kinase activity. EMBO J. *8*, 3059–3069.

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

Galaktionov, K., and Beach, D. (1991). Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell *67*, 1181–1194.

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 p34cdc2. Cell *67*, 197–211.

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 Thr 167 is required for fission yeast $p34^{cdc2}$ function. EMBO J. *10*, 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.

Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25-C by cdc2/cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J. *12*, 53–63.

Honda, R., Ohba, Y., and Yasuda, H. (1992). The cell cycle regulator, human $p50^{wee1}$, is a tyrosine kinase and not a serine/threonine kinase. Biochem. Biophys. Res. Commun. *186*, 1333–1338.

Huchon, D., Ozon, R., and Demaille, J.G. (1981). Protein phosphatase-1 is involved in *Xenopus* oocyte maturation. Nature *294*, 358–359.

Hunt, T. (1991). Cyclins and their partners: from a simple idea to complicated reality. Semin. Cell Biol. *2*, 213–222.

Igarashi, M., Nagata, A., Jinno, S., Suto, K., and Okayama, H. (1991). $Wee1^+$-like gene in human cells. Nature *353*, 80–83.

Izumi, T., Walker, D.H., and Maller, J.L. (1992). Periodic changes in the phosphorylation of the Xenopus cdc25 phosphatase regulate its activity. Mol. Biol. Cell *3*, 927–939.

Jessus, C., and Beach, D. (1992). Oscillation of MPF is accompanied by periodic association between cdc25 and cdc2-cyclin B. Cell *68*, 323–332.

Karsenti, E., Newport, J., Hubble, R., and Kirschner, M. (1984). Interconversion of metaphase and interphase microtubule arrays, as studied by the injection of centrosomes and nuclei into Xenopus eggs. J. Cell Biol. *98*, 1730–1745.

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.

Kinoshita, N., Yamano, H., Le Bouffant-Sladeczek, F., Kurooka, H., Ohkura, H., Stone, E.M., Takeuchi, M., Toda, T., Yoshida, T., and Yanagida, M. (1991). Sister-chromatid separation and protein dephosphorylation in mitosis. Cold Spring Harbor Symp. Quant. Biol. *56*, 621–628.

Krek, W., and Nigg, E.A. (1991a). Differential phosphorylation of vertebrate p34cdc2 kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO J. *10*, 305–316.

Krek, W., and Nigg, E.A. (1991b). Mutations of $p34^{cdc2}$ phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to $p34^{cdc2}$ kinase activation in vertebrates. EMBO J. *10*, 3331–3341.

Krek, W., and Nigg, E.A. (1992). Cell cycle regulation of vertebrate p34cdc2 activity: identification of Thr161 as an essential in vivo phosphorylation site. New Biol. *4*, 323–329.

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.

Kumagai, A., and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. Cell *70*, 139–151.

Langan, T.A., Gautier, J., Lohka, M., Hollingsworth, R., Moreno, S., Nurse, P., Maller, J., and Sclafani, R.A. (1989). Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells. Mol. Cell. Biol. *9*, 3860–3868.

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.J., and Kirschner, M.W. (1991). INH, a negative regulator of MPF, is a form of protein phosphatase 2A. Cell *64*, 415–423.

Lehner, C.F., and O'Farrell, P.H. (1989). Expression and function of Drosophila cyclin A during embryonic cell cycle progression. Cell *56*, 957–968.

Lehner, C.F., and O'Farrell, P.H. (1990). The roles of Drosophila cyclins A and B in mitotic control. Cell *61*, 535–547.

Lorca, T., Labbé, J.-C., Devault, A., Fesquet, D., Capony, J.-P., Cavadore, J.-C., Le Bouffant, F., and Doreé, M. (1992). Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. EMBO J. *11*, 2381–2390.

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

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., and Codd, G.A. (1990). Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. *264*, 187–192.

McGowan, C.H., and Russell, P. (1993). Human wee1 kinase inhibits cell division by phosphorylating $p34^{cdc2}$ exclusively on Tyr15. EMBO J. *12*, 75–85.

Millar, J.B.A., Blevitt, J., Gerace, L., Sadhu, K., Featherstone, C., and Russell, P. (1991a). $p55^{CDC25}$ is a nuclear protein required for the initiation of mitosis in human cells. Proc. Natl. Acad. Sci. USA *88*, 10500–10504.

Millar, J.B.A., McGowan, C.H., Jones, R., Sadhu, K., Bueno, A., Richardson, H., and Russell, P. (1991b). cdc25 M-phase inducer. Cold Spring Harbor Symp. Quant. Biol. *56*, 577–584.

Millar, J.B.A., McGowan, C.H., Lenaers, G., Jones, R., and Russell, P. (1991c). p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast. EMBO J. *10*, 4301–4309.

Minshull, J., Golsteyn, R., Hill, C.S., 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.

Murray, A.W. (1992). Creative blocks: cell-cycle checkpoints and feedback controls. Nature *359*, 599–604.

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

Murray, A.W., Solomon, M.J., and Kirschner, M.W. (1989). The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature *339*, 280–286.

Norbury, C., Blow, J., and Nurse, P. (1991). Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates. EMBO J. *10*, 3321–3329.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. Nature *344*, 503–508.

Ohkura, H., Noriyuki, K., 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.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. EMBO J. *11*, 961–971.

Parker, L.L., Atherton-Fessler, S., and Piwnica-Worms, H. (1992). $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. Proc. Natl. Acad. Sci. USA *89*, 2917–2921.

Parker, L.L., and Piwnica-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. Science *257*, 1955–1957.

Philpott, A., Leno, G.H., and Laskey, R.A. (1991). Sperm decondensation in Xenopus egg cytoplasm is mediated by nucleoplasmin. Cell *65*, 569–578.

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 Hunt, T. (1987). Molecular cloning and characterization of the mRNA for cyclin from sea urchin eggs. EMBO J. *6*, 2987–2995.

Pines, J., and Hunter, T. (1991). Cyclin-dependent kinases: a new cell cycle motif? Trends Cell Biol. *1*, 117–121.

Russell, P., and Nurse, P. (1987a). The mitotic inducer nim1+ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. Cell *49*, 569–576.

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

Sadhu, K., Reed, S.I., Richardson, H., and Russell, P. (1990). Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc. Natl. Acad. Sci. USA *87*, 5139–5143.

Smith, D.B., and Johnson, K.S. (1988). Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-S-transferase. Gene *67*, 31–40.

Smythe, C., and Newport, J.W. (1992). Coupling of mitosis to the completion of S phase in Xenopus occurs via modulation of the tyrosine kinase that phosphorylates $p34^{cdc2}$. Cell *68*, 787–797.

Sola, M.M., Langan, T., and Cohen, P. (1991). $p34^{cdc2}$ phosphorylation sites in histone H1 are dephosphorylated by protein phosphatase 2A1. Biochim. Biophys. Acta *1094*, 211–216.

Solomon, M.J., 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. (1992). Role of phosphorylation in $p34^{cdc2}$ activation: identification of an activating kinase. Mol. Biol. Cell *3*, 13–27.

Strausfeld, U., Labbé, J.C., Fesquet, D., Cavadore, J.C., Picard, A., Sadhu, K., Russell, P., and Dorée, M. (1991). Dephosphorylation and activation of a $p34^{cdc2}$/cyclin B complex in vitro by human CDC25 protein. Nature *351*, 242–245.

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

Tung, H.Y.L., Resink, 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., DePaoli-Roach, A.A., and Maller, J.L. (1992). Multiple roles for protein phosphatase 1 in regulating the Xenopus early embryonic cell cycle. Mol. Biol. Cell *3*, 687–698.

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

Yanagida, M., Kinoshita, N., Stone, E.M., and Yamano, H. (1992). Protein phosphatases and cell division cycle control. In: Ciba Foundation Symposium 170: Regulation of the eukaryotic cell cycle, vol. 170, Chichester: Wiley, 130–146.