Cyclin A Potentiates Maturation-promoting Factor Activation in the Early *Xenopus* Embryo via Inhibition of the Tyrosine Kinase That Phosphorylates CDC2

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Abstract. We have produced human cyclin A in *Escherichia coli* and investigated how it generates H1 kistone kinase activity when added to cyclin-free extracts prepared from parthenogenetically activated *Xenopus* eggs. Cyclin A was found to form a major complex with cdc2, and to bind cdk2/Eg1 only poorly. No lag phase was detected between the time when cyclin A was added and the time when H1 histone kinase activity was produced in frog extracts, even in the presence of 2 mM vanadate, which blocks cdc25 activity. Essentially identical results were obtained using extracts prepared from starfish oocytes. We conclude that formation of an active cyclin A–cdc2 kinase during early development escapes an inhibitory mechanism that delays formation of an active cyclin B–cdc2 kinase. This inhibitory mechanism involves phosphorylation of cdc2 on tyrosine 15. Okadaic acid (OA) activated cyclin B–cdc2 kinase and strongly reduced tyrosine phosphorylation of cyclin B–associated cdc2, even in the presence of vanadate. 6-dimethylaminopurine, a reported inhibitor of serine-threonine kinases, suppressed OA-dependent activation of cyclin B–cdc2 complexes. This indicates that the kinase(s) which phosphorylate(s) cdc2 on inhibitory sites can be inactivated by a phosphorylation event, itself antagonized by an OA-sensitive, most likely type 2A phosphatase.

We also found that cyclin B– or cyclin A–cdc2 kinases can induce or accelerate conversion of the cyclin B–cdc2 complex from an inactive into an active kinase. Cyclin B–associated cdc2 does not undergo detectable phosphorylation on tyrosine in egg extracts containing active cyclin A–cdc2 kinase, even in the presence of vanadate. We propose that the active cyclin A–cdc2 kinase generated without a lag phase from neo-synthesized cyclin A and cdc2 may cause a rapid switch in the equilibrium of cyclin B–cdc2 complexes to the tyrosine-dephosphorylated and active form of cdc2 during early development, owing to strong inhibition of the cdc2-specific tyrosine kinase(s). This may explain why early cell cycles are so rapid in many species.

ENTRY into mitosis depends on the activation of maturation-promoting factor (MPF),[1] a stoichiometric complex between cdc2, a protein kinase catalytic subunit and cyclin B, a regulatory subunit (for reviews, see Nurse, 1990; and Dorée, 1990). The pathway of MPF activation is not fully understood, although there is evidence that newly synthesized cyclin B binds to inactive monomeric cdc2, promoting a series of posttranslational modifications of cdc2 that lead first to accumulation of the complex in an inactive form (Solomon et al., 1990). These modifications include phosphorylation of Tyr 15 and Thr 14 (Gould and Nurse, 1989; Norbury et al., 1991; Krek and Nigg, 1991). Conversion into an active kinase is due to the activity of cdc25, which dephosphorylates Tyr 15 (Strausfeld et al., 1991; Gautier et al., 1991; Dunphy and Kumagai, 1991; Millar et al., 1991; Lee et al., 1992) and probably Thr 14.

In most animals, including *Xenopus* and *Drosophila*, early development is characterized by a series of rapid and synchronous cell cycles which takes place after fertilization. These cell cycles are believed to be a sequence of S phase and M phase without intervening G phases. After this an abrupt transition, the midblastula transition (Signoret and Lefresne, 1971) takes place, leading to more complex cell cycles similar to those observed in somatic cells. After this transition, divisions become asynchronous and slower, owing to the addition of G phases. Oscillations of MPF activity,

Alain Devault and Didier Fesquet contributed equally to this work.

1. *Abbreviations used in this paper*: HSA, human serum albumin; MPF, maturation-promoting factor; OA, okadaic acid; TLC, thin layer plates; TLE, thin layer electrophoresis.

periodic duplication of centrosomes and changes in the network of microtubules, as well as cleavages, are independent of DNA replication in early embryos; they continue in enucleated eggs (Briggs et al., 1951; Picard et al., 1988; Dabauvalle et al., 1988) and in the presence of the replication inhibitor aphidicolin (Kimelman et al., 1987; Picard et al., 1987; Raff and Glover, 1988). After the midblastula transition however, cell cycle arrests if DNA replication is incomplete.

Mitotic cyclin cDNAs have been cloned and sequenced from a variety of organisms, and on the basis of sequence homologies in the most conserved central 200 amino acids (the cyclin box), have been subdivided into two classes, the A type and the B type (for review see Nugent et al., 1991). Both types of cyclins also contain a conserved but slightly different stretch of aminoacids (the destruction box) in its NH_2-terminal region, whose integrity is required for cell cycle-regulated proteolysis by a ubiquitin-dependent system (Glotzer et al., 1990; Lorca et al., 1991). Although cyclin A has also been reported to be required for DNA replication in somatic cells (Girard et al., 1991; Zindy et al., 1992; Pagano et al., 1992) and to maintain dependence of mitosis on completion of DNA replication (Walker and Maller, 1991) observations that cyclin A mutants in *Drosophila* arrest at G2 of the sixteenth cell cycle (Lehner and O'Farrell, 1990), and that truncated cyclin A, like truncated cyclin B (Murray et al., 1989; Ghiara et al., 1991) arrests Spisula embryos at M phase (Luca et al., 1991; Lorca et al., 1992) argue that cyclin A is required for and plays an essential role in mitosis, perhaps more specifically during early embryogenesis.

Cyclin B-cdc2 kinase activity rises more smoothly than that of the cyclin A-cdc2 complex during the first mitotic cell cycle in *Xenopus*, (Minshull et al., 1990) and probably does so in other early embryos (Westendorf et al., 1989). This suggested that the mechanism of formation of active cyclin A-cdc2 kinase might not be the same as that of cyclin B-cdc2 kinase. In the present work we found that recombinant cyclin A forms extremely rapidly an active complex with cdc2 in egg extracts, and this even in conditions which prevent cdc25 to dephosphorylate inhibitory residues in cdc2. This indicates that cdc2 undergoes limited phosphorylation on inhibitory residues in cyclin A, as compared to cyclin B complexes. We also found that cyclin A-cdc2 kinase can trigger activation of the cyclin B-cdc2 complex in such extracts, at least partially by inhibiting a tyrosine kinase which phosphorylates cdc2 on inhibitory residues. This suggests that at least during early embryogenesis, cyclin A potentiates activation of MPF by limiting the phosphorylation of cyclin B-associated cdc2 on inhibitory residues.

Materials and Methods

Egg Extracts

Interphase *Xenopus* extracts were prepared as follows: unfertilized eggs were dejellied in 2% cysteine, and transferred in MMR/2 (50 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05 mM Na-EGTA, 2.5 mM Na-Hepes, pH 7.7). Cycloheximide (100 μg/ml) was added and the eggs were electrically activated 15 min later. Extracts were prepared 25 min after activation, as described by Murray and Kirschner (1989), except that cycloheximide (20 μg/ml) was present throughout the procedure. Metaphase extracts were prepared the same way from cycloheximide-treated but unactivated eggs.

Starfish extracts were prepared from oocytes arrested at G_2 as described elsewhere (Labbé et al., 1991), and centrifuged at 105,000 *g* for 1 h before using supernatants for experiments.

CDC2 Kinase and Recombinant Proteins

Starfish cyclin B-cdc2 kinase was prepared as previously described (Labbé et al., 1989*a*, 1991).

Human cyclins A (both the full-length and a Δ46-truncated protein), the starfish Δ58 cyclin B fused at its COOH terminus with the human serum albumin (HSA) binding site of streptococcal protein G, and human CDC25 were produced as described (Lorca et al., 1992; Strausfeld et al., 1991). Plasmids encoding *Xenopus* cyclin A and cdc2 were generous gifts of Dr. Karsenti (EMBL, Heidelberg, Germany) and Jean Gautier (University of California, San Francisco, CA).

Immunodetection and Affinity Purification

Immunoprecipitations were performed after diluting samples in RIPA buffer (150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM Na-glycerophosphate, 0.5% Na-deoxycholate, 1% NP-40, 10 mM Na-phosphate, pH 7.6) and immune complexes isolated on protein A-Sepharose. In some experiments protein A-Sepharose beads cross-linked to affinity-purified antibodies were used (Schneider et al., 1982). For Western blots we used a chemiluminescence detection system (Amersham International, Amersham, England). Unless otherwise specified complexes containing the starfish fusion protein were isolated on HSA-Sepharose beads after dilution in RIPA buffer. For fixation of cdc2-containing complexes on $p13^{suc1}$-Sepharose, samples were diluted 10-fold in a buffer containing 80 mM β-glycerophosphate, 20 mM EGTA, 10 mM DTT, 5 mM EDTA and 50 mM NaF at pH 7.3 before adding the beads, prepared as already described (Labbé et al., 1991).

Proteolytic Cleavages

Tryptic digestion was performed in 0.1% (wt/vol) ammonium bicarbonate with 0.1 mg of TPCK-trypsin (Serva, Heidelberg, Germany) at 37°C. After 16 h, TPCK-trypsin was added a second time. After 8 h the gel piece was removed and the supernatant lyophilized.

The resulting peptide mixture was further digested in 0.1% ammonium bicarbonate by endoproteinase-Glu-C (Boehringer Mannheim GmbH, Mannheim, Germany), from *Staphylococcus aureus* V8 (0.02 mg) overnight at 37°C. The digestion was stopped by lyophilization.

Phosphoamino Acids Analysis and Peptide Mapping

Phosphoamino acids analysis was carried out according to Cooper et al. (1983). Phosphopeptide mapping was carried out either by monodimensional thin layer electrophoresis (TLE) or by TLE followed by ascending chromatography on cellulose thin layer plates (TLC). Monodimensional electrophoresis was run at pH 1.9 in formic acid-acetic acid-water (50-156-1794, vol/vol/vol/vol) according to Boyle et al. (1991). For bidimensional peptide mapping, electrophoresis was at pH 3.5 in pyridine-acetic acid-water (10-100-1890, vol/vol/vol) with migration toward the cathode followed by ascending chromatography right angle in isobutyric acid-n butanol-pyridine-acetic acid-water (1250-38-96-58-558, vol/vol/vol/vol/vol) as in Boyle et al. (1991).

Phosphopeptide Synthesis

Solid phase synthesis of the Val Tyr Thr His Glu peptide was performed on a 9050 Milligen Synthetizer (Watford, U.K.), with the use of pep Syn KA resin and FMOC as temporary α amino acid protection. Upon completion of the peptide chain assembly the only free hydroxyl group of Thr residue was phosphorylated according to Otvos et al. (1989), cleaved from the resin with a TFA-water-phenol mixture and further purified by ionic exchange chromatography on a Mono Q HR 5/5 column eluted with a 0–0.5 M ammonium bicarbonate linear gradient and lyophilization of the phosphopeptide-containing fractions.

H1 Histone Kinase Assays

Aliquots of extracts or affinity-purified cyclin-cdc2 complexes were diluted in 10 vol of a buffer containing 20 mM Hepes, pH 7.4, 10 mM

$MgCl_2$, 1 mg/ml H1 histone (Boehringer Mannheim GmbH) and 100 μM ATP (200 cpm/pmol) and incubated at 25°C. Reactions were stopped after 5 or 10 min either with Laemmli's buffer or by spotting on pieces of P81 phosphocellulose paper and extensive dilution in water.

Results

Formation of Inactive Cyclin B–cdc2 complex in Xenopus Eggs Extracts

Pioneer experiments by Murray and Kirschner (1989) demonstrated that addition of truncated sea urchin cyclin B (Δ90 and Δ13) to an interphase *Xenopus* egg extract activates MPF and drives the extract into a mitotic state. It was later shown that the heterologous cyclin binds monomeric *Xenopus* cdc2, promoting a series of posttranslational modifications of the chimeric kinase that lead to the transient accumulation of an inactive complex with cdc2 phosphorylated on tyrosine and threonine inhibitory residues. Activation of this complex was blocked by vanadate, which inhibits phosphatase activity of cdc25 (Solomon et al., 1990).

We also obtained such results in some interphase frog extracts (see Materials and Methods) using a tagged protein containing residues 59-388 of starfish cyclin B fused at its COOH-terminus with the HSA binding domain of streptococcal protein G (Fig. 1, type 2 extracts). In other interphase extracts however (type 1 extracts, Fig. 1), cyclin B–cdc2 complexes were formed but they remained clamped in an inactive state, even in the absence of vanadate. The same result has been obtained occasionally by others using either the sea urchin Δ90 cyclin (see Fig. 3 in Glotzer et al., 1991) or recombinant *Xenopus* B1 cyclin (Clarke, P., and Karsenti, E., personal communication). In our hands, frequency of extracts behaving in the second way was highest (near 75%) when eggs were preincubated with cycloheximide before parthenogenetic activation, preventing protein synthesis to occur during preparation of extracts. Cdc2 kinase remained inactive for hours in such extracts, even when cdc2 was saturated by adding 240 nM cyclin B. As expected, cyclin was not phosphorylated, and cdc2 phosphorylated on threonine and tyrosine residues in the inactive complex (data not shown).

Besides Tyr 15 and Thr 14, binding to cyclin induces the phosphorylation of cdc2 on another residue, Thr 161 (Krek and Nigg, 1991; Solomon et al., 1992; Lorca et al., 1992) or its homologue Thr 167 in fission yeast (Gould et al., 1991). Phosphorylation of Thr 161 on cdc2 is believed to be required for either stabilization (Ducommun et al., 1991) or alternatively to confer H1 kinase activity to cyclin–cdc2 complexes (Solomon et al., 1992). To investigate whether Thr 161 was phosphorylated in inactive cyclin B–cdc2 complexes, cyclin B was added together with γ-^{32}P-ATP (6 mCi/ml) to interphase extracts. For this and the following experiments we used extracts which did not support "spontaneous" activation of cyclin B–cdc2 complexes, even after a lag phase. 30 min later cyclin B–containing complexes were immunoprecipitated and either treated for 10 min in the absence of ATP with recombinant human cdc25, or left untreated. In both cases, complexes were eluted, components separated by SDS-PAGE and analyzed by autoradiography. As shown in Fig. 2 *a*, cdc2 shifted almost quantitatively to a higher mobility form after cdc25 treatment, indicating that dephosphorylation of inhibitory residues had occurred. Nonetheless 25–45% of the initial radioactivity were still associated with cdc2 after cdc25 treatment (Fig. 2 *b*), and assigned to phosphothreonine by phosphoamino acids analysis (not shown). To map the phosphorylated threonine residue, cdc2 was cut out from the gel, treated with both trypsin and the V_8 protease, and the resulting material submitted to bidimensional analysis and autoradiography. A single spot was detected after treatment with cdc 25, which migrated as the synthetic phosphopeptide Val-Tyr-Thr(P)-His-Glu (Fig. 2 *c*). This sequence is that of the peptide spanning Thr 161 in cdc2 that would be expected to be generated by proteolysis under such conditions. We conclude that even in its inactive form, the complex which accumulates in interphase extracts upon cyclin B addition contains a Thr 161-phosphorylated cdc2 subunit.

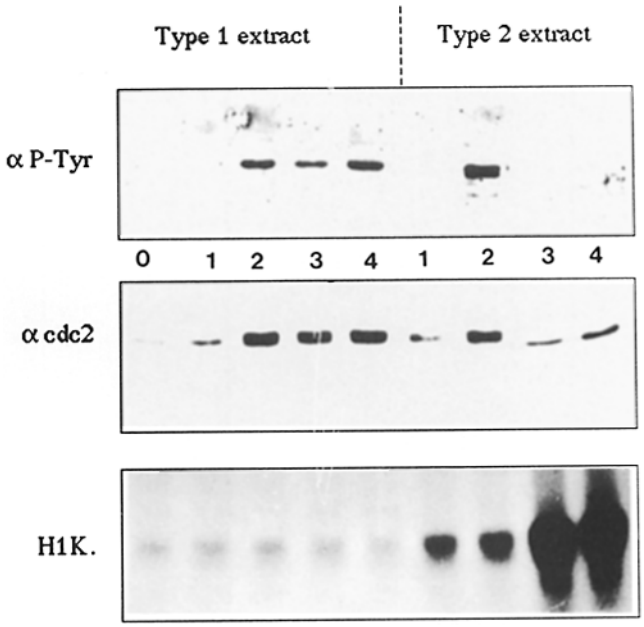

Figure 1. Starfish cyclin B can generate either inactive or active complexes with *Xenopus* cdc2, depending on extracts. Recombinant starfish cyclin B (160 nM) was added to two interphase frog extracts prepared in the same way (see Material and Methods) but from two different females. Aliquots were taken as a function of time and treated with the same amount of p13^{suc1}-Sepharose beads. The retained material was either eluted with Laemmli's buffer, analyzed by SDS-PAGE, and Western blotted or transferred with the beads in a reaction mix designed to measure H1 kinase activity (see Materials and Methods). Blots were probed first with anti-phosphotyrosine antibodies (*top*) then with antibodies (called NMPF) directed against a 12 amino acid peptide corresponding to the conserved NH_2-terminal sequence of cdc2 (*middle*). The lower panel shows an autoradiogram of H1 kinase activities in the different aliquots. Aliquots were taken before (*0*) or 5 (*1*), 15 (*2*), 60 (*3*) and 90 min (*4*) after cyclin B addition. Cyclin B–cdc2 kinase reached its maximal activity within 30 min and remained stable thereafter because the cyclin B fusion protein lacks the destruction box and does not undergo utiquitin-dependent proteolysis. Note that the dephosphorylated cdc2 monomer does not bind efficiently p13^{suc1}, as already reported (Devault et al., 1991).

Potentiation of Cyclin B–cdc2 Complex Activation by Cyclin A– or Cyclin B–cdc2 Kinases

The inactive cyclin B–cdc2 complex formed in interphase

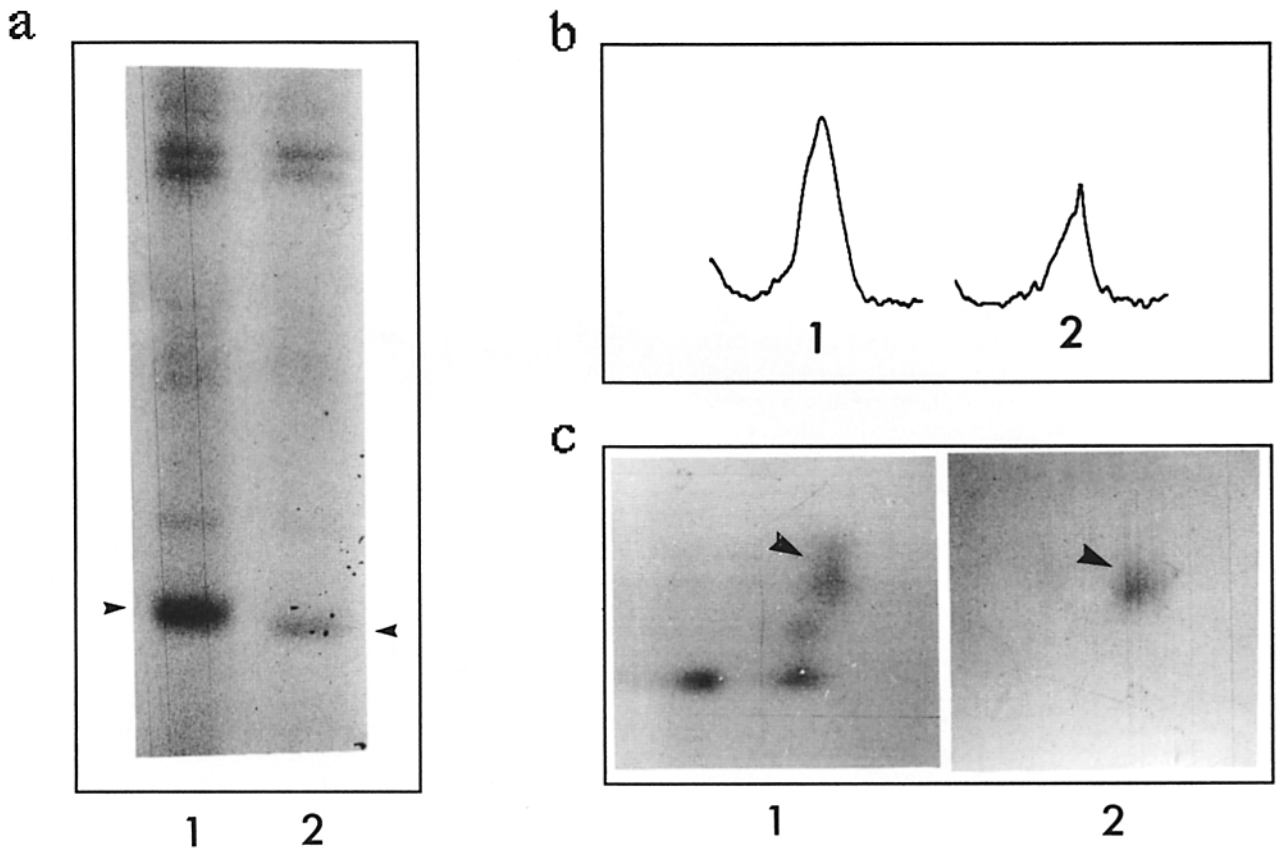

Figure 2. Cdc2 is phosphorylated on Thr 161 in inactive cyclin B–cdc2 complexes. Cyclin B (160 nm) was added together with γ-32P-ATP (6 mCi/ml) to an interphase extract. 30 min later cyclin B–containing complexes were immunoprecipitated with anti-starfish cyclin B antibodies and the immunoprecipitate split into two equal parts. One was treated for 10 min in the absence of ATP with recombinant human cdc25. Both the cdc25-treated and non-treated immunoprecipitates were eluted with Laemmli's buffer and proteins separated by SDS-PAGE. (*a*) Autoradiogram of the untreated (*1*) and the cdc25-treated aliquots (*2*). Arrowheads point to cdc2. (*b*) Densitometric scanning of cdc2 in lanes *1* and *2* from the autoradiogram. (*c*) The cdc2 bands were cut out from lanes *1* and *2*, digested with both trypsin and the V8 protease, and the resulting material submitted to bidimensional analyis and autoradiography. The arrowhead on each autoradiogram points to the position of the Val-Tyr-Thr(P)-His-Glu phosphopeptide (detected with a ninhydrin spray) run simultaneously with the labeled samples.

frog extracts appeared to be similar, if not identical, to pre-MPF, the inactive cyclin B–cdc2 complex which accumulates in G2-arrested oocytes of at least some species (Gautier and Maller, 1991; Kumagai and Dunphy, 1991; Strausfeld et al., 1991). Indeed both complexes can be activated even in the absence of ATP by cdc25, which dephosphorylates the inhibitory residues in cdc2. One of the most characteristic properties of MPF is its ability to be amplified in serial transfers in which the recipient of a transfer becomes the donor of the next one (Masui and Markert, 1971; Kishimoto et al., 1982). During this process, the microinjected cyclin B–cdc2 kinase converts pro-MPF into active cyclin B–cdc2 kinase (Labbé et al., 1987 and 1989). The amplification loop becomes activated when cyclin B–cdc2 kinase activity exceeds a threshold value, which depends on species and can be lowered by inhibiting type 2A phosphatase activity (Dorée et al., 1991; Picard et al., 1991).

This led us to investigate the effect of adding active cyclin B–cdc2 kinase to interphase extracts containing the inactive complex. Highly purified starfish cyclin B–cdc2 kinase (Labbé et al., 1989) was added to the final H1 histone kinase activity of 8 pmol/min/μl to interphase extracts containing an inactive cyclin B–cdc2 complex generated by adding 70 nM starfish cyclin B. 15 min later cdc2-containing complexes were recovered on p13^{suc1}-Sepharose beads and either assayed for H1 histone kinase activity or Western blotted and analyzed with anti-phosphotyrosine antibodies. A threefold higher kinase activity was recovered when starfish cdc2 kinase was added to extracts containing the inactive complex, and this was associated with disappearance of phosphotyrosine from cdc2 (not shown). We conclude that cyclin B–cdc2 kinase can activate in frog extracts an amplification loop which converts the in vitro assembled complex from an inactive into an active protein kinase, as it does in oocytes.

Cyclin A microinjection also readily activates MPF in G2-arrested oocytes (Swenson et al., 1986) and it does so even more efficiently than cyclin B (Roy et al., 1991). In the next experiments, we investigated whether cyclin A–cdc2 kinase could trigger conversion of inactive cyclin B–cdc2 kinase in frog extracts. We could not use starfish cyclin A–cdc2 kinase, because starfish cyclin A has not yet been characterized. Therefore we generated a chimeric complex between recombinant human cyclin A, produced in *Escherichia coli* (see Materials and Methods) and frog cdc2 in interphase egg extracts. Besides forming a protein kinase complex with cdc2, as cyclin B does, cyclin A has been shown to associate

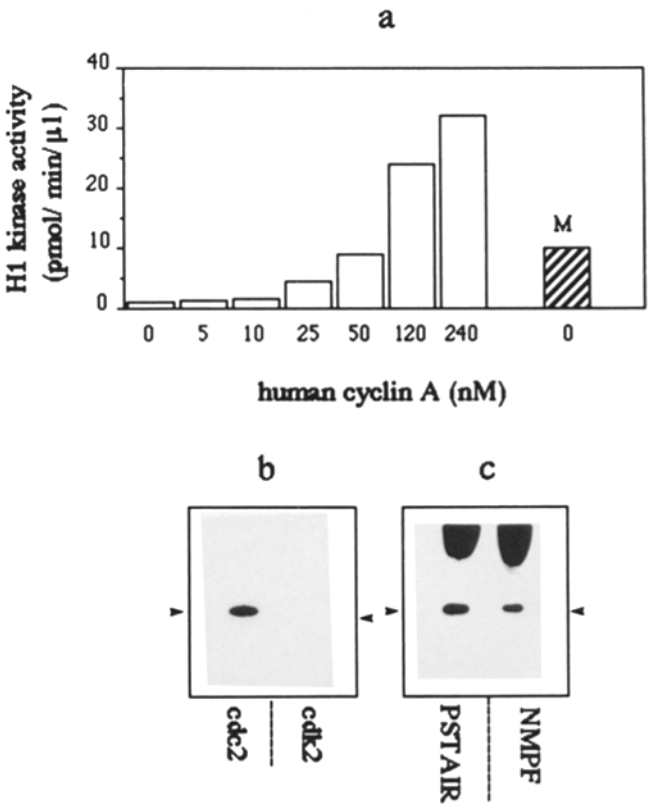

Figure 3. Bacterially produced human cyclin A forms an active complex with cdc2 in interphase frog extracts. (*a*) Aliquots were taken 30 min after adding increasing amounts of human cyclin A to interphase extracts and H1 histone kinase activities measured in anti-cyclin A immunoprecipitates. *M* indicates the level of H1 histone kinase activity found in the equivalent volume of crude extract prepared from metaphase II-arrested eggs. (*b*) 2 µl of reticulocyte lysate containing equal amounts of either ^{35}S-labeled human cdc2 or *Xenopus* cdk2/Eg1 (~10^4 cpm) were added to 20 µl of interphase extract. 5 min later, cyclin A (*120 nM*) was added. After incubation for 30 min, cyclin A-containing complexes were isolated on anti-cyclin A beads and analyzed by SDS-PAGE and fluorography. In control experiments performed in the absence of cyclin A, neither cdc2 nor cdk1/Eg1 were found to bind to the anti-cyclin A beads (not shown). Arrowheads point to the expected positions of $p34^{cdc2}$ (*left*) and $p32^{cdk2}$ (*right*). (*c*) The material immunoprecipitated by anti-cyclin A antibodies after cyclin A addition to *Xenopus* extracts was analyzed by Western blotting with two antibodies: (*PSTAIR*) Recognizes both cdc2 and cdk2/Eg1; and (NMPF) Recognizes cdc2 and does not cross-react with Eg1. Arrowheads point to $p34^{cdc2}$.

in somatic cells with cdk2, a related protein containing the PSTAIR epitope found in the cdc2 family (Paris et al., 1991; Pines and Hunter, 1990; Elledge and Spottswood, 1991). In the active H1 kinase complex which formed in egg extracts, however, most cyclin A was associated to cdc2, not cdk2, for the following reasons: (*a*) cdk2 is about 20-fold less abundant than cdc2 in frog extract (Gabrielli et al., 1992). Therefore it could not contribute a significant part of the large H1 kinase activity produced at saturating cyclin A concentration, which reached approximately threefold the level found in the equivalent volume of crude extract prepared from metaphase II-arrested eggs (Fig. 3 *a*); (*b*) when cyclin A was added to extracts containing identical amounts of ^{35}S-labeled human cdc2 or *Xenopus* cdk2 proteins, both translated in the reticulocyte system, much more radioactivity was co-immunoprecipitated with cyclin A in the first than in the second case, although cdk2 contains a similar number of methionine residues as cdc2 (Fig. 3 *b*); and (*c*) anti-cyclin A immunoprecipitates contained a single major protein (34 kD) cross-reacting with both an antibody against the PSTAIR peptide, which recognizes both cdc2 and cdk2, and an antibody directed against the 12 amino acids NH_2-terminal sequence of cdc2, which does not cross-react with cdk2 (Fig. 3 *c*).

An inactive cyclin B-cdc2 complex was isolated by affinity on HSA-Sepharose beads from a frog extract complemented with the starfish cyclin B fusion protein and treated with another aliquot of the same extract containing the active human cyclin A-cdc2 kinase. 30 min later, the cyclin B-cdc2 complex was checked both for H1 histone kinase activity and tyrosine phosphorylation of the cdc2 subunit. As shown in Fig. 4 *a* and *b*, both tyrosine dephosphorylation and kinase activation had occurred. No cyclin B-cdc2 kinase activation occurred when the complex was treated with an extract lacking cyclin A. The possibility that the HSA-Sepharose matrix used to recover the cyclin B-cdc2 complexes had been contaminated by the cyclin A-cdc2 kinase was ruled out by showing that no cyclin A had relocated from the extract to the HSA-Sepharose beads (Fig. 4 *d*) and that no loss of H1 kinase activity had occurred in the extract after incubation with the beads (Fig. 4 *a*, compare lanes *3* and *4*). It could also be argued that activation of the cyclin B-cdc2 complexes involved the exchange of cdc2 subunits between both types of cdc2 complexes. This was most unlikely since extracts in which all the available cdc2 subunits had been engaged in inactive complexes with cyclin B did neither support formation of cyclin A-cdc2 complexes (Fig. 4 *e*) nor H1 histone kinase activation (data not shown) upon cyclin A addition. This confirms that both cyclin A and cyclin B kinase complexes share in common cdc2 as the catalytic subunit and shows in addition that cyclin A-cdc2 kinase, not merely cyclin A, is required for activation of the amplification loop. This experiment also excludes the possibility that despite its low-abundancy cyclin A-cdk2 might be the active complex catalyzing pre-MPF activation because cyclin B does not bind and therefore would not be expected to deplete cdk2 from extracts.

We conclude that both active cyclin B- and cyclin A-cdc2 kinases can trigger conversion of the inactive cyclin B-cdc2 complex into an active complex. As described above (Fig. 1), some interphase frog extracts support "spontaneous" activation of cyclin B-cdc2 kinase after a lag phase. This lag phase was shortened when active cyclin B- or cyclin A-cdc2 kinases were added to such extracts (data not shown).

Cyclin A-cdc2 Complex Does Not Require cdc25 for Its Activation

One of the most intriguing differences between cyclin A and cyclin B is the timing of activation of their associated protein kinases during cell cycle. In the early *Xenopus* embryo, cyclin A-cdc2 kinase reaches a maximum when cyclin B-cdc2 kinase activity is just beginning to rise (Minshull et al., 1990). This led us to investigate the kinetics of cyclin A-cdc2 kinase activation in egg extracts.

As shown on Fig. 5 *a*, no lag phase could be demonstrated

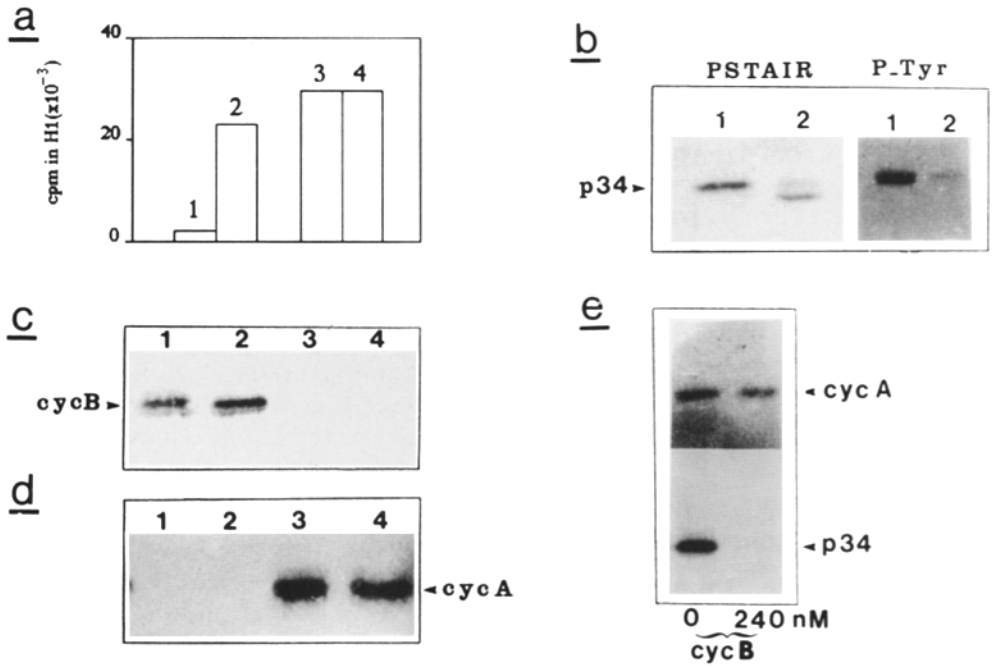

Figure 4. Cyclin A induces cyclin B–cdc2 complex activation in interphase frog extracts. An inactive cyclin B–cdc2 complex, made in a frog extract by adding starfish cyclin B fusion protein (160 nm), was affinity-purified on HSA-Sepharose (see Fig. 2). It was transferred in aliquots of frog extract precincubated for 30 min in the presence (*2*) or absence (*1*) of cyclin A (120 nM). 30 min later it was recovered and checked for cdc2 dephosphorylation and kinase activation. (*a*) H1 histone kinase activities were measured in purified cyclin B–cdc2 complex (*1* and *2*) as well as in frog extracts (*3* and *4*) before adding (*3*) or after depleting (*4*) the cyclin B–cdc2 complexes. (*b*) The purified cyclin B–cdc2 complex was Western blotted and analyzed for $p34^{cdc2}$ dephosphorylation with affinity-purified antibodies against either PSTAIR (*left*) or phosphotyrosine (*right*). (*c* and *d*) Affinity-purified B–cdc2 complexes, isolated either before (*1*) or after activation (*2*), as well as the material immunoprecipitated from cyclin A–containing frog extracts with purified antibodies against human cyclin A, either before adding (*3*) or after depleting (*4*) the cyclin B–cdc2 complexes, were run on polyacrylamide gels. Western blots were analyzed for the presence of either starfish cyclin B or human cyclin A with the corresponding antibodies (*c*, anti-cyclin B; *d*, anti-cyclin A). (*e*) Human cyclin A (120 nM) was added in a frog extract containing either no starfish cyclin B, or 240 nM starfish cyclin B. 30 min later cyclin A was immunoprecipitated as above and the immunoprecipitated materials were run on polyacrylamide gels. Western blots were analyzed for the presence of either cyclin A or $p34^{cdc2}$ with the corresponding antibodies.

between the time when human cyclin A was added and the time when H1 kinase activity was produced in frog extracts. Nonetheless, a faint amount of tyrosine-phosphorylated cdc2 was detected in immunoaffinity-purified cyclin A–cdc2 complex. It was removed when immunoprecipitates were treated with bacterially produced cdc25 (Fig. 5 *b*). Absence of a detectable lag phase could be due to a very short half life of the inactive and tyrosine-phosphorylated complex. However, vanadate did not prevent cyclin A–cdc2 kinase activation (Fig. 5 *a*), rather suggesting that direct formation of the active complex bypassed the step of tyrosine phosphorylation. Similar results were obtained using recombinant *Xenopus* cyclin A (data not shown).

To test the generality of the rapid and vanadate-insensitive

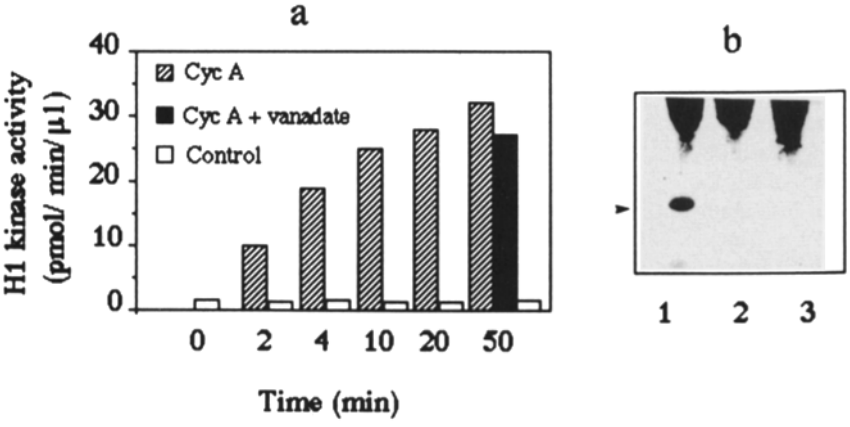

Figure 5. Mechanism of formation of active cyclin A–cdc2 kinase in frog extracts. (*a*) Time course of cyclin A–cdc2 kinase activation in interphase frog extracts after addition of recombinant human cyclin A (120 nM). Samples were taken at the indicated times from extracts receiving or not (*control*) cyclin A at zero time, diluted 30-fold in water and immediately frozen in liquid nitrogen. H1 histone kinase activities were measured subsequently in the thawed samples. An experiment was run in parallel with 2 mM vanadate included in the extract, to block cdc25 activity. This did not block cyclin A kinase activation. (*b*) The material immunoprecipitated with antibodies against human cyclin A from samples taken at time 10 min in experiment depicted in panel a was analyzed by immunoblotting with anti-phosphotyrosine antibodies. (*1*) Sample containing cyclin A. (*2*) Sample without cyclin A. (*3*) Sample containing cyclin A but the immunoprecipitate was treated with cdc25 before running the gel.

activation of cyclin A–cdc2 kinase during early development, we also used extracts prepared from starfish oocytes. Immature starfish oocytes arrested at G2 contain an inactive cyclin B–cdc2 complex (Strausfeld et al., 1991). This complex could be converted to its active form in the extracts by adding bacterially produced cdc25, and as expected this was blocked by 2 mM vanadate. When cyclin A was added in a parallel experiment to such extracts, H1 kinase activity rapidly formed, even without cdc25 addition. Moreover this cyclin A–cdc2 kinase activity was generated without delay even in the presence of 2 mM vanadate (data not shown).

Failure of vanadate to suppress or delay formation of active cyclin A–cdc2 complexes in egg extracts prepared from various species indicates that cdc25 activity is not required for cyclin A to form an active complex with cdc2, at least in the early embryo.

We demonstrated in the previous section that addition to *Xenopus* egg extracts of either cyclin A–cdc2 or cyclin B–cdc2 active kinases can turn on an amplification loop which converts the cyclin B–cdc2 complex into an active protein kinase complex. The finding that cyclin A–cdc2 kinase forms without lag phase in egg extracts, and thus escapes the inhibitory mechanism that delays cyclin B–cdc2 kinase activation, strongly suggests that cyclin A–cdc2 kinase may actually potentiate MPF activation in the early embryo.

Cyclin A–cdc2 Kinase Inhibits Tyrosine Phosphorylation of cdc2 in Cyclin B–cdc2 Complexes

Since the cyclin B–cdc2 complex generated by adding starfish cyclin B to interphase frog extracts is already phosphorylated on Thr 161 (Fig. 2), its conversion to an active form due to the presence of active cyclin A–cdc2 kinase in egg extracts could be due to a reduction in the activity of kinase(s) which phosphorylate(s) cdc2 on inhibitory residues, to an increase in the activity of the activating phosphatase (cdc25), or to a combination of both events.

To detect the putative inhibition of a kinase phosphorylating Tyr 15, we examined the time course of cdc2 tyrosine phosphorylation following addition of recombinant cyclin B to frog extracts containing or not the active cyclin A–cdc2 kinase. The inclusion of sodium vanadate, an inhibitor of cdc25, ensured that changes in the accumulation of phosphotyrosine were due to the tyrosine kinase alone. Since the cdc2 monomer is dephosphorylated and phosphorylation of cdc2 in cyclin–cdc2 complexes changes its electrophoretic mobility on SDS gels, the rate of tyrosine phosphorylation could be compared to the rate of complex formation. As shown in Fig. 6 (*top*), accumulation of phosphotyrosine in cyclin B–associated cdc2 roughly paralleled formation of the complex in the absence of cyclin A. In contrast phosphotyrosine was not detectable at any time in cyclin B–associated cdc2 when egg extracts contained active cyclin A–cdc2 kinase. We conclude that cyclin A–cdc2 kinase activity strongly reduces activity of the kinase which phosphorylates cdc2 on Tyr 15 and maintains the cyclin B–cdc2 complex in an inactive state. As expected, H1 kinase activity of cyclin B–cdc2 complexes was much higher in vanadate containing extracts after activation with cyclin A–cdc2 kinase (Fig. 6, *bottom*).

Finally, we investigated whether, once modified by cyclin A–cdc2 kinase, the egg extract remained able at least for

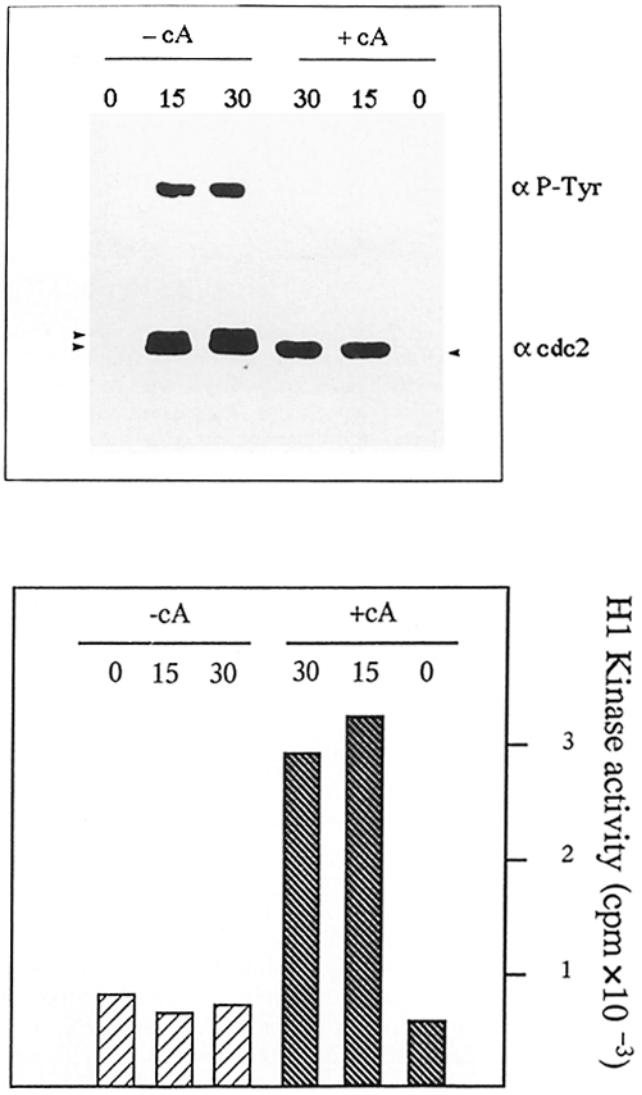

Figure 6. Cyclin A–cdc2 kinase inhibits the tyrosine kinase that phosphorylates cdc2 in cyclin B–cdc2 complexes. (*Top*) Recombinant cyclin B was added (160 nM) to a frog extract containing (*+cA*) or not (*−cA*) active cyclin A–cdc2 kinase (12 pmol/min/μl), generated by adding human cyclin A (60 nM) in frog extracts. Sodium vanadate (2 mM) was added before cyclin B addition to inhibit cdc25. HSA-Sepharose beads were added to aliquots taken at the indicated times (min) and diluted 10-fold in a buffer containing 80 mM β-glycerophosphate, 20 mM EGTA, 10 mM DTT, 5 mM EDTA, and 50 mM NaF at pH 7.3. Identical amounts of affinity-purified cyclin B–containing complexes were analyzed by SDS-PAGE, Western blotted, and the piece of nitrocellulose containing cdc2 probed first with antibodies directed against phosphotyrosine (*αP-Tyr*) or with NMPF antibodies directed against cdc2 (*αcdc2*). Note that only the higher mobility form of cdc2 is detected in extracts containing cyclin A–cdc2 kinase (*arrowhead, left*). To load identical amounts of cdc2 in each lane, *Xenopus* cdc2 was translated in vitro and the resulting ^{35}S-labeled protein used as a tracer to quantify the amount of cdc2 recovered on the affinity matrices. (*Bottom*) Same experiment as in upper panel, but affinity-purified cyclin B–cdc2 complexes were assayed for H1 kinase activity as described in Materials and Methods.

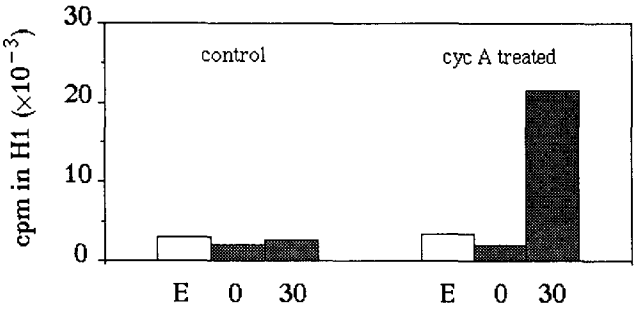

Figure 7. Cyclin A-cdc2 kinase can be removed prior to cyclin B-cdc2 complex addition and still promotes its activation in frog extracts. Cyclin A-cdc2 kinase, covalently bound to Sepharose beads, was added or not (*control*) to a frog extract, and removed 30 min later. 2 min later (zero time) aliquots of an inactive cyclin B-cdc2 complex, generated in a parallel experiment in a non-cyclin A-treated interphase extract and purified by affinity on HSA-Sepharose, was added either in the cyclin A-treated or in the non cyclin A-treated extract (*control*). H1 kinase activities of the cyclin B-cdc2 complexes were assayed either before (0) or 30 min after transfer. (*E*) Basal H1 kinase activities of the extract (no cyclin B-cdc2 complex added) as measured either before addition (*control*) or after removal of cyclin A-cdc2 kinase.

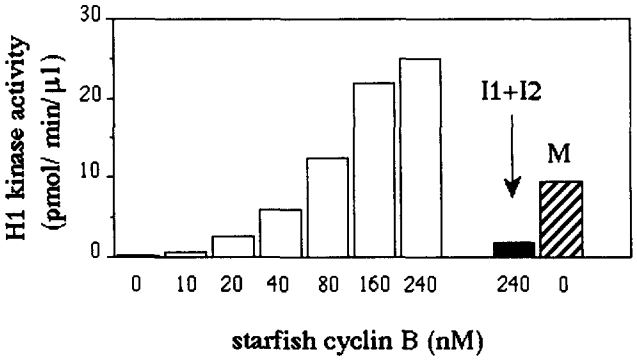

Figure 8. OA induces cyclin B-cdc2 complex activation in interphase frog extracts. OA (1 μM) was added to frog extracts containing increasing amounts of the cyclin B fusion protein, and H1 histone kinase was monitored 30 min later. Controls: H1 histone kinase activities in interphase frog extract containing both 240 nM cyclin B and a mixture of inhibitors 1 and 2 (both 0.25 μM), but no OA. (*M*) Metaphase extract without addition of cyclin or phosphatase inhibitor.

some time to convert the inactive cyclin B-cdc2 complex into an active protein kinase complex, even after removing cyclin A-cdc2 kinase. For this experiment, human cyclin A was covalently linked to Sepharose beads and added to a frog extract. The immobilized cyclin A bound cdc2 in the extract, and generated an active H1 kinase. 30 min later the beads were removed. No significant H1 kinase activity was left in the extract. 2 min after cyclin A removal an inactive cyclin B-cdc2 complex, produced in another aliquot of the same frog extract was added to the cyclin A-treated aliquot. It readily underwent activation within the next 30 min. No activation was observed when the cyclin B-cdc2 complex was transferred to non-cyclin A-treated extracts (Fig. 7).

An Okadaic Acid-Sensitive Phosphatase Activates the Tyrosine Kinase that Phosphorylates cdc2

Solomon et al. (1990) showed that duration of the lag phase before activation of cyclin B-cdc2 kinase in frog extracts was shortened by okadaic acid (OA) and lengthened by adding purified type 2A phosphatase. We confirmed that OA or microcystin (both 1 μM) readily induces activation of the in vitro produced cyclin B-cdc2 complex in *Xenopus* extracts, whilst inhibitors 1 and 2 had no such effects (Fig. 8). Phosphorylation of Thr 161 is absolutely required for cdc2 kinase activation (Gould and Nurse, 1991; Ducommun et al., 1991; Solomon et al., 1992; Lorca et al., 1992). We showed that Thr 161 is already phosphorylated before activation in cyclin B-cdc2 complexes (Fig. 2) and although this was not directly determined, we believe that this phosphorylation is stoichiometric because complexes were activated to the same extent by adding recombinant cdc25 in the presence or absence of ATP. Although type 2A phosphatase was reported to dephosphorylate a mitosis-specific threonine residue, presumably Thr 161, on cdc2 (Lee et al., 1991), this could therefore not be its unique target in inhibiting kinase activation.

To determine whether activation of the cyclin B-cdc2 complex by OA involved inhibition of a kinase phosphorylating Tyr 15, we examined the time course of tyrosine phosphorylation on cdc2 after the addition of recombinant cyclin B to frog extracts, in the presence or absence of 1 μM OA. The inclusion of sodium vanadate ensured that changes in the accumulation of phosphotyrosine were due to the tyrosine kinase alone. When OA was present in the extract to block activity of type 2A phosphatase, tyrosine phosphorylation of cdc2 still occurred to some extent, but it was reduced with respect to the untreated extract (Fig. 9 *a*). In the absence of OA, two bands of tyrosine-phosphorylated cdc2 were observed. The lower mobility band, which reflects incremental phosphorylation of cdc2 on Thr 14, was not observed in OA-treated extracts. OA had no effect when it was added only after cdc2 had shifted to its lower mobility form. We conclude that inhibition of an OA-sensitive phosphatase strongly reduces activity of the kinase(s) which phosphorylate(s) Tyr 15 and Thr 14 on cyclin B-associated cdc2.

The above results suggested that type 2A phosphatase counteracts a serine-threonine kinase inactivating the cdc2-specific tyrosine kinase in frog extracts. A reported protein kinase inhibitor, 6-dimethyl-aminopurine (6-DMAP) has been shown to reversibly inhibit cdc2 kinase activation without inhibiting tyrosine phosphorylation of cdc2 (Neant and Guerrier, 1988; Jessus et al., 1991). In the next experiment we took advantage of the apparent selectivity of the protein kinase inhibitor. Interphase extracts, containing 2 mM vanadate and 1 μM OA, were treated with 0.5 mM DMAP, or not, before adding the recombinant starfish cyclin B. 30 min later, aliquots were taken and examined for tyrosine phosphorylaton of cdc2. As shown in Fig. 9, *b-d*, and in agreement with the above hypothesis, the serine/threonine kinase inhibitor suppressed the effect of OA and restored the inhibitory phosphorylation of cdc2 on tyrosine. In this experiment OA completely suppressed tyrosine phosphorylation of cdc2 in the absence of DMAP.

Discussion

In the present work, we found that cdc25 activity is not re-

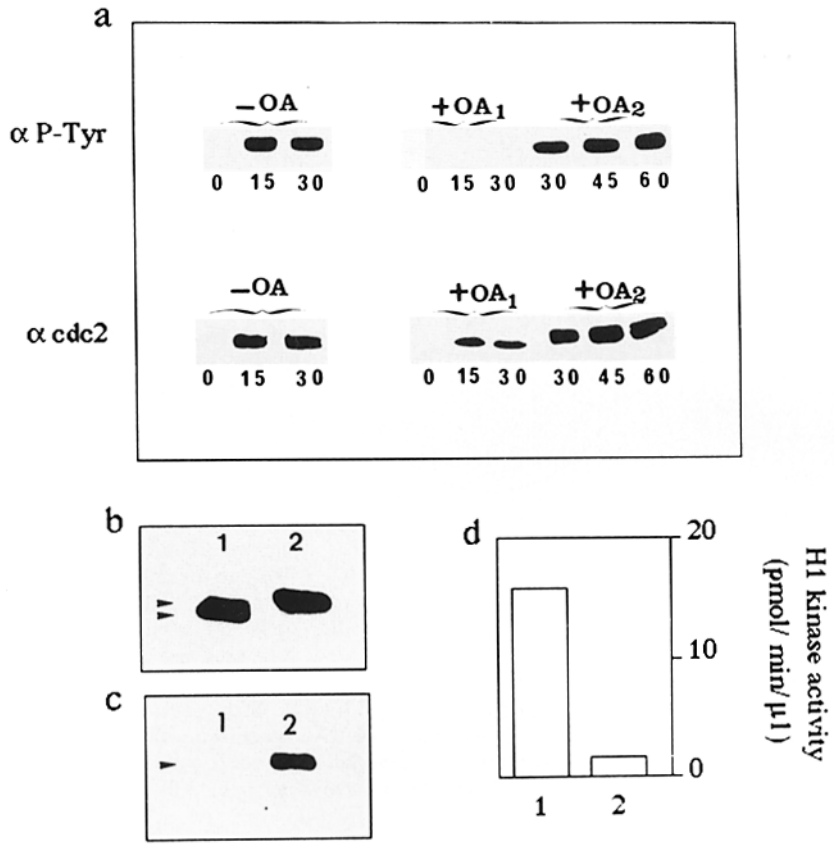

Figure 9. The tyrosine kinase that phosphorylates cdc2 is activated by an okadaic-sensitive phosphatase and inhibited by a DMAP-sensitive serine/threonine kinase. (*a*) 1 μM OA was added (*+OA*) or not (*−OA*) either simultaneously (*OA1*) or 30 min after (*OA2*) adding at zero time the starfish cyclin B fusion protein (100 nM) to an interphase frog extract containing 2 mM vanadate (to block cdc25). Aliquots were taken at the indicated times (minutes), and the material retained on HSA-Sepharose beads was analyzed by SDS-PAGE, Western-blotted and the pieces of nitrocellulose containing cdc2 probed either with anti-phosphotyrosine (*αP-Tyr*), or with anti-NMPF antibodies (*αcdc2*). All samples contained identical amounts of cdc2 (quantified as described in legend to Fig. 6). In a parallel experiment OA was added 10 min before cyclin B to an extract containing either vanadate alone (*1*) or both vanadate and 0.5 mM DMAP (*2*). Aliquots were taken 30 min after cyclin B addition and the affinity-purified cyclin B–containing complexes either Western blotted and probed with anti-NMPF (*b*) and anti-phosphotyrosine antibodies (*c*) or assayed for H1 kinase activity (*d*).

quired for cyclin A to form an active kinase complex with cdc2 in a variety of extracts prepared from oocytes or early embryos. This correlates well with the observation that only a small fraction of cyclin A–associated cdc2 is ever phosphorylated on tyrosine in *Xenopus* extracts. It may explain why cyclin A–cdc2 kinase activation is so rapid relative to that of cyclin B–cdc2 kinase which is delayed, if not blocked, by quantitative phosphorylation of Tyr-15 of cdc2. It may be worth emphasizing that, whilst Solomon et al. (1990) clearly showed that cdc2 undergoes tyrosine phosphorylation only after its binding with cyclin B, they did not formally demonstrate that tyrosine phosphorylation occurs before kinase activation after binding of cdc2 with cyclin B. Rather they consistently observed a low level of H1 histone kinase in the presence of vanadate, as well as at early times after cyclin B was added in interphase frog extracts, suggesting that at least some newly formed cyclin B–cdc2 complexes underwent mitotic phosphorylation (at Thr 161) to the exclusion of inactivating phosphorylations (at Thr 14 and Tyr 15). Thus, both cyclin A– and cyclin B–cdc2 complexes may be formed essentially according to the same mechanisms, although cyclin A–associated cdc2 appears to be a relatively poor substrate for the tyrosine kinase which phosphorylates and inactivates cdc2.

Interestingly, we found that cyclin B– or cyclin A–cdc2 kinases can induce or accelerate conversion of the cyclin B–cdc2 complex from an inactive into an active kinase. This is in keeping with results obtained by Solomon et al. (1990), who showed that tyrosine dephosphorylation of cdc2 proceeds faster in mitotic than interphase frog extracts. This response appears to be closely related to the process known in oocytes as MPF amplification, which allows a small amount of MPF to be rapidly amplified in serial transfer of cytoplasm in which the recipient of a transfer becomes the donor to the next one, and this even in the absence of protein synthesis (Wasserman and Masui, 1975; Dorée, 1982). We propose that the active cyclin A–cdc2 kinase generated without a lag phase from neo-synthesized cyclin A and cdc2 may cause a rapid switch in the equilibrium of cyclin B–cdc2 complexes to the tyrosine-desphosphorylated and active form of cdc2.

The cdc2-specific tyrosine kinase could be the target of cyclin A–cdc2 kinase in potentiating cyclin B–cdc2 kinase activation. In agreement with this view, we showed in the present work that cyclin B–associated cdc2 did not undergo detectable phosphorylation on tyrosine in egg extracts containing active cyclin A–cdc2 kinase, even when cdc25 activity was inhibited by vanadate. This was not due to modification of the cyclin B–cdc2 complex because of its phosphorylation by cyclin A–cdc2 kinase, because cyclin A–cdc2 kinase could be removed from the extract before adding cyclin B.

Several observations indicate that completion of DNA replication and initiation of mitosis are coupled during cell cycle. For example, if inhibitors of DNA synthesis are added to dividing cells, these cells will not enter mitosis until the inhibitors are removed and replication is completed. Also, when cells in S phase are fused to cells in the G2 phase of the cell cycle, the G2 nucleus is delayed from entering mitosis until the S phase nucleus has completed replication (Rao and Johnson, 1970). Inhibition of MPF activation by unreplicated DNA has been shown to be mediated by phosphorylation of its cdc2 subunit on inhibitory residues, in-

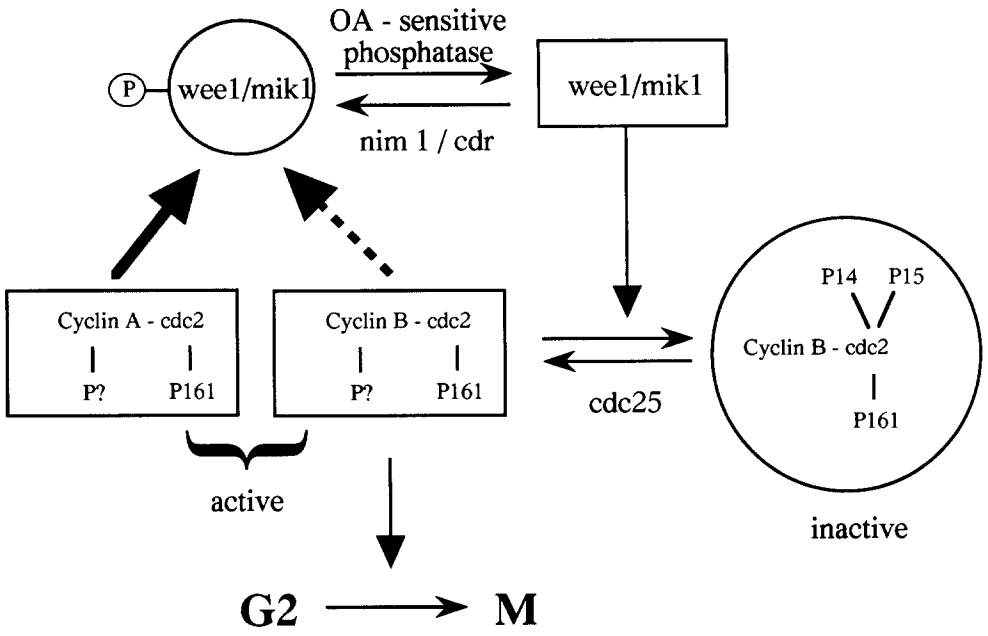

Figure 10. Model for cyclin A potentiation of MPF activation via inhibition of the tyrosine/threonine kinases that phosphorylate cdc2. Cyclin B–cdc2 kinase (MPF) exists in either of two major forms, an inactive one with cdc2 phosphorylated on residues 14, 15, and 161, and an active one, with cdc2 phosphorylated on threonine 161 only, which drives the cell from G2 to M phase. The equilibrium between both forms depends on the activity of homologues of the fission yeast wee 1/mik 1 and cdc25 gene products, which respectively promote phosphorylation and dephosphorylate Thr 14 and Tyr 15. The wee 1/mik 1 kinases are themselves negatively controlled by a phosphorylation event catalyzed by homologues of the fission yeast nim1/cdr kinase, which is antagonized by an OA-sensitive, most likely type 2A, phosphatase. Cyclin A– and cyclin B–cdc2 complexes accumulate progressively after their destruction at metaphase of the preceding cell cycle. Both cyclin A– and cyclin B–cdc2 kinases activities can in principle displace the wee 1/mik 1 equilibrium from its active to its inactive state (by an unknown mechanism), but in contrast to cyclin B–cdc2, most cyclin A–cdc2 complex escapes inhibitory phosphorylation on cdc2, and once formed it gains without lag phase kinase activity. This explains why it plays the major (the earlier) role in inactivating wee 1/mik 1.

cluding Tyr 15 (Kumagai and Dunphy, 1991; Meijer et al., 1991). It seems that cdc25 is part of the linkage between DNA replication and mitosis: indeed fission yeast mutants in cdc2 that are insensitive to cdc25 divide in the presence of hydroxyurea, an inhibitor of DNA replication (Enoch and Nurse, 1990).

Recently, it was reported that ablation of cyclin A overcame an aphidicolin block in *Xenopus* extracts containing sperm chromatin, and thereby induced premature entry into mitosis (Walker and Maller, 1991). Ablation of cyclin A had no such effect in the absence of added nuclei. This was taken as indicating that association of cyclin A with the nucleus was required for unreplicated DNA to block MPF activation. Actually, cyclin A rapidly shuttles in the nucleus of somatic cells after its translation which starts at late G1 (Pines and Hunter, 1991; Girard et al., 1991), in contrast to cyclin B which does not appear to enter the nucleus before prophase. Since cdc25 protein is also primarily localized in the nucleus during interphase (Millar et al., 1991), this suggested that cyclin A could act in the nucleus, most likely in association with either cdc2 or cdk2, and prevent cdc25 from activating cyclin B–cdc2 kinase.

Cyclin A may however play a different role in early embryos where the dependence of M phase on S phase is not observed. The rate of sequestration of cyclin A by the nucleus would be expected to be reduced in early embryos, owing to their high ratio of cytoplasm to nuclei. In agreement with this view, cyclin A was shown to be cytoplasmic during interphase in the early *Drosophila* embryo, and to relocate to the nucleus only at late prophase (Lehner and O'Farrell, 1989; Maldonado-Codina and Glover, 1992). This may also hold true for cdc25, although information is lacking. Interestingly, null mutants of cyclin A were found to arrest at G2 before the sixteenth mitosis, after maternal cyclin A had been exhausted, even though they accumulated cyclin B. This suggested that cyclin A could be required for MPF activation in early embryos, even though it may not play this role in somatic cells, owing to its rapid sequestration in the nucleus.

We propose, therefore, that in early embryos cyclin A, being mostly cytoplasmic, forms spontaneously an active complex with cdc2 kinase that, by inactivating the cdc2-specific tyrosine kinase, prevents the inactivation of cyclin B–cdc2 kinase (Fig. 10). This might explain why early cell cycles are so rapid in most animals and would be in agreement with earlier reports that no tyrosine phosphorylation of cdc2 could be detected in *Xenopus* embryos during the second to the twelfth cell cycle (Ferrell et al., 1991) and that the tyrosine kinase that phosphorylates cdc2 functions in the cytoplasm (Kumagai and Dunphy, 1991). Yet, we do not know how cyclin A–cdc2 kinase acts to inactivate the cdc2-specific tyrosine kinase. Nonetheless, we demonstrated that okadaic acid strongly reduced phosphorylation of both Tyr 15 and Thr 14 on cyclin B–associated cdc2, even in the presence of vanadate, which suppresses cdc25 activity. This suggested that the kinase(s) which phosphorylate(s) cdc2 on inhibitory sites could be inactivated by a phosphorylation event, itself antagonized by an OA-sensitive phosphatase. In agreement with this view, we showed that 6-DMAP, a reported inhibitor of serine-threonine kinases, suppresses OA-dependent activation of cyclin B–cdc2 complexes.

In fission yeast, two protein kinases, wee 1 and mik 1, act together to maintain cdc2 in the tyrosine phosphorylated, inactive state (Russell and Nurse, 1987; Lundgren et al., 1991). The wee 1 kinase is able to phosphorylate both seryl and tyrosyl residues in vitro (Featherstone and Russell, 1991). Although wee 1 has not been shown to phosphorylate

cdc2 directly, co-expression of cdc2 with wee 1 in insect cells using a baculovirus vector results in phosphorylation of cdc2 on tyrosine, and this phosphorylation is greatly stimulated by co-expressed cyclin (Parker et al., 1991). These results stongly suggest that wee 1 and mik 1, and their homologues in higher eukaryotes, catalyze phosphorylation of Tyr 15 and Thr 14 on cyclin-associated cdc2.

Interestingly, a gene encoding a putative serine/threonine kinase, nim 1/cdr, has been described as a mitotic inducer which operates through negative regulation of wee 1 (Russell and Nurse, 1987; Feilotter et al., 1991). To our knowledge, it has not been investigated whether mutations in nim 1/cdr are epistatic to mutations in ppa2, the gene encoding the major type 2A phosphatase in fission yeast (Kinoshita et al., 1990). Our results suggest that type 2A phosphatase might antagonize the products of nim1/cdr homologues, if they exist in higher eukaryotes.

Although cdc25 is actually phosphorylated and thus potentially regulated during cell cycle (Moreno et al., 1990), it is unclear whether phosphorylation of cdc25 might be a late event in the process of cdc2 kinase activation during early development. Indeed the inactive cyclin B–cdc2 complex stored in starfish or *Xenopus* oocytes activates either spontaneously in crude extracts depleted of ATP (Labbé et al., 1988, 1989*b*) or after purification to near homogeneity when non-phosphorylated recombinant cdc25 is added (Strausfeld et al., 1991). Moreover cdc25 extracted from G2-arrested oocytes is reported to be biologically as active as that from metaphase-arrested eggs (Jessus and Beach, 1992). Phosphorylation of cdc25 might serve other purposes and regulate for example its nuclear localization or its association with other components during cell cycle. Nonetheless we did not address in the present work the question as to whether cyclin A–cdc2 kinase also activates cdc25 in egg extracts, besides inactivating the tyrosine kinase which phosphorylates cdc2 on inhibitory sites. This remains open for future investigations.

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