Phosphorylation and Activation of the *Xenopus* Cdc25 Phosphatase in the Absence of Cdc2 and Cdk2 Kinase Activity

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The M-phase inducer, Cdc25C, is a dual-specificity phosphatase that directly phosphorylates and activates the cyclin B/Cdc2 kinase complex, leading to initiation of mitosis. Cdc25 itself is activated at the G₂/M transition by phosphorylation on serine and threonine residues. Previously, it was demonstrated that Cdc2 kinase is capable of phosphorylating and activating Cdc25, suggesting the existence of a positive feedback loop. In the present study, kinases other than Cdc2 that can phosphorylate and activate Cdc25 were investigated. Cdc25 was found to be phosphorylated and activated by cyclin A/Cdk2 and cyclin E/Cdk2 in vitro. However, in interphase *Xenopus* egg extracts with no detectable Cdc2 and Cdk2, treatment with the phosphatase inhibitor microcystin activated a distinct kinase that could phosphorylate and activate Cdc25. Microcystin also induced other mitotic phenomena such as chromosome condensation and nuclear envelope breakdown in extracts containing less than 5% of the mitotic level of Cdc2 kinase activity. These findings implicate a kinase other than Cdc2 and Cdk2 that may initially activate Cdc25 in vivo and suggest that this kinase may also phosphorylate M-phase substrates even in the absence of Cdc2 kinase.

INTRODUCTION

Cdc25 is a dual-specificity phosphatase that directly activates the Cdc2 kinase at the G₂/M transition by dephosphorylating Tyr15 and probably Thr14 in Cdc2 (for review see Millar and Russell, 1992; Dunphy, 1994; Maller, 1994). Although in *Schizosaccharomyces pombe* and *Drosophila*, Cdc25 accumulation acts in a dose-dependent manner to promote entry into mitosis (Russell and Nurse, 1986; Edgar and O'Farrell, 1989), in vertebrate cells Cdc25 levels are invariant throughout the cell cycle (Millar *et al.*, 1991; Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). This implies that in higher eucaryotes the regulation of Cdc25 involves post-translational modifications. Indeed, the intrinsic Cdc25 phosphatase activity has been shown to be directly increased by phosphorylation, with a high in M-phase and a low in interphase (Izumi *et al.*, 1992;

Kumagai and Dunphy, 1992). One of the kinases that activates Cdc25, at least in vitro, is the cyclin B/Cdc2 complex, a target of Cdc25 (Hoffman *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994). This has suggested the existence of a positive feedback loop between Cdc2 and Cdc25, which may explain the autoamplification of maturation-promoting factor (MPF)¹ activity in oocytes injected with catalytic amounts of either MPF or Cdc25. The positive feedback loop may also contribute to the irreversible nature of Cdc2 activation at the G₂/M transition, and perhaps the abruptness of entry into M-phase. Because Cdc2 and Cdc25 are reported to form a protein complex in vivo, at least in M-phase (Gautier *et al.*, 1991; Jessus and Beach, 1992), it is likely that Cdc25 is a physiological substrate for Cdc2. However, the ini-

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¹ Abbreviations used: CaMKII, calmodulin-dependent kinase II; CSF, cytotostatic factor; DAPI, 4',6-diamidino-2-phenylindole-dihydrochloride; GST, glutathione S-transferase; MPF, maturation-promoting factor; NEBD, nuclear envelope breakdown.

tial trigger that causes Cdc25 to become active at the G₂/M transition remains unknown.

Here we investigated whether other kinases exist that can phosphorylate and activate Cdc25. For this analysis, we have utilized *Xenopus* egg extracts (CSF extracts) that carry out cell cycle transitions in vitro. Such extracts begin in metaphase with high total H1 kinase activity, progress into S-phase with low H1 kinase activity, and then cycle back into M-phase with high H1 kinase activity, nuclear breakdown, and chromosome condensation. In the presence of sufficient nuclei, the DNA polymerase inhibitor aphidicolin will block re-entry into mitosis by activating the checkpoint that ensures the dependence of mitosis on completion of DNA replication (Dasso and Newport, 1990). One reason for suspecting that kinases other than Cdc2 exist that act on Cdc25 is that premature initiation of mitosis by okadaic acid (OA) in aphidicolin-blocked extracts results in substantial activation of Cdc25 before activation of cyclin A/or cyclin B/Cdc2 complexes (Izumi *et al.*, 1992). In addition, two-dimensional phosphopeptide maps of Cdc25 during mitosis have additional spots compared with maps of Cdc25 phosphorylated in vitro by cyclin B/Cdc2 (Izumi *et al.*, 1992). Here we report that both cyclin A/Cdk2 and cyclin E/Cdk2 can phosphorylate and activate Cdc25 in vitro. Furthermore, in interphase extracts devoid of Cdc2 and Cdk2, we detected another protein kinase that could phosphorylate and activate Cdc25 in the presence of the phosphatase inhibitor microcystin. Although the nature and the physiological significance of this kinase remain unknown, it may have an important role both as a trigger for entry into mitosis and for phosphorylation of various M-phase substrates.

MATERIALS AND METHODS

Xenopus Egg Extracts

Metaphase II-arrested cytosolic factor (CSF) extracts were prepared as described (Murray, 1991) except that leupeptin was omitted from all buffers. Interphase extracts were derived from CSF extracts by addition of 150 μ M emetine, 100 μ M puromycin, and 400 μ M CaCl₂ to release metaphase II arrest, followed by incubation at 23°C for 40 min. In these extracts, protein synthesis was totally suppressed as judged by ³⁵S-methionine incorporation. Routinely, 3 μ M microcystin and extraction buffer (EB) (80 mM β -glycerophosphate, pH 7.3, 15 mM MgCl₂, and 20 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid [final concentration]) were added to the interphase extracts at 40 min after CaCl₂ addition. Although the addition of EB was not necessary to detect the effects of microcystin on the shift of Cdc25 and other mitotic phenomena, EB was added simultaneously with microcystin in most of the experiments described here to accelerate the effects of microcystin. Demembrated sperm nuclei were added at a concentration of 300/ μ l and visualized by fluorescence microscopy with the DNA-binding dye 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) (1 μ g/ml).

Cdc2- and Cdk2-depleted egg extracts were prepared as follows: CSF extracts were mixed with 1 vol of EB and centrifuged at 15 000 $\times$ g for 10 min at 4°C. The cytosol fraction derived from 200 μ l of original extract was incubated (four times for 45 min each time) with either control beads or p13^{suc1}-Sepharose beads (60 μ l vol of beads,

20 mg p13/ml resin) at 4°C under constant agitation. The beads were removed each time by centrifugation at 2000 $\times$ g for 1 min.

Antibodies

Immunoblotting and immunoprecipitation were done using polyclonal antibodies directed toward either bacterially expressed cyclin A, B1, B2, or Cdc25 (Izumi and Maller, 1991; Izumi *et al.*, 1992), or peptides encoding the C-terminus of Cdc2 (CDLKDSSLDPNQIRN) or Cdk2 (THPFFRDVSRPTPHLI) (Gabrielli *et al.*, 1992). *Xenopus* lamin III was monitored with monoclonal antibody L6-5D5 (Stick, 1988).

Assays of Cdc25 Activity

Endogenous Cdc25 activity was measured in immunocomplexes by its ability to dephosphorylate and activate tyrosine-phosphorylated cyclin B/Cdc2. These experiments were done using an antibody against a peptide encoding the C-terminus of Cdc25 (CKTSVG-DRKRREQVA) as described previously (Izumi *et al.*, 1992), except that tyrosine-phosphorylated cyclin B/Cdc2 was isolated from stage VI oocytes and immunoprecipitation was done in the presence of 1 μ M microcystin. Recombinant Cdc25 protein was expressed in baculovirus-infected Sf9 cells as a fusion protein with glutathione S-transferase (GST), with a hemagglutinin (HA) tag at the C-terminus. Both wild-type and mutant T3 (Thr48, Thr67, and Thr138 mutations) and T3S2 (T3 plus Ser205 and Ser285 mutations) GST-Cdc25-C1HA proteins were produced in baculovirus-infected Sf9 cells and purified as described previously (Izumi and Maller, 1993). The activity of recombinant GST-Cdc25 proteins was assayed after recovery on glutathione-agarose beads as described (Izumi and Maller, 1993).

Kinase Assays and Analysis of Phosphorylation

Histone H1 kinase assays were performed at 30°C for 15 min, and phosphorylation of GST-Cdc25 protein in vitro was done as described previously (Izumi and Maller, 1993). The p13^{suc1}-precipitable H1 kinase assay was done using p13^{suc1}-Sepharose beads as previously described (Brizuela *et al.*, 1987; Gabrielli *et al.*, 1992). Kinase assays with CaMKII were done in the presence of 300 μ M CaCl₂ and 30 μ g/ml calmodulin. ³²P-labeling of egg extracts and two-dimensional tryptic phosphopeptide mapping were performed as described (Izumi *et al.*, 1992; Izumi and Maller, 1993).

RESULTS

Cdc25 Can Be Phosphorylated and Activated without Cyclin Synthesis or Cdc2

Previous studies have shown that an embryonic cell cycle of alternating M-phase and S-phase can be reconstituted in a cell-free system from *Xenopus* eggs (CSF extracts, Lohka and Maller, 1985; Murray and Kirschner, 1989). These cell cycles require the synthesis of cyclin B and remain in interphase after completion of DNA synthesis if cyclin B is absent. To examine whether there are kinase(s) other than Cdc2 that are able to activate Cdc25, metaphase II-arrested CSF extracts were treated with emetine and puromycin to inhibit protein synthesis before release of metaphase arrest by calcium. Upon calcium addition, pre-existing M-phase A- and B-type cyclins are degraded, and no new cyclins can be synthesized. Such extracts contain no detectable cyclin A, B1, or B2 as judged by Western blotting. Because of the inhibition of cyclin synthesis,

Figure 1. Phosphorylation and activation of Cdc25 without Cdc2 kinase activity. Metaphase-arrested CSF egg extracts were incubated with or without emetine and puromycin to inhibit protein synthesis. Forty minutes after Ca^{2+} addition to release extracts from metaphase arrest, 1600 U/ μl of recombinant Inh-2 or 3 μM microcystin with EB was added to samples of the emetine- and puromycin-treated extracts. Immunoblotting of Cdc25 (A) or the activity of Cdc25 (B) is shown. Cdc2 immunocomplex H1 kinase activity (C) and p13^{suc1}-precipitable H1 kinase activity (D) are also shown in the emetine- and puromycin-treated extracts with microcystin added at 40 min. (E) Recombinant wild-type (lanes 1–4), mutant T3 (lanes 5–8), and T3S2 (lanes 9–12) GST-Cdc25-C1HA proteins (final concentration 5 ng/ μl) were incubated with either metaphase (M) (lanes 1, 2, 5, 6, 9, and 10) or emetine- and puromycin-treated interphase extracts (I) (lanes 3, 4, 7, 8, 11, and 12) with (lanes 2, 4, 6, 8, 10, and 12) or without (lanes 1, 3, 5, 7, 9, and 11) microcystin. Immunoblotting of Cdc25 is shown. Note that endogenous Cdc25 and its shift were also detected.

entry into M-phase is blocked after completion of DNA synthesis and Cdc25 remains in its faster-migrating electrophoretic form at 74 kDa (Figure 1A, lanes 5 and 6), a position characteristic of interphase (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). The M-phase phosphorylation sites of Cdc25 are substrates for both protein phosphatase 1 and 2A in vitro (Izumi

et al., 1992), and OA or microcystin, inhibitors of both phosphatases, have been shown to cause electrophoretic shifts and activation of interphase Cdc25 (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). OA or microcystin also cause premature entry into mitosis under some conditions, so it seemed possible either could lead to activation of Cdc25 kinases. To investi-

gate this possibility, microcystin was added to emetine- and puromycin-treated extracts at 40 min after calcium addition, when almost all of the pre-existing mitotic cyclins had been degraded as judged by immunoblotting and when DNA synthesis had been completed. EB was also added simultaneously at 40 min because EB has been found to enhance the electrophoretic shift of Cdc25 at M-phase (J. Kuang, personal communication). Within 20 min after addition of microcystin, Cdc25 was shifted up to 96 kDa, the position seen in control M-phase extracts (Figure 1A, lanes 9 and 10). Furthermore, the protein phosphatase activity of Cdc25 was comparable to that of control M-phase extracts (Figure 1B). In contrast, recombinant phosphatase inhibitor-2 (Inh-2), a specific inhibitor of protein phosphatase 1, had no effect on phosphorylation and activation of Cdc25 (Figure 1A, lanes 7 and 8; Figure 1B). This result suggests a role for protein phosphatase 2A rather than protein phosphatase 1 to inactivate by dephosphorylation either Cdc25 itself or Cdc25-activating kinase(s), although the existence of forms of protein phosphatase 1 resistant to Inh-2 cannot be excluded. Cdc2 immunocomplex kinase assays detected little histone H1 kinase activity in these extracts even after the addition of microcystin (less than 5% of that in an M-phase extract) (Figure 1C). $p13^{suc1}$ -precipitable H1 kinase activity showed slightly greater activity compared with Cdc2 kinase activity in interphase extracts (about 10% of that in M-phase) (Figure 1D). This probably reflects cyclin E/Cdk2 activity, which also has affinity for $p13^{suc1}$ (Minshull *et al.*, 1990; Gabrielli *et al.*, 1992), because cyclin E is not degraded upon M-phase exit, and cyclin E/Cdk2 shows significant activity even in interphase extracts treated with emetine (about one-third of M-phase Cdk2 activity) (Rempel *et al.*, 1995). These results indicate that Cdc25 can be completely phosphorylated, shifted, and activated in the almost total absence of Cdc2 kinase activity after addition of microcystin.

The experiments described above analyzed endogenous Cdc25. Previously, we have expressed in baculovirus-infected Sf9 cells both wild-type Cdc25 and mutants with substitutions in the phosphorylation sites required for activation (Izumi and Maller, 1993). Therefore, we also incubated exogenous recombinant GST-Cdc25 proteins in these extracts. Wild-type GST-Cdc25 was shifted from 104 kDa, the position of the unphosphorylated form, to 128 kDa and 132 kDa in control and microcystin-treated M-phase extracts, respectively (Figure 1E, lanes 1 and 2). It was also shifted up to 128 kDa in microcystin-treated interphase extracts, in contrast to control interphase extracts (105 kDa) (Figure 1E, lanes 3 and 4). Previously, mutant T3 and T3S2 Cdc25 (in which the Ser/Pro and Thr/Pro sites conserved between human and *Xenopus* Cdc25-C have been mutated) exhibited greatly reduced electrophoretic shifts and activation both in

Figure 2. Phosphorylation and activation of Cdc25 by cyclin A/Cdk2 and cyclin E/Cdk2 in vitro. GST-Cdc25-C1HA-W (0.3 μ g) was phosphorylated without any additions (lane 7) or with GST-cyclin E/Cdk2 (lanes 1 and 5), GST-cyclin A/Cdk2 (lanes 2 and 4), cyclin B/Cdc2 (MPF) (lane 3), or CaMKII (lane 6). The amount of kinase in each assay was 39 U, 3 U, 23 U, 11 U, and 10 U toward histone H1 (lanes 1–5), and 4 U toward synapsin I (lane 6), respectively (1 U is the amount of enzyme that catalyzes incorporation of 1 pmol of phosphate per minute into each substrate). Recombinant GST-cyclin E/Cdk2 and GST-cyclin A/Cdk2 expressed in baculovirus-infected Sf9 cells were generous gifts from Dr. Helen Piwnicka-Worms (Washington University). Purified CaMKII from bovine brain was a generous gift of Dr. Michael D. Browning (University of Colorado). An autoradiogram of the gel is shown. The arrow indicates the position of Coomassie blue-stained unphosphorylated GST-cdc25C1HA-W.

M-phase extracts and after phosphorylation by cyclin B/Cdc2 in vitro (Izumi and Maller, 1993). In the present experiments, these mutants again showed much smaller shifts in both control M-phase and microcystin-treated interphase extracts (111 kDa) (Figure 1E, lanes 5, 8, 9, and 12). The similar behavior of the T3 and T3S2 mutant proteins in the two extracts suggests that the T3S2 sites are the targets of phosphorylation and activation in both cases. However, it should be noted that the shift of the wild-type Cdc25 protein was somewhat greater in extracts as compared with that after phosphorylation by cyclin B/Cdc2 in vitro (Izumi and Maller, 1993; see also Figure 2), and greatly reduced, but still detectable, shifts of the T3 and T3S2 mutant Cdc25 proteins were evident in microcystin-treated interphase extracts (Figure 1E). These findings suggest that there exists a kinase(s) other than Cdc2 that can phosphorylate Cdc25 at both the T3S2 sites and other sites.

Cdc25 Can Be Phosphorylated and Activated by Cyclin E/Cdk2, Cyclin A/Cdk2, and CaMKII In Vitro

Because there is significant Cdk2 activity in emetine- and puromycin-treated extracts (Gabrielli *et al.*, 1992;

Rempel *et al.*, 1995), we next examined whether Cdk2 can phosphorylate and activate Cdc25 in vitro, although a previous study reported that Cdk2 is unable to phosphorylate human Cdc25 in vitro (Hoffman *et al.*, 1993). We found that baculovirus-expressed Cdk2 complexed with either cyclin E or cyclin A could phosphorylate and shift Cdc25 in vitro (Figure 2, lanes 1 and 2). Although cyclin E/Cdk2 and cyclin A/Cdk2 showed less activity toward Cdc25 as compared with cyclin B/Cdk2 when each kinase activity was normalized with respect to activity toward histone H1 (Figure 2), other data indicate phosphopeptide maps produced after phosphorylation by each kinase are almost indistinguishable. Furthermore, other experiments showed that both cyclin A/Cdk2 and cyclin E/Cdk2 could activate Cdc25 in vitro. Combined with previous results showing that cyclin A/Cdk2 also can phosphorylate and activate Cdc25 in vitro (Izumi and Maller, 1993), these data suggest that every physiological set of cyclins and cdks (cyclin B/Cdk2, cyclin A/Cdk2, cyclin A/Cdk2, and cyclin E/Cdk2) can activate Cdc25 through phosphorylation of the same sites. Therefore, it seems unlikely that cyclin components of Cdk complexes confer different substrate specificity upon the complex; rather they may have other roles such as the targeting of their kinase partner to different subcellular locations.

Interestingly, CaMKII could also phosphorylate and shift GST-Cdc25 in vitro (Figure 2, lane 6). Preliminary results show that CaMKII also activates Cdc25 in vitro. Moreover, other experiments indicate that this kinase can shift mutant T3 and T3S2 Cdc25 protein to almost the same extent as wild-type Cdc25. This is consistent with the different consensus sites phosphorylated by CaMKII as compared with Cdc2. However, considering that these mutant Cdc25 proteins do not shift in extracts that undergo cell cycle transitions (Izumi and Maller, 1993; see also Figure 1E), Cdc25 is unlikely to be a physiological substrate for CaMKII or any other kinase with the same substrate specificity. Furthermore, because microcystin-induced Cdc25 phosphorylation was augmented in the presence of EB containing 20 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (J. Kuang, personal communication, and our unpublished results), it is unlikely that CaMKII, which requires Ca^{2+} for its activity, is responsible for microcystin-induced Cdc25 phosphorylation (Figure 1A). However, the possibility remains that this kinase is involved in Cdc25 activation in other cell types or under other conditions, perhaps with different Cdc25 isoforms. In any case, this finding indicates that a kinase with a substrate specificity completely different from that of cyclin-dependent kinases can phosphorylate, shift, and activate Cdc25.

Cdc25 Can Be Phosphorylated and Activated without Cdc2 and Cdk2 Activity in Extracts

It is possible that the Cdc2-independent phosphorylation and activation of Cdc25 in extracts is mediated by Cdk2 because there is significant cyclin E/Cdk2 activity in microcystin-treated interphase extracts (Gabrielli *et al.*, 1992; Rempel *et al.*, 1995), and this kinase can phosphorylate and activate Cdc25 in vitro (Figure 2). To evaluate possible effects of Cdk2 toward phosphorylation of Cdc25, emetine- and puromycin-treated interphase extracts were incubated repeatedly with p13^{suc1}-beads. Both Cdc2 and Cdk2 were completely depleted in p13^{suc1}-treated extracts as judged by immunoblotting with high-affinity antibodies (Figure 3A). Because a significant increase of total H1 kinase activity was observed in emetine- and puromycin-treated interphase extracts after addition of microcystin, total H1 kinase activity was also examined in Cdc2- and Cdk2-depleted extracts after addition of microcystin. Although control interphase extracts still showed a significant increase in H1 kinase activity with microcystin, Cdc2- and Cdk2-depleted extracts showed no increase (Figure 3B). Importantly, however, in these Cdc2- and Cdk2-depleted extracts, Cdc25 was still phosphorylated and shifted after addition of microcystin to a level similar to that found with microcystin addition to control interphase extracts (Figure 3C). There was a consistent subtle difference in the maximal shifts between these two extracts (96 kDa and 94 kDa in control and Cdc2- and Cdk2-depleted extracts, respectively) (Figure 3C), suggesting that Cdc2 and/or Cdk2 may be required for maximal phosphorylation of Cdc25 in microcystin-treated interphase extracts. Nevertheless, most of the phosphorylation and shift of Cdc25 could be attained in the absence of both Cdc2 and Cdk2. Other data show that Cdc25 activity in Cdc2- and Cdk2-depleted extracts treated with microcystin is also increased to the level observed without p13^{suc1}-bead treatment, as expected. This indicates that the kinase(s) responsible for most of the phosphorylation of Cdc25 in microcystin-treated interphase extracts is distinct from Cdc2 and Cdk2, although it appears to have a similar specificity for the T3S2 sites. We have named this novel activity Kinase X.

Partial Chromosome Condensation and Nuclear Envelope Breakdown in Microcystin-treated Interphase Extracts

Previously it was reported that OA, another inhibitor of protein phosphatase 1 and 2A, induces premature mitotic events such as chromosome condensation, the production of MPM-2 antigens, and dispersion of nuclear lamin in BHK21 cells (Yamashita *et al.*, 1990). In this cell line, cyclin B is already synthesized and complexed with Cdc2 in S-phase, and premature activa-

Figure 3. Phosphorylation and activation of Cdc25 after p13^{suc1}-bead depletion. Interphase extracts were treated with either control beads or p13^{suc1} beads as described in MATERIALS AND METHODS. (A) Cdc2 (lanes 1 and 2) and Cdk2 (lanes 3 and 4) were immunoblotted from either control bead-treated (lanes 1 and 3) or p13^{suc1} bead-treated extracts (lanes 2 and 4). Both Cdc2 and Cdk2 were efficiently depleted to undetectable levels after four successive incubations with p13^{suc1}-Sepharose beads. (B and C) Control-depleted (squares in B and lanes 1–5 in C) and Cdc2- and Cdk2-depleted (diamonds in B and lanes 6–10 in C) interphase extracts were incubated at 23°C with 3 μ M microcystin in the presence of emetine and puromycin. Samples were removed for total histone H1 kinase assay (B) at the indicated times and immunoblotted for

tion of Cdc2 kinase is correlated with mitotic phenomena after addition of OA, suggesting that Cdc2 activity is required for OA-induced premature mitosis (Yamashita *et al.*, 1990). Because in the present study Cdc25 phosphorylation characteristic of M-phase was found in microcystin-treated extracts even in the absence of Cdc2 kinase activity, we next examined whether other M-phase events also occur in these extracts. In emetine- and puromycin-treated extracts, sperm nuclei still acquired a nuclear envelope (Figure 4, panel a). With time they increased in size, reflecting the import of nuclear materials from the extract (Figure 4, panels e and f). Chromosome condensation also occurred at least partially, although it took longer to develop compared with a control extract (compare Figure 4, panels b–f). However, nuclear envelope breakdown (NEBD) never occurred in these extracts (Figure 4, panels e and f). In contrast, the addition of microcystin promptly (within 20 min) caused chromosome condensation and NEBD to occur (Figure 4, panel g). After microcystin treatment, chromosomes tended to be smaller and less condensed compared with those in control M-phase extracts, probably because of premature entry into mitosis (compare Figure 4, panels d and g–i). However, some of the chromosomes in microcystin-treated extracts were almost indistinguishable from those in control M-phase extracts (Figure 4, panels j and k). This observation indicates that NEBD and at least partial chromosome condensation can occur in the presence of microcystin without Cdc2 kinase activity. The addition of partially purified MPF (active cyclin B/Cdc2) to a *Xenopus* egg extract causes activation of nuclear disassembly factors (Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985). Under physiological conditions, MPF may induce NEBD by activating other kinases, inactivating protein phosphatases, or directly phosphorylating substrates involved in specific mitotic events such as NEBD. Our results suggest that MPF may have an important role in activating other kinases that can carry out specific mitotic events. It is possible that other kinases are also responsible for M-phase phenomena in BHK21 cells treated with OA (Yamashita *et al.*, 1990).

We examined the state of the microtubules in these extracts by both immunofluorescence staining and polarization microscopy. Microcystin caused almost complete disassembly of microtubule structures in both control M-phase and interphase extracts (G. Sluder, T.I., and J.L.M., unpublished observation),

Cdc25 (C) at the following times: 0 min, lanes 1 and 6; 10 min, lanes 2 and 7; 20 min, lanes 3 and 8; 30 min, lanes 4 and 9; 40 min, lanes 5 and 10. All the total H1 kinase assays were done in the presence of 3 μ M microcystin to make certain phosphatases were fully inactivated in all samples.

Figure 4. Nuclear morphology in microcystin-treated extracts in the presence of emetine and puromycin. Extracts were made as in Figure 1. Demembrated sperm nuclei were visualized by fluorescence microscopy with the DNA-binding dye DAPI. At the times indicated, samples were withdrawn from control extracts (panels b-d) or from emetine- and puromycin-treated extracts without (panels a, e, and f) or with (panels g-k) the addition of microcystin and EB at 40 min, and monitored by both phase-contrast (top) and DAPI fluorescence microscopy (bottom). As described in the text, some of the chromosomes in microcystin-treated extracts (panels g-i) tended to be smaller and less condensed compared with those in control M-phase extracts (panel d), but many were almost indistinguishable from those in M-phase (panels j and k).

consistent with results in OA-treated BHK21 cells (Yamashita *et al.*, 1990) and in starfish eggs and embryos (Picard *et al.*, 1989). Therefore, we could not evaluate mitotic spindle morphology in microcystin-treated extracts.

M-Phase Phosphorylation of Lamin L_{III} Does Not Occur in Cdc2- and Cdk2-depleted Extracts

Nuclear morphology is difficult to assess after p13^{suc1}-bead depletion of Cdc2 and Cdk2 because the successive centrifugation steps in the depletion protocol remove particulate components essential for nuclear envelope formation (Lohka and Masui, 1984). Therefore we examined other biochemical M-phase characteristics, such as the phosphorylation state of lamins in microcystin-treated extracts. Lamin, which polymerizes to form the lamina lying on the inner side of the nuclear membrane, is heavily phosphorylated at mi-

toxis (Gerace and Blobel, 1980), and this phosphorylation is believed to cause disassembly of the nuclear envelope. Cdc2 can phosphorylate lamin in vitro at sites phosphorylated in vivo during M-phase (Peter *et al.*, 1990). Furthermore, analysis of lamin mutants has shown that two in vitro phosphorylation sites for Cdc2 in human lamin, Ser22 and Ser392, are important for disassembly of lamin filaments (Heald and McKeon, 1990; Ward and Kirschner, 1990). However, the relative intensities of phosphorylation of individual peptides are not identical between lamin phosphorylated by Cdc2 in vitro and that phosphorylated in vivo during M-phase (Peter *et al.*, 1990; Lüscher *et al.*, 1991), and some additional phosphopeptides can be detected only in vivo (Lüscher *et al.*, 1991), suggesting the existence of kinase(s) other than Cdc2 that can phosphorylate lamin. The idea that other kinases are important for lamin phosphorylation is also suggested

by the finding that the rate of rat liver lamin phosphorylation by Cdc2 in vitro is only 1% of that of H1 (Gabrielli *et al.*, 1992). This situation is similar to that of Cdc25 phosphorylated by Cdc2 in vitro compared with that phosphorylated in M-phase extracts. In the case of *Xenopus* Cdc25, the phosphopeptides of Cdc25 phosphorylated in M-phase extracts include most of those phosphorylated by cyclin B/Cdc2 in vitro, but several additional phosphopeptides are also evident (Izumi and Maller, 1993). Moreover, the crucial activating T3S2 sites in Cdc25 are not the sites most heavily phosphorylated by Cdc2 in vitro (Izumi and Maller, 1993).

Xenopus eggs contain a special form of lamin called L_{III} (Stick, 1988). This lamin has two Ser/Pro sites homologous to those in human lamin, which are important for disassembly (Stick, 1988; Heald and McKeon, 1990). As expected, lamin L_{III} was heavily phosphorylated in control M-phase extracts, whereas it was barely phosphorylated in interphase (Figure 5A, lanes a and b). In microcystin-treated interphase extracts, lamin L_{III} also appeared heavily phosphorylated when Cdc2 kinase activity was absent (Figure 5A, lane c). In interphase, lamin was phosphorylated mainly on one site (Figure 5B, panel b, spot 3), whereas it was phosphorylated on two major sites in M-phase (Figure 5B, panel a, spots 1 and 2). Interestingly, phosphorylation of an interphase site (spot 3) was largely suppressed in M-phase. On the other hand, in microcystin-treated interphase extracts, lamin was phosphorylated on both interphase (spot 3) and M-phase (spots 1 and 2) sites in addition to one new site (spot 4) (Figure 5B, panel c). Because the MAP kinase/S6 kinase pathway was also activated in these microcystin-treated extracts (B. VanRenterghem, T.I., and J.L.M., unpublished observation), and S6 kinase II (rsk) has been reported to phosphorylate lamin in vitro (Ward and Kirschner, 1990), phosphorylation of this new site may be induced by S6 kinase II. However, lamin L_{III} was not phosphorylated at M-phase-specific sites after microcystin treatment of extracts depleted of Cdc2 and Cdk2 by p13 beads (Figure 5B, panels d and e). This finding that lamin L_{III}, unlike Cdc25, is not phosphorylated at M-phase-specific sites in microcystin-treated Cdc2- and Cdk2-depleted extracts strongly suggests the involvement of Cdc2 and/or Cdk2 in lamin phosphorylation in vivo. Furthermore, Cdc2/Cdk2 are also strongly implicated as physiological lamin kinases because M-phase-specific sites were more highly phosphorylated in control M-phase extracts than in microcystin-treated interphase extracts (Figure 5B, panels a and c). This result also provides additional evidence that Kinase X activity is distinct from Cdc2/Cdk2.

Figure 5. Phosphorylation of lamin L_{III} in microcystin-treated extracts. (A) Extracts were incubated with 5 mCi/ml [γ -³²P]ATP for 30 min prior to CSF release. Lamin L_{III} was immunoprecipitated with monoclonal antibody L6-5D5 (Stick, 1988) from control M-phase (no CSF release) (lane a), interphase extracts without (lane b), or with 3 μ M microcystin and EB for 40 min (lane c). The immunoprecipitate was resolved by SDS gel electrophoresis and autoradiographed. (B) Two-dimensional tryptic phosphopeptide mapping was performed on lamin L_{III} immunoprecipitated from control M-phase extracts (panel a), interphase extracts without (panel b) or with microcystin and EB (panel c), and Cdc2- and Cdk2-depleted interphase extracts with microcystin (panel d). In panel e, two-dimensional phosphopeptide mapping of a mixture of samples a and d is shown.

DISCUSSION

This study has examined how Cdc2 kinase contributes to M-phase phosphorylation using extracts in which new protein synthesis is inhibited and in which Cdc2 and Cdk2 are depleted. The initial focus was on Cdc25, a direct activator of cyclin B/Cdc2 that is itself activated by phosphorylation in M-phase (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992). Although the

physiological kinase(s) responsible for initial Cdc25 activation has not been established, cyclin B/Cdc2 itself has been thought to be at least partly responsible for M-phase Cdc25 phosphorylation, based on the following five lines of evidence. First, cyclin B/Cdc2 can activate Cdc25 in vitro by phosphorylation, accompanied by a characteristic mobility shift in gels and a change in protein phosphatase activity similar to that found in M-phase in vivo (Hoffman *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994). Second, human Cdc25-C is phosphorylated on the same sites at mitosis in vivo and by cyclin B/Cdc2 in vitro (Hoffman *et al.*, 1993; Strausfeld *et al.*, 1994), although the phosphopeptide maps are very different in these two studies. In the case of *Xenopus* Cdc25, the phosphopeptides derived from Cdc25 in M-phase extracts include most of those phosphorylated by cyclin B/Cdc2 in vitro, but there are also several additional sites (Izumi and Maller, 1993). Third, Cdc2 and Cdc25 have been reported to be associated in M-phase in vivo (Gautier *et al.*, 1991; Jessus and Beach 1992). Fourth, prior thiophosphorylation of recombinant Cdc25 by cyclin B/Cdc2 is reported to be necessary to induce M-phase in *Xenopus* oocytes and mammalian cells (Hoffman *et al.*, 1993; Strausfeld *et al.*, 1994), although other work indicates no need for such prior phosphorylation (Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Lee *et al.*, 1992; Izumi and Maller, 1993). Finally, *Xenopus* Cdc25 T3S2 mutants, in which the putative Cdc2 phosphorylation sites that are conserved in human Cdc25 have been replaced, fail to undergo electrophoretic shifts or activation either in egg extracts or after phosphorylation by cyclin B/Cdc2 in vitro (Izumi and Maller, 1993). These mutants also exhibit less ability to induce M-phase in Cdc25-dependent oocyte extracts (Izumi and Maller, 1993), making positive feedback an important aspect of the mechanism for entry into mitosis. However, it is still unclear what the trigger of Cdc25 activation is that initiates the positive feedback loop. Our hypothesis is that another kinase may be the trigger, based on the peptide maps described above and because substantial activation of Cdc25 occurs before Cdc2 activation in aphidicolin-blocked egg extracts treated with OA (Izumi *et al.*, 1992).

To investigate phosphorylation and activation of Cdc25 without Cdc2 activity, extracts were treated with protein synthesis inhibitors to block cyclin A and B synthesis in interphase. Upon addition of microcystin, Cdc25 was almost fully activated as well as fully shifted by phosphorylation. However, these extracts still contain detectable Cdk2 activity (Rempel *et al.*, 1995) although it is less than 5% of M-phase Cdc2 activity. To remove any contribution of these activities, both Cdc2 and Cdk2 were completely removed from interphase extracts by p13^{suc1}-bead treatment (Figure 3A). Microcystin was still able to effectively

cause the phosphorylation, shift, and activation of Cdc25 in these depleted extracts. Although this result does not exclude the physiological involvement of Cdc2 and Cdk2 in Cdc25 phosphorylation in vivo, it indicates that a distinct Kinase X exists that can phosphorylate and activate Cdc25. This kinase would appear to have a substrate specificity related to but distinct from Cdc2 because it acts on the T3S2 sites without an increase in either H1 kinase activity (Figure 3) or in lamin mitotic site phosphorylation (Figure 5), suggesting its site specificity is more restricted compared with Cdc2. Purification and cloning of Kinase X will be required for absolute clarification of its relationship to Cdc2/Cdk2.

OA has been reported to induce premature mitosis and Cdc2 kinase activation, suggesting that Cdc2 is required for OA-induced premature mitosis (Yamashita *et al.*, 1990). However, it would also be predicted that the unknown *Xenopus* Kinase X described here would also phosphorylate and activate Cdc25 during OA-induced premature mitosis in the presence or absence of Cdc2 and Cdk2. Because Inh-2 was unable to induce Cdc25 activation, protein phosphatase 2A may be involved in regulation of this kinase. If this kinase is a physiological trigger for mitosis, it could be hypothesized that protein phosphatase 2A activity should change during mitotic initiation. Currently, there is no rigorous evidence for regulation of protein phosphatase 2A activity in the cell cycle. However, the length of the lag period between accumulation of cyclin to a threshold concentration and actual Cdc2 activation is regulated by the activity of protein phosphatase 2A in *Xenopus* egg extracts (Solomon *et al.*, 1990). If reduction of protein phosphatase 2A activity is indeed a part of the physiological trigger for mitosis, the unknown kinase toward Cdc25 identified here should have a role in inducing M-phase. From this standpoint, Lee *et al.* (1994) recently reported that the addition of protein phosphatase 2A affects Thr161 phosphorylation, not Tyr15 dephosphorylation, of Cdc2 during the lag period, and they suggest that Tyr 15 phosphorylation determined by the balance of Cdc25 and Wee1 activity is not part of the initial trigger for mitosis. However, it is not surprising that interphase Cdc25, which has minimal activity (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992), is not suppressed further by the addition of protein phosphatase 2A. Furthermore, there is no evidence for the existence of phosphorylation sites in interphase that have negative effects on Cdc25 activity (Izumi *et al.*, 1992). To evaluate the hypothesis of Lee *et al.* (1994), it should be investigated whether the addition of OA or microcystin influences Tyr15 dephosphorylation during the lag period after addition of cyclin B. On the basis of previous results indicating that both OA and vanadate (an inhibitor of tyrosine protein phosphatases) alter the length of the lag pe-

riod (Solomon *et al.*, 1990) and that OA causes activation of Cdc25 before cyclin B/Cdc2 activation (Izumi *et al.*, 1992), it is likely that the balance between Cdc25 and Wee1 activities determines the timing of the induction of M-phase in cycles where Tyr15 is phosphorylated.

Recently, M-phase Cdc25 has been reported to be one of the MPM-2 antigens (Kuang *et al.*, 1994). MPM-2 antibodies are believed to recognize a highly conserved epitope shared by many mitotic phosphoproteins (Davis *et al.*, 1983). Interestingly, the *Xenopus* kinases phosphorylating the MPM-2 epitope on interphase proteins are reported to be distinct from Cdc2 (Kuang and Ashorn, 1993), although it is thought that the substrate specificity is overlapping in some cases (J. Kuang, personal communication). It is possible that Kinase X, which phosphorylates Cdc25 in Cdc2- and Cdk2-depleted extracts, might be an MPM-2 epitope kinase. Protein kinase(s) that phosphorylate Cdc25 may also have other important substrates. *S. pombe* Wee1 has been reported to be inactivated by M-phase phosphorylation in *Xenopus* egg extracts, and its N-terminal half contains numerous Ser/Pro and Thr/Pro sites like those seen in Cdc25 (Tang *et al.*, 1993). *S. pombe* Wee1 can be phosphorylated and shifted up in Cdc2-depleted extracts to an extent similar to that in control extracts after addition of OA (Tang *et al.*, 1993), although the physiological relevance to *Xenopus* Wee1 remains to be clarified. Therefore, it is possible that the same Kinase X, distinct from Cdc2 and Cdk2, that activates Cdc25 could also trigger and/or maintain M-phase by inactivating Wee1.

Previously we have shown that cyclin A/Cdc2 can phosphorylate and activate Cdc25 in vitro (Izumi and Maller, 1993). In this study both cyclin E/Cdk2 and cyclin A/Cdk2 were also shown to phosphorylate and activate Cdc25 in vitro. Although the physiological role of these kinases for Cdc25 activation is unknown, they might have a role in cyclin B/Cdc2 activation and M-phase induction because they are activated in M-phase before cyclin B/Cdc2 is activated (Minshull *et al.*, 1990; Gabrielli *et al.*, 1992; Rempel *et al.*, 1995). In any case, these results clearly suggest that differences in kinase or cyclin components are unlikely to confer completely different substrate specificity on cyclin-dependent kinases. The reason for the discrepancy between our results and those of Hoffman *et al.* (1993), who failed to detect kinase activity toward Cdc25 with either cyclin A/Cdc2 or cyclin A/Cdk2, is unknown. However, other investigators have recently confirmed that both cyclin A/Cdc2 and cyclin A/Cdk2 can phosphorylate human Cdc25C in vitro (Strausfeld *et al.*, 1994).

In addition to Cdc25 phosphorylation, we observed some M-phase-like phenomena such as chromosome condensation, NEBD, and lamin phosphorylation in

Figure 6. Model for M-phase induction. The key features of this model are as follows: Cdc25 is activated by both Cdc2 and another unknown kinase (Kinase X) that is not Cdk2. Kinase X may also work in M-phase to directly phosphorylate M-phase substrates. The substrate specificity of Kinase X is expected to be similar to but distinct from that of Cdc2. Kinase X is proposed to be negatively regulated by either phosphatase 1 or 2A. The existence of a negative regulatory pathway from Cdc2 to phosphatases 1 and 2A has not yet been established.

the absence of cyclin synthesis after microcystin treatment. Among these phenomena, microcystin could not bypass a requirement for Cdc2/Cdk2 in the M-phase-specific phosphorylation of lamin, indicating that Cdc2/Cdk2 and/or other kinase(s) dependent on their activity are responsible for lamin phosphorylation in vivo. This is in agreement with previous results that show Cdc2 kinase can phosphorylate other forms of lamin (Peter *et al.*, 1990; Ward and Kirschner, 1990). Because of the technical difficulties in obtaining nuclear formation in Cdc2- and Cdk2-depleted extracts, it remains to be clarified whether chromosome condensation and NEBD after microcystin treatment are directly mediated by kinase(s) other than Cdc2 and Cdk2. Nevertheless, these microcystin-induced M-phase-like phenomena are different from those observed in BHK21 cells after OA treatment (Yamashita *et al.*, 1990), because they occurred without obvious cyclin B synthesis and Cdc2 activation in egg extracts. We consistently observed partial chromosome condensation in emetine- and puromycin-treated extracts even without addition of microcystin. These observations suggest that there are kinase(s) other than Cdc2 important for structural changes in the nucleus at mitosis. Our model for M-phase induction is presented in Figure 6. Currently it is unknown whether the activity of this kinase toward Cdc25 is negatively regulated by protein phosphatase 1 or 2A, or whether the inhibition

of both phosphatases is enough to cause increased phosphorylation of Cdc25 with a basal level of Kinase X activity.

The NimA protein kinase has been reported to be directly involved in some mitotic phenomena such as organization of the nuclear envelope and the mitotic spindle in *Aspergillus nidulans* (Osmani *et al.*, 1991b). However, Kinase X is unlikely to be a *Xenopus* homologue of NimA for the following reasons. First, NimA protein kinase has a very different substrate specificity from that of Cdc2 kinase (Osmani *et al.*, 1991b). The analysis of phosphorylation of Cdc25 at the T352 sites suggests that the substrate specificity of the kinase identified here should be very similar to that of Cdc2 kinase. Second, NimA alone cannot initiate mitotic events in the absence of Cdc2 (Osmani *et al.*, 1991a), in contrast to the results presented here, which show that certain mitotic events can be induced in the absence of Cdc2. Third, NimA is unable to phosphorylate *Xenopus* Cdc25 in vitro (S. Osmani, personal communication). Finally, at least some MPM-2 detected phosphorylation occurs without NimA protein kinase activity (Osmani *et al.*, 1991b).

Previously we and others have suggested that positive feedback between Cdc2/Cdk2 and Cdc25 is important for entry into M-phase. The present finding of a novel kinase that can also phosphorylate and activate Cdc25 does not necessarily reduce the importance of positive feedback. However, the ability of Kinase X to phosphorylate and activate Cdc25 in the absence of Cdc2 and Cdk2 may be important for the initial triggering of M-phase, which would tip the kinase/phosphatase balance in favor of the positive feedback loop. Currently we are purifying and characterizing this kinase from Cdc2-depleted extracts to gain further information about its physiological role in the induction of M-phase.

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REFERENCES

- Brizuela, L., Draetta, G., and Beach, D.H. (1987). p13^{suc1} acts in the fission yeast cell division cycle as a component of the p34^{cdc2} protein kinase. *EMBO J.* 6, 3507–3514.
- 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.
- Davis, F.M., Tsao, T.Y., Fowler, S.K., and Rao, P.N. (1983). Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci. USA* 80, 2926–2930.
- Dunphy, W.G. (1994). The decision to enter mitosis. *Trends Cell Biol.* 4, 202–207.
- Edgar, B.A., and O'Farrell, P.H. (1989). Genetic control of cell division patterns in the *Drosophila* embryo. *Cell* 57, 177–187.
- Gabrielli, B.G., Roy, L.M., Gautier, J., Philippe, M., and Maller, J.L. (1992). A cdc2-related kinase oscillates in the cell cycle independently of G2/M cyclins and cdc2. *J. Biol. Chem.* 267, 1969–1975.
- Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F., and Kirschner, M.W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34^{cdc2}. *Cell* 67, 197–211.
- Gerace, L., and Blobel, G. (1980). The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell* 19, 277–287.
- Heald, R., and McKeon, F. (1990). Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* 61, 579–589.
- Hoffman, 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.
- Izumi, T., and Maller, J.L. (1991). Phosphorylation of *Xenopus* cyclins B1 and B2 is not required for cell cycle transitions. *Mol. Cell. Biol.* 11, 3860–3867.
- Izumi, T., and Maller, J.L. (1993). Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M-phase. *Mol. Biol. Cell* 4, 1337–1350.
- Izumi, T., Walker, D.H., and Maller, J.L. (1992). Periodic changes in phosphorylation of the *Xenopus* cdc25 phosphatase regulate its activity. *Mol. Biol. Cell* 3, 927–939.
- Jessup, C., and Beach, D. (1992). Oscillation of MPF is accompanied by periodic association between cdc25 and cdc2-cyclin B. *Cell* 68, 323–332.
- Kuang, J., and Ashorn, C.L. (1993). Identification of two kinases which phosphorylate the MPM-2 epitope during *Xenopus* oocyte maturation. *J. Cell Biol.* 123, 859–868.
- Kuang, J., Ashorn, C.L., Gonzalez-Kuyvenhoven, M., and Penkala, J.E. (1994). cdc25 is one of the MPM-2 antigens involved in the activation of maturation-promoting factor. *Mol. Biol. Cell* 5, 135–154.
- Kumagai, A., and Dunphy, W.G. (1991). The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell* 6, 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.
- 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., Turck, C., and Kirschner, M.W. (1994). Inhibition of cdc2 activation by INH/PP2A. *Mol. Biol. Cell* 5, 323–388.

- Lohka, M., and Maller, J.L. (1985). Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. *J. Cell Biol.* 101, 518–523.
- Lohka, M., and Masui, Y. (1984). Roles of cytosol and cytoplasmic particles in nuclear envelope assembly and sperm pronuclear formation in cell-free preparations from amphibian eggs. *J. Cell. Biol.* 98, 1220–1230.
- Lüscher, B., Brizuela, L., Beach, D., and Eisenman, R.N. (1991). A role for the p34^{cdc2} kinase and phosphatases in the regulation of phosphorylation and disassembly of lamin B₂ during the cell cycle. *EMBO J.* 10, 865–875.
- Maller, J.L. (1994). Biochemistry of cell cycle checkpoints at the G2/M and metaphase/anaphase transitions. *Semin. Dev. Biol.* 5, 3061–3068.
- Miake-Lye, R., and Kirschner, M. (1985). Induction of early mitotic events in a cell-free system. *Cell* 1, 165–175.
- Millar, J.B.A., Blevitt, J., Gerace, L., Sadhu, K., Featherstone, C., and Russell, P. (1991). 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., and Russell, P. (1992). The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell* 68, 07–10.
- 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. (1991). Cell cycle extracts. *Methods Cell Biol.* 36, 581–605.
- Murray, A.W., and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. *Nature* 339, 275–280.
- Osmani, A.H., McGuire, S.L., and Osmani, S.A. (1991a). Parallel activation of the NimA and p34^{cdc2} cell cycle-regulated protein kinases is required to initiate mitosis in *A. nidulans*. *Cell* 67, 283–291.
- Osmani, A.H., O'Donnell, K., Pu, R.T., and Osmani, S.A. (1991b). Activation of the nimA protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the bimE checkpoint. *EMBO J.* 10, 2669–2679.
- Peter, M., Nakagawa, J., Dorée, M., Labbé, J.C., and Nigg, E.A. (1990). In vitro disassembly of the nuclear lamina and M-phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* 61, 591–602.
- Picard, A., Capony, J.P., Brautigan, D.L., and Dorée, M. (1989). Involvement of protein phosphatases 1 and 2A in the control of M-phase-promoting factor activity in starfish. *J. Cell Biol.* 109, 335–337.
- Rempel, R.E., Sleight, S.B., and Maller, J.L. (1995). Maternal Cdk2/cyclin E complexes function during meiotic and early embryonic cell cycles that lack a G₁ phase. *J. Biol. Chem. (in press)*.
- Russell, P., and Nurse, P. (1986). cdc25⁺ functions as an inducer in the mitotic control of fission yeast. *Cell* 45, 145–153.
- Solomon, M.J., Glotzer, M., Lee, T., Philippe, M., and Kirschner, M.W. (1990). Cyclin activation of p34^{cdc2}. *Cell* 63, 1013–1024.
- Stick, R. (1988). cDNA cloning of the developmentally regulated lamin L_{III} of *Xenopus laevis*. *EMBO J.* 7, 3189–3197.
- Strausfeld, U., Fernandez, A., J.-Capony, P., Girard, F., Lautredou, N., Derancourt, J., Labbe, J.-C., and Lamb, N.J.C. (1994). Activation of p34^{cdc2} protein kinase by microinjection of human cdc25C into mammalian cells. *J. Biol. Chem.* 269, 5989–6000.
- Tang, Z., Coleman, T.R., and Dunphy, W.G. (1993). Two distinct mechanisms for negative regulation of the Wee1 protein kinase. *EMBO J.* 12, 3427–3436.
- Ward, G.E., and Kirschner, M.W. (1990). Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell* 61, 561–577.
- Yamashita, K., Yasuda, H., Pines, J., Yasumoto, K., Nishitani, H., Ohtsubo, M., Hunter, T., Sugimura, T., and Nishimoto, T. (1990). Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO J.* 9, 331–338.