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Entry into mitosis without Cdc2 kinase activation

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SUMMARY

Mouse FT210 cells at 39°C cannot enter mitosis but arrest in G_2 phase, because they lack Cdc2 kinase activity as a result of a temperature-sensitive lesion in the *cdc2* gene. Incubation of arrested cells with the protein phosphatase 1 and 2A inhibitor okadaic acid induces morphologically normal chromosome condensation. We now show that okadaic acid also induces two other landmark events of early mitosis, nuclear lamina depolymerization and centrosome separation, in the absence of Cdc2 kinase activity. Okadaic acid-induced entry into mitosis is accompanied by partial activation of Cdc25C and may be prevented by tyrosine phosphatase inhibitors and by the protein kinase inhibitor staurosporine, suggesting that Cdc25C and kinases distinct from Cdc2 are required for these mitotic events. Using in-gel assays, we show that a 45-kDa protein kinase normally activated at mitosis is also activated by okadaic acid independently of Cdc2 kinase. The 45-kDa kinase can utilize GTP, is stimulated by spermine and is inhibited by heparin. These properties are characteristic of the kinase CK2, but immunoprecipitation studies indicate that it is not CK2. The data underline the importance of a tyrosine phosphatase, possibly Cdc25C, and of kinases other than Cdc2 in the structural changes the cell undergoes at mitosis, and indicate that entry into mitosis involves the activation of multiple kinases working in concert with Cdc2 kinase.

Key words: Cdc25, In-gel kinase assay, Lamin, Microtubule, Okadaic acid, Mouse FT210 cell

INTRODUCTION

Cells undergo dramatic structural changes when they enter mitosis: their shape changes as the cytoskeleton is reorganised, the nuclear envelope breaks down, a spindle is assembled, and chromatin condenses into chromosomes. Many of these changes are controlled by reversible protein phosphorylation (Nigg, 1993). To understand how mitosis is regulated requires identification of the protein kinases and phosphatases involved and determination of how they are coordinated.

A large body of evidence has demonstrated a key role for Cdc2 kinase activity in committing cells to enter mitosis. Cdc2 kinase also plays a role in subsequent mitotic events by phosphorylating target proteins directly, or indirectly by phosphorylating other kinases or phosphatases (reviewed by Norbury and Nurse, 1992). Activation of Cdc2 kinase is effected by association of the $p34^{cdc2}$ catalytic subunit with a positive regulatory subunit, cyclin B, by phosphorylation of Thr161 of the $p34^{cdc2}$ subunit by Cdk activating kinase, and by dephosphorylation of Tyr 15 and Thr 14 of the $p34^{cdc2}$ subunit by the Cdc25C phosphatase (reviewed by Coleman and Dunphy, 1994). Cdc25C phosphatase activity is also regulated by reversible phosphorylation (reviewed by Coleman and Dunphy, 1994). The active form has an extensively phosphorylated amino-terminal domain and this phosphorylation can be carried out by Cdc2 kinase in vitro. It has been suggested that a modest amount of active Cdc2 kinase would self-catalyze through Cdc25C activation, causing rapid mitotic entry, and that the 'trigger' for entry into mitosis could be an upstream event resulting in the initial activation of Cdc2 kinase or Cdc25C (Hoffman et al., 1993; Izumi and Maller, 1993, 1995). However, in at least one physiological experiment this simple autocatalytic loop could not be demonstrated (Norbury et al., 1991). Furthermore, other kinases appear to be required for Cdc25C activation. Plx1 kinase, a member of the Polo family of kinases, can also phosphorylate and activate Cdc25C in vitro, but the mitotic phosphorylation pattern of Cdc25C reveals phosphorylation sites in addition to those produced by Plx1 and Cdc2 (Kumagai and Dunphy, 1996). The activity of Cdc2 kinase and Cdc25C is also negatively controlled by protein phosphatase 2A, which dephosphorylates both (Felix et al., 1990; Clarke et al., 1993; Lee et al., 1994).

In the filamentous fungus *Aspergillus nidulans*, activation of Cdc2 kinase is not sufficient to trigger mitosis; another kinase, NIMA, is hyperphosphorylated and activated downstream of Cdc2 kinase (Ye et al., 1995), but is essential for initiation of mitosis (Osmani et al., 1991). Overexpression of NIMA in interphase human cells produces an abnormal condensation of chromatin reminiscent of apoptosis, suggesting that human cells may possess NIMA-related pathways (Lu and Hunter, 1995; O'Connell et al., 1994). However, none of the members of the mammalian family of Nek kinases, identified on the basis of their sequence similarity to NIMA (Letwin et al., 1992; Levedakou et al., 1994; Schultz and Nigg, 1993), play such a significant role. They may be involved in other aspects of mitosis, as indicated by association of human Nek2 with the

centrosome which gives rise to the poles of the mitotic spindle (Fry et al., 1998).

The importance of kinases that can be activated independently of Cdc2 kinase has been revealed in experiments employing protein phosphatase inhibitors such as microcystin, okadaic acid or fostriecin. Microcystin treatment of interphase *Xenopus* egg extracts, which have no detectable Cdc2 kinase, activates a distinct kinase that can phosphorylate and activate Cdc25, and induce nuclear envelope breakdown and chromosome condensation (Izumi and Maller, 1995). Murine FT210 cells, which bear a temperature-sensitive lesion in the *cdc2* gene (Th'ng et al., 1990), arrest in G_2 in the absence of Cdc2 kinase activity at the restrictive temperature, but when treated with okadaic acid or fostriecin undergo chromosome condensation (Guo et al., 1995).

Other kinases with demonstrated roles in mitosis include the βII form of PKC (Goss et al., 1994) and Plk1 (Nigg et al., 1996). Plk1 is likely to be dependent on the activity of Cdc2 kinase since it is phosphorylatable by Cdc2 kinase (Hamanaka et al., 1995) and it is not activated by okadaic acid in FT210 cells in the absence of Cdc2 kinase activity (P.G., unpublished data). Several other kinases also become activated at mitosis (Halleck et al., 1984; Stukenberg et al., 1997; Taylor and Shalloway, 1996) and many proteins, such as histone H3, MP70, MP105 and glial fibrillary acidic protein, are phosphorylated at mitosis by kinases distinct from cyclin-dependent kinases (Guo et al., 1995; Matsuoka et al., 1992; Stukenberg et al., 1997).

In this study, we have defined further the mitotic events which can be triggered by okadaic acid without a requirement for Cdc2 kinase and examined their control by protein kinases and protein tyrosine phosphatases. We have also identified a 45-kDa protein kinase normally activated at mitosis, which can be activated independently of Cdc2 kinase. The results are consistent with a model whereby Cdc25C controls entry into mitosis by activating multiple protein kinases.

MATERIALS AND METHODS

Cell culture and synchronization of FT210 cells

Mouse FT210 mammary carcinoma cells were grown at 32°C as in Guo et al. (1995). To synchronize cells to S and early G_2, they were treated for 16 hours with 1.25 μg/ml aphidicholin (Sigma) to arrest them at the G_1/S boundary, released from the aphidicholin block for 9 hours, and incubated again with aphidicholin for another 16 hours. Cells were released from the second aphidicholin block for 3 hours or 6 hours and designated the S and S/G_2 populations, respectively. Synchronized G_2 and mitotic cell populations were obtained by incubating cells in isoleucine-deficient RPMI-1640 medium supplemented with 10% heat-inactivated, dialyzed fetal calf serum (Gibco) for 16 hours, followed by release into complete medium. Released cells were incubated in the presence of 50 ng/ml nocodazole (Sigma) for 16 hours to obtain a mitotic population. To obtain a G_2 population lacking Cdc2 kinase, released cells were incubated in the presence of 1.25 μg/ml aphidicholin for 9 hours, and then in aphidicolin-free medium at 39°C for 16 hours. Flow cytometry was performed as in Anderson et al. (1998).

Immunoprecipitation

Immunoprecipitations were performed as described previously (Penner et al., 1997). Briefly, 5×10^6 cells were arrested in G_2 at 39°C or in mitosis with nocodazole. Cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 30 μg/ml DNase I and 30 μg/ml RNase A for 30 minutes on ice and passed through a 26-gauge needle. Lysates were cleared by centrifugation at 16,000 ***g*** for 10 minutes. Supernatants were collected and precleared with rabbit pre-immune serum and 20 μl of protein A-agarose. Cleared lysates were incubated for 2 hours at 4°C with the appropriate antiserum: 1 μl anti-Cdc2 (Guo et al., 1995), 5 μg anti-Cdc25C (Santa Cruz) or 1 μl anti-CK2α serum (Penner et al., 1997). The lysates were further incubated with 50 μl of 50% protein A-agarose for 1 hour. Antigen-antibody complexes were collected by centrifugation and washed ×5 with lysis buffer.

Cdc2 kinase activity assay

Immunoprecipitated Cdc2 complexes were equilibrated in histone H1 kinase buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mM ATP) for 10 minutes at 25°C. The supernatants were removed and beads were incubated in 30 μl histone H1 kinase buffer supplemented with 1 mM sodium orthovanadate, 1 μM okadaic acid, 0.5 mg/ml histone H1 (Gibco) and 0.2 μCi/μl [γ-^{32}P]ATP for 10 minutes at 30°C. Reactions were stopped by heating for 5 minutes at 95°C in sodium dodecyl sulfate (SDS) sample buffer. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry using a PhosphorImager.

Cdc25C activity assay

Immunoprecipitated Cdc25C complexes were equilibrated in phosphatase assay buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM dithiothreitol) for 10 minutes. Samples were then mixed in phosphatase assay buffer for 1 hour at 25°C with equivalent amounts of immunoprecipitated Cdc2 kinase complexes from FT210 cells treated with γ irradiation to inactivate the Cdc2 kinase. The beads were collected by centrifugation, washed ×3 in histone H1 kinase buffer suppplemented with 2 mM sodium orthovanadate, and a Cdc2 kinase activity assay was performed as described above.

Western blotting

Immunoprecipitated CK2α complexes were boiled in SDS sample buffer and used for in-gel kinase assays (see below) or western blotting. For western blotting, samples were resolved by 10% SDS-PAGE, transferred to nitrocellulose and blocked for 2 hours in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl) and 10% non-fat dry milk. The nitrocellulose sheet was incubated with a CK2α monoclonal antibody (Transduction Laboratories) diluted 1:1000 in TBS, 0.1% Tween-20, 1% non-fat dry milk. Antigen-antibody complexes were detected using a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Gibco) and enhanced chemiluminescence (SuperSignal, Pierce).

In-gel kinase assays

Cells were collected and washed with cold PBS. They were either solubilized directly in SDS sample buffer or lysed on ice for 30 minutes in 50 mM Tris-HCl, pH 8.0, 1% Nonidet P40, 1 mM dithiothreitol, 2 mM ethylene diamine-tetraacetic acid, 5 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethysulphonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 30 μg/ml Dnase I and 30 μg/ml RNase A. Cellular debris was removed by centrifugation at 16,000 ***g*** for 20 minutes. Protein concentration was determined using the Bradford assay (Bradford, 1976).

Equal amounts of protein were separated by SDS-PAGE. The protein kinase substrate was polymerized directly into the separating gel at a concentration of 0.5 mg/ml. The substrate used was a modified sequence of amino acids 29-139 of the murine Ets-1 transcription factor. The sequence contains two consensus sites each for protein kinase C and CK2, numerous sites for proline-directed kinases, such

as the cyclin-dependent family of kinases, and a MAP kinase site, PXTP, which was created by mutating the leucine residue at position 36 to a proline.

Following electrophoresis, SDS was removed by washing the gel ×3 for 20 minutes each in 50 mM Tris-HCl, pH 8.0, 20% 2-propanol. Proteins were completely denatured by incubating in 50 mM Tris-HCl, pH 8.0, 6 M guanidine hydrochloride for 1 hour. Renaturation of the separated proteins was achieved through successive washes with 50 mM Tris-HCl, pH 8.0, 0.4% Tween-40 and 5 mM β-mercaptoethanol at 4°C for 16 hours.

After preincubation of the gel in kinase buffer (40 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 2 mM dithiothreitol) for 10 minutes, the assay was started by adding 10 μCi/ml [γ-^{32}P]ATP and continued for 1 hour with gentle shaking. Excess radioactivity was removed by washing in 5% (w/v) trichloroacetic acid, 1% sodium pyrophosphate until radioactivity was no longer detectable in the wash solution using a Geiger counter. The gel was then dried and the phosphorylated bands viewed and quantitated using a PhosphorImager (Molecular Dynamics).

Other procedures

The relative number of cells in mitosis was determined by an enzyme-linked immunosorbent assay (ELISA) as described by Roberge et al. (1998) or by microscopy of mitotic spreads as described by Guo et al. (1995). Immunofluorescence microscopy using monoclonal antibody E7 to β-tubulin (developed by M. Klymkowsky and obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa) and lamin monoclonal antibody E6, was carried out as in Anderson et al. (1997).

RESULTS

Induction of mitotic events by phosphatase inhibitors in the absence of Cdc2 kinase activity

We have previously shown that the protein phosphatase inhibitors fostriecin and okadaic acid can induce morphologically normal chromosome condensation in cells lacking Cdc2 kinase activity (Guo et al., 1995). These experiments used FT210 cells, which have a temperature-sensitive Cdc2 kinase (Th'ng et al., 1990). FT210 cells grow normally at 32°C, but at the restrictive temperature of 39°C they lose Cdc2 kinase activity and arrest in G_2. We used this experimental system to determine whether additional mitotic events can take place in the absence of Cdc2 kinase activity, and to study their control. Cells were synchronized to S or S/G_2 at 32°C using aphidicolin, arrested in G_2 by subsequent incubation at 39°C, or arrested in mitosis at 32°C using the microtubule inhibitor nocodazole (see Materials and methods). The cell cycle profile of these synchronized populations was determined by flow cytometry of DNA content and is shown in Fig. 1.

Cells at different stages of the cell cycle were prepared for immunofluorescence microscopy to visualize the chromosomes using the DNA dye Hoechst 33258, the nuclear lamina using an antibody to nuclear lamins, and the microtubules using an antibody to β-tubulin (Fig. 2). Interphase cells or cells arrested in G_2 at 39°C had diffuse chromatin staining in the nucleus, a bright ring of anti-lamin immunofluorescence around the nucleus indicating an intact nuclear lamina, and a single dot of bright β-tubulin immunofluorescence, from which microtubules radiate, representing the interphase centrosome (Fig. 2A-D). Cells

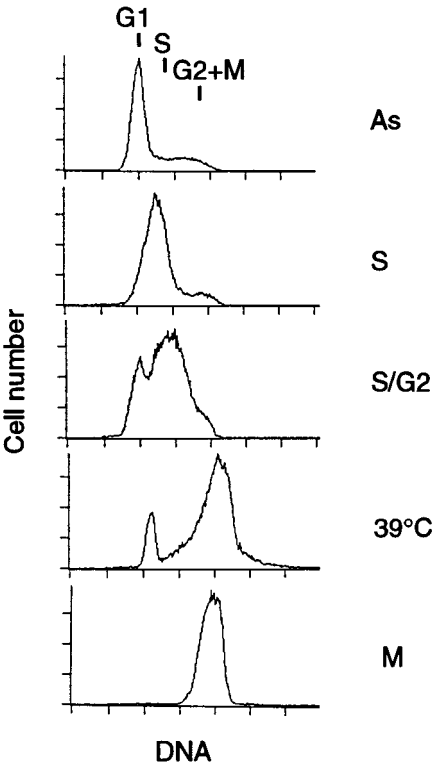

Fig. 1. Distribution of cells in different phases of the cell cycle. Asynchronously dividing cells (As) or cells synchronised to S, S/G_2, G_2 without Cdc2 kinase activity (39°C) and mitotic (M) phases of the cell cycle were analysed by flow cytometry. The *x*-axis shows the relative DNA content of the cells determined by intensity of propidium iodide fluorescence. The DNA content corresponding to G_1, S and G_2+M cells is indicated.

arrested in mitosis with nocodazole had highly condensed chromosomes, a depolymerized nuclear lamina, as seen by the disappearance of the perinuclear ring and the appearance of diffuse cytoplasmic lamin immunofluorescence, and two dots of β-tubulin immunofluorescence resulting from centrosome separation (Fig. 2M-P). In the presence of nocodazole, the mitotic spindle does not form because microtubules depolymerize**.** Cells arrested in G_2 at 39°C had fewer than 2% mitotic cells. When treated with different concentrations of okadaic acid for 2 hours, 20-60% of the cells showed condensed chromosomes, as previously observed (Guo et al., 1995), and also showed nuclear lamina depolymerization (Fig. 2E,F). Most cells also showed two dots of β-tubulin immunofluorescence indicative of centrosome separation but, even in the absence of nocodazole, a mitotic spindle was not formed and the chromosomes were not aligned (Fig. 2G,H). Additional microtubule nucleation centers were also occasionally observed (not shown).

To confirm the absence of Cdc2 kinase activity in cells at the restrictive temperature, Cdc2 kinase was immunoprecipitated from cells arrested at 39°C and treated or not with okadaic acid, and from nocodazole-blocked mitotic cells. Cdc2 kinase activity was then measured using histone H1 as a substrate (Fig. 3A). Cells arrested at 39°C had little or no Cdc2 kinase activity, and treatment with okadaic acid caused no significant increase, as previously described (Guo et al.,

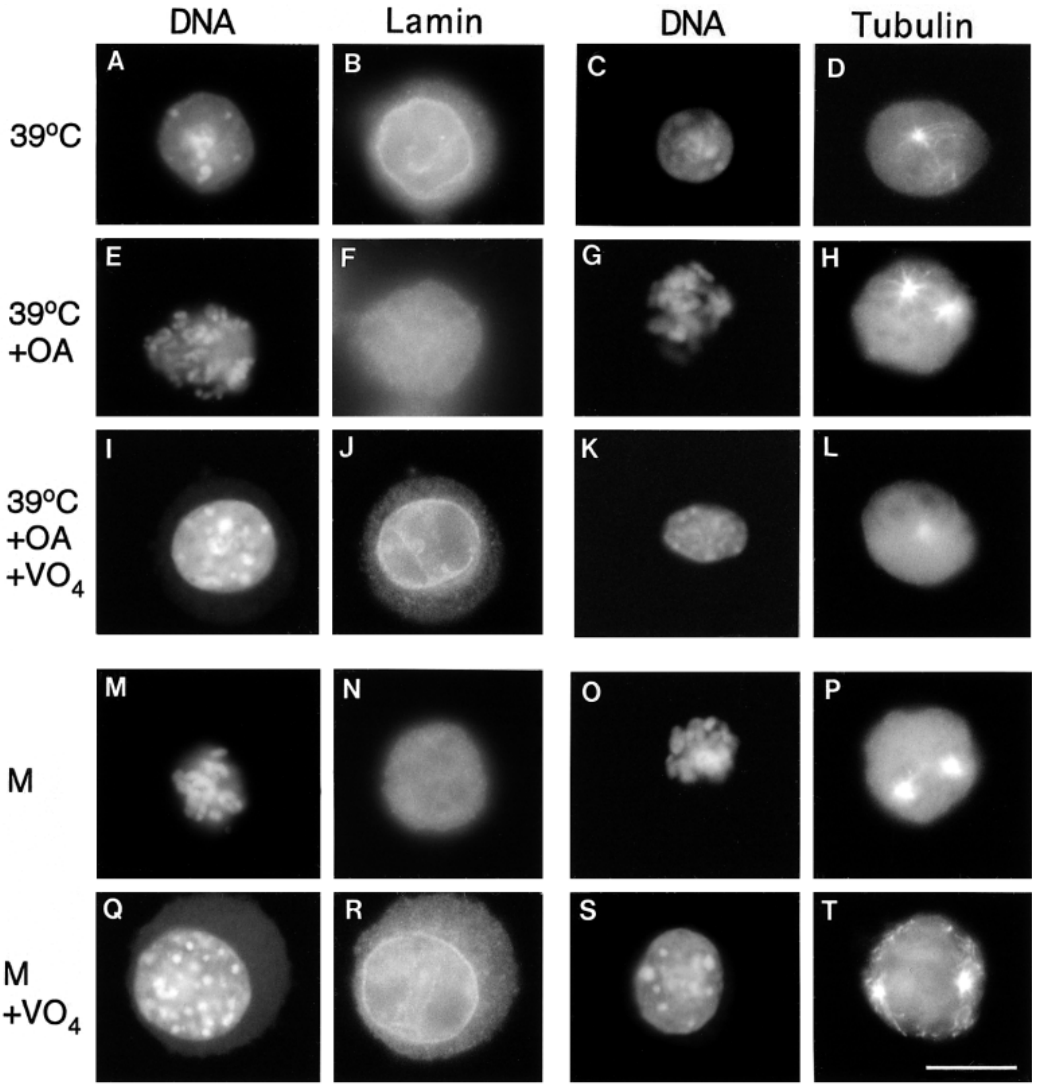

Fig. 2. Mitotic events induced by okadaic acid and their inhibition by sodium orthovanadate. Cells were arrested in G_2 without Cdc2 kinase and left untreated (39°C), treated with 0.5 μM okadaic acid for 2 hours (39°C +OA), or treated with 0.1 mM sodium orthovanadate for 2 hours before addition of okadaic acid (39°C +OA+VO_4). Cells were arrested in mitosis using nocodazole and left untreated (M) or were treated with sodium orthovanadate (M+VO_4). The panels show paired micrographs of a representative cell stained for both DNA and lamins (left two rows) or stained for DNA and β-tubulin (right two rows). Bar, 20 μm.

1995). In comparison, cells arrested in mitosis with nocodazole showed high Cdc2 kinase activity. It has previously been shown that p34^{cdc2} becomes degraded during incubation of FT210 cells at 39°C and that the activity of Cdc2 kinase isolated from FT210 cells is thermolabile at 39°C (Th'ng et al., 1990; Guo et al., 1995), and that >90% of cellular cyclin A and cyclin B are degraded during 2 hours incubation with 0.5 μM okadaic acid (Guo et al., 1995; our unpublished observations). In addition, histone H1, a well established substrate of Cdc2 kinase, does not become phosphorylated during incubation of FT210 cells with okadaic acid at 39°C (Guo et al., 1995), confirming the in vitro results. Moreover, the okadaic acid-induced mitotic events described above were also observed when cells arrested at 39°C were preincubated with the Cdc2 kinase inhibitor olomoucine at a concentration (100 μM) that prevents normal entry into mitosis. Therefore, the observed mitotic events can take place in cells containing little or no Cdc2 kinase activity and without the increase in Cdc2 kinase activity normally observed at mitosis.

These results establish that two major mitotic events, chromosome condensation and nuclear lamina depolymerization, can take place normally in the absence of Cdc2 kinase activity and that a third, the formation of the mitotic spindle, is initiated by separation of the centrosomes but is not completed.

Stimulation of Cdc25C activity by okadaic acid treatment of G_2-arrested cells

In *Xenopus* egg extracts devoid of Cdc2 and Cdk2 kinase activity, microcystin causes Cdc25 activation as well as chromosome condensation and nuclear envelope breakdown (Izumi and Maller, 1995). We investigated whether okadaic acid has a similar effect in FT210 cells in the absence of Cdc2 kinase activity. Cells were arrested in G2 at 39°C and treated or not with okadaic acid for 2 hours, or were arrested in mitosis with nocodazole. Cdc25C was isolated by immunoprecipitation and its activity was measured by its ability to activate Cdc2 kinase isolated from cells arrested in G_2 by DNA damage (Poon et al., 1997). As shown in Fig. 3B, Cdc25C activity was low in cells arrested in G_2 and about eightfold higher in cells arrested in mitosis, as observed by others (Poon et al., 1997). Treatment of cells arrested in G_2 at 39°C with okadaic acid stimulated Cdc25C 3- to 4-fold (Fig. 3B). Purified *Xenopus* Cdc25C was used as a positive control (Fig. 3B).

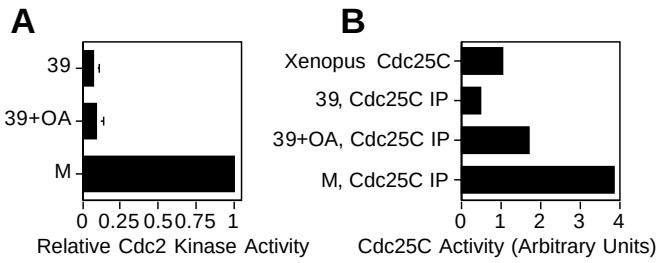

Fig. 3. Effect of okadaic acid on Cdc2 and Cdc25C activity. (A) The histone H1 kinase activity of Cdc2 kinase immunoprecipitated from cells arrested in G_2 at 39°C (39), cells arrested at 39°C and treated with okadaic acid (39+OA) or cells arrested in mitosis using nocodazole (M) is expressed as a fraction of the activity measured in nocodazole-arrested cells. Error bars show s.d. obtained from 3 separate experiments. (B) Cdc25C was immunoprecipitated from cells treated as in A and its activity was measured by its ability to stimulate Cdc2 kinase isolated from cells arrested in G_2 after DNA damage. Relative Cdc25C activity units were calculated as follows: $(Activity_{Cdc25C} - Activity_{buffer})/Activity_{buffer}$, where $Activity_{Cdc25C}$ = activity of Cdc2 incubated with Cdc25C and $Activity_{buffer}$ = activity of Cdc2 incubated with buffer. Recombinant *Xenopus* Cdc25C (0.5 μg) was used as a positive control. IP, immunoprecipitate.

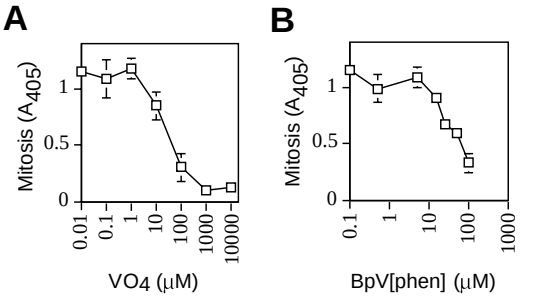

Fig. 4. Inhibition of mitosis by tyrosine phosphatase inhibitors measured by ELISA. Cells arrested in mitosis were treated with different concentrations of sodium orthovanadate (VO_4) (A) or BpV[phen] (B) for 4 hours and mitosis was determined by ELISA.

These results show that okadaic acid treatment causes Cdc2 kinase-independent activation of Cdc25C, raising the possibility that the observed mitotic events could be triggered by Cdc25C activation.

Tyrosine phosphatase inhibitors cause chromosome decondensation and nuclear envelope reformation in okadaic acid-treated and mitotic cells

We next investigated the effects of tyrosine phosphatase inhibitors on mitosis. Sodium orthovanadate is a powerful inhibitor of many tyrosine phosphatases (Swarup et al., 1982). Potassium bisperoxo(1,10-phenanthroline)oxovanadate (BpV[phen]) appears to be more specific for Cdc25 because it causes a late G_2 arrest in cycling cells as well as inhibiting Cdc25 in vitro (Faure et al., 1995). Cells arrested in mitosis with nocodazole were treated with different concentrations of these two inhibitors for 4 hours. Both sodium orthovanadate and BpV[phen] caused mitotic cells to revert to an interphase-like state, as measured by the loss of a mitosis-specific antigen (Anderson et al., 1998) determined by ELISA (Fig. 4) and by microscopy, where the percentage of cells with condensed chromosomes decreased from 46% to 7% after treatment with 0.1 mM sodium vanadate and to 8% after treatment with 0.1 mM BpV[phen] (Fig. 5). This effect was concentration-dependent, with an IC_{50} of 40-50 μM for sodium orthovanadate and 25-30 μM for BpV[phen] (Fig. 4).

To examine this inhibition more closely at the morphological level, cells blocked in mitosis with nocodazole were treated with sodium orthovanadate or BpV[phen] for 4 hours and the chromosomes, nuclear lamina and microtubules were examined by fluorescence microscopy. Both inhibitors caused chromosome decondensation. In most instances, decondensation was partial, with masses of condensed chromatin interspersed with interphase-like chromatin in a well defined round nucleus (Fig. 2Q,S), without nuclear division. The inhibitors also caused the reformation of the nuclear lamina, as monitored by the appearance of a bright ring of lamin immunofluorescence around the nucleus (Fig. 2R), but there was no reduction in the number of β-tubulin nucleation centers (Fig. 2T).

To determine the effects of the tyrosine phosphatase inhibitors on entry into mitosis induced by okadaic acid, cells were arrested at 39°C and incubated with sodium orthovanadate for 2 hours and then treated or not with okadaic acid for 2 hours. Sodium orthovanadate prevented the induction of mitosis by okadaic acid, as shown by the absence of chromosome condensation (Fig. 2I,K), the presence of an intact nuclear envelope (Fig. 2J) and of a single dot of β-tubulin immunofluorescence (Fig. 2L). This inhibition was complete (Fig. 5). Similar results were obtained with BpV[phen] (not shown).

These results indicate that the activity of a tyrosine phosphatase, possibly Cdc25C, is required for cells to enter mitosis without Cdc2 kinase activity, and is also required to maintain cells in the mitotic state.

Mitotic protein kinases activated by okadaic acid independently of Cdc2 kinase

Chromosome condensation induced by okadaic acid or fostriecin in cells lacking Cdc2 kinase activity is inhibited by the general protein kinase inhibitor staurosporine (Guo et al., 1995), suggesting that it is induced by the activation of protein kinases. To identify protein kinases activated specifically at mitosis, we carried out in-gel kinase assays on extracts from cells at different phases of the cell cycle and compared their patterns of protein kinase activity. In-gel kinase assays (Heider et al., 1994; Hutchcroft et al., 1991) are based on the

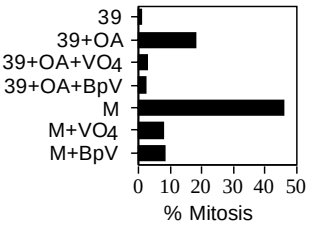

Fig. 5. Inhibition of mitosis by tyrosine phosphatase inhibitors measured by microscopy. Cells were treated as described in the legends to Figs 2,4 and the percentage of cells in mitosis was determined microscopically.

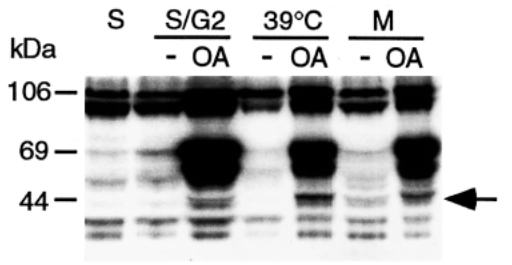

Fig. 6. In-gel kinase assay. Synchronized cells were treated with 0.5 μM okadaic acid (OA) for 2 hours or not (–). Cell extracts were subjected to in-gel kinase assay. Active kinases are detected as radioactive bands. The arrow indicates the position of the 44-kDa and 45-kDa mitotic kinases. The positions of marker proteins are shown.

observation that many protein kinases can be renatured after separation by SDS-PAGE. By providing a general substrate within the gel, in this case a protein that contains consensus phosphorylation sites for several protein kinases, including protein kinase C, CK2 and proline-directed kinases, and supplying [γ-^{32}P]ATP, the presence of several active kinases can be detected as radioactive bands representing phosphorylation of the incorporated substrate or kinase autophosphorylation. An advantage of this technique is that prior knowledge of the nature and properties of the kinases is not required. However, in-gel kinase assays do not provide a complete profile of the active kinases in an extract since they detect only those kinases that do not require association with a regulatory subunit for activity and that can withstand denaturation and renaturation.

Extracts were prepared from cells synchronized as in Fig. 1 and were subjected to the in-gel kinase assay. Most radioactive bands were present at about the same intensity in extracts from all cell cycle phases except for two closely migrating bands at 44 kDa and 45 kDa, which were detected only in the cells arrested in mitosis (Fig. 6, arrow). Samples of synchronized cells were also treated with 0.5 μM okadaic acid for 2 hours. Treatment of cells in S/G_2, or cells arrested in G_2 at 39°C with okadaic acid also induced the activation of the 44-kDa and 45-kDa kinases normally active only at mitosis and of additional kinases (Fig. 6). This shows that treatment of 39°C-arrested cells with okadaic acid can activate protein kinases normally active only during mitosis.

Inhibition of the 44-kDa and 45-kDa kinases by treatment of mitotic cells with staurosporine and tyrosine phosphatase inhibitors

Since tyrosine phosphatase inhibitors and staurosporine inhibit the induction of mitosis by okadaic acid, we next determined whether these compounds affect the activity of the two mitotic kinases identified above. Cells arrested in mitosis were treated for 2 hours with either staurosporine, the cyclin-dependent kinase inhibitor olomoucine, the MAP kinase inhibitor PD09805 or the PKC inhibitors UCN-01 and GF109203X, sodium orthovanadate or BpV[phen]. Cell lysates were prepared and in-gel kinase assays were performed. Of the kinase inhibitors only staurosporine (130 nM) caused the disappearance of the 44-kDa and 45-kDa bands (Fig. 7A). Sodium orthovanadate and BpV[phen] also inhibited these two kinases (Fig. 7B) with a concentration dependence similar to that of mitotic inhibition (Fig. 4). The other kinases in the gel were not inhibited by sodium orthovanadate and BpV[phen] (Fig. 7B). These results are consistent with an involvement of these kinases in the mitotic events induced by okadaic acid.

Characterization of the mitotic 44-kDa and 45-kDa protein kinases

We investigated the substrate requirements of the mitotic kinases. Cells were arrested in mitosis at 32°C with nocodazole, or in G_2 at 39°C, and in-gel kinase assays were performed using either [γ-^{32}P]ATP or [γ-^{32}P]GTP. Remarkably, the 44- and 45-kDa kinases were able to use both ATP and GTP as phosphate donors (Fig. 8A). The extracts contained few other kinases capable of using GTP, a major one at about 42 kDa and a minor one at about 38 kDa, and these were active in both G_2 and mitotic cells (Fig. 8A). Phosphoamino acid analysis showed that the 44- and 45-kDa kinases are serine/threonine protein kinases (not shown). Very few Ser/Thr kinases are known which can use both ATP and GTP as phosphate donors and of these the best characterized is CK2 (reviewed in (Allende and Allende, 1995)).

CK2 is usually isolated as a heterodimer of two catalytic subunits (α and/or α') and two noncatalytic regulatory subunits (β) in tight association (Allende and Allende, 1995). The α and α' subunits are encoded by distinct genes and have molecular masses of approximately 42-44 kDa and 38 kDa, respectively, and the β subunit has a molecular mass of 26 kDa (Allende and Allende, 1995). CK2 is also unusual among protein kinases in that its activity in vitro can be inhibited by heparin, which

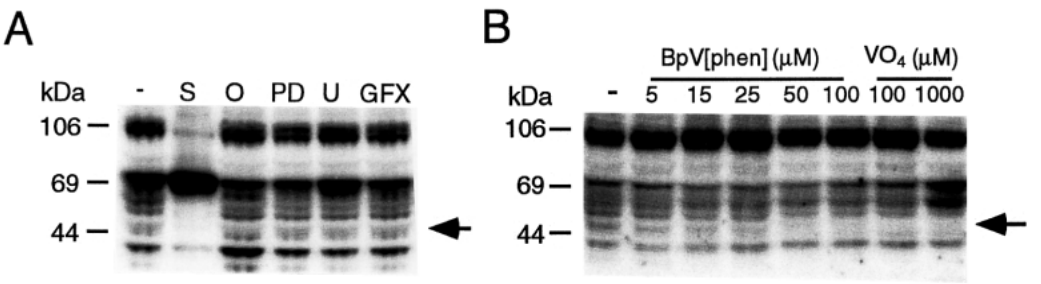

Fig. 7. Effect of protein kinase inhibitors and tyrosine phosphatase inhibitors on the activity of the 44-kDa and 45-kDa kinases. In-gel kinase assay on extracts obtained from cells arrested in mitosis and treated without (–) or with 130 nM staurosporine (S), 100 μM olomoucine (O), 25 μM PD098059 (PD), 100 nM UCN-01 (U) or 1 μM GF109203X (GFX) for 2 hours (A) or with different concentrations of BpV[phen] or sodium orthovanadate (VO_4) for 4 hours (B). The arrow indicates the position of the 44-kDa and 45-kDa kinases. The positions of marker proteins are shown.

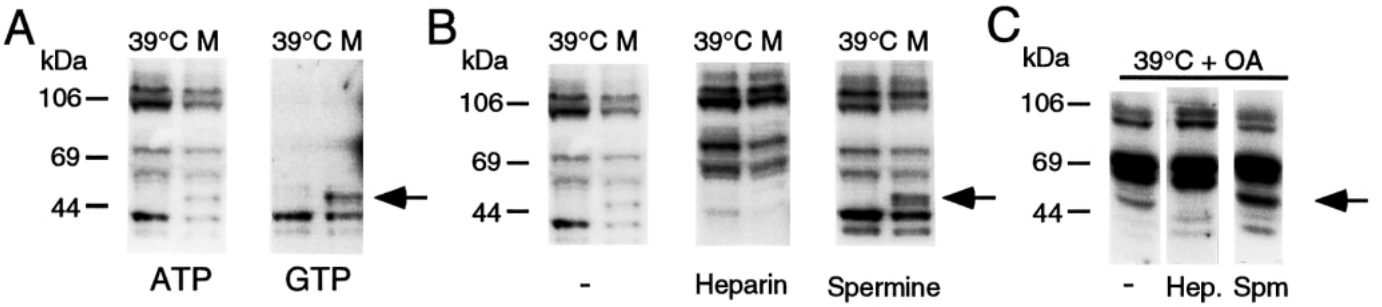

Fig. 8. In vitro characterization of the activity of the 44-kDa and 45-kDa kinases. (A) In-gel kinase assays were performed on extracts from cells arrested in G_2 (39°C) or in mitosis (M) using 32 nM [γ-^{32}P]ATP or [γ-^{32}P]GTP as phosphate donor sources. (B) In-gel kinase assays were performed after preincubation of the gels with buffer (–), 20 μg/ml heparin or 2 mM spermine for 1 hour. (C) In-gel kinase assays were performed as in B using extracts from cells arrested at 39°C and treated with okadaic acid. The arrow indicates the position of the 44-kDa and 45-kDa kinases. The positions of marker proteins are shown.

binds to the α subunits (Hathaway et al., 1980). To examine further the relationship between the 38-, 42-, 44- and 45-kDa kinases and CK2 α and α′, we examined the effect of heparin on their activity in the in-gel kinase assay. Cell lysates prepared from cells arrested in G_2 at 39°C or arrested in mitosis were subjected to an in-gel kinase assay in the presence or absence of 20 μg/ml heparin for 1 hour at 25°C. All four kinases were inhibited by heparin (Fig. 8B). Heparin also inhibited the 44- and 45-kDa kinases that had been activated in G_2-arrested cells by treatment with okadaic acid (Fig. 8C). It has previously been shown that human CK2α (43 kDa) and CK2α′ (38 kDa) can be detected by in-gel kinase assay (Lin et al., 1996). It is therefore probable that the 38-kDa and 42-kDa kinases are CK2α′ and CK2α, respectively. The 44-kDa and 45-kDa bands have properties resembling those of the CK2 catalytic subunits.

Another unusual property of CK2 is that its activity in vitro can be enhanced by polyamines such as spermine, reportedly through binding to the β subunit (Leroy et al., 1997). Cell lysates obtained as above were also subjected to the in-gel kinase assay in the presence of 2 mM spermine. Spermine stimulated the activity of the 38-kDa (CK2α′) and 42-kDa (CK2α) kinases active during interphase and mitosis, and of the 44- and 45-kDa kinases active during mitosis and in cells arrested in G_2 at 39°C and then treated with okadaic acid (Fig. 8B,C). Other active kinases in the gel were not stimulated by spermine. This property has not previously been assigned to the catalytic subunits of CK2.

To investigate further the nature of the 44- and 45-kDa kinases, cell lysates were prepared from cells arrested in G_2 at 39°C, or in mitosis. They were then immunoprecipitated or not with a CK2α-specific antiserum (Penner et al., 1997) and analysed by in-gel kinase assay. As shown before, active 44- and 45-kDa kinases were detected in the mitotic extract but not in the G_2 extract (Fig. 9, left panel). Active 42-kDa kinase was detected in the CK2α immunoprecipitate from the G_2 extract, confirming that this band is indeed CK2α (Fig. 9, right panel). The antibody immunoprecipitated both CK2α and the 44-kDa kinase from the mitotic extract (Fig. 9, right panel). The sum of the activity of CK2α and 44-kDa bands recovered from the mitotic cells was approximately equal to that of the CK2α recovered from the G_2 cells. CK2α is known to become hyperphosphorylated at mitosis and to migrate more slowly in SDS-PAGE (Bosc et al., 1995; Litchfield et al., 1992). This result indicates that the 44-kDa kinase is not a protein kinase specifically activated at mitosis, but rather the phosphorylated, slower migrating form of active CK2α. The 45-kDa kinase active at mitosis was not detected in CK2α immunoprecipitates from the mitotic extract (Fig. 9, right panel).

The results show that a 45-kDa protein kinase is activated at mitosis and by treatment of cells arrested at 39°C with okadaic acid. This kinase is directly or indirectly sensitive to staurosporine and to tyrosine phosphatase inhibitors. The strict correlation between the requirements for the induction of mitotic entry in the absence of Cdc2 kinase and for the activation of the 45-kDa protein kinase suggests that it may play a role in controlling mitosis.

DISCUSSION

We have used a temperature-sensitive Cdc2 kinase cell line to demonstrate that in addition to chromosome condensation (Guo et al., 1995), nuclear lamina depolymerization and centrosome separation can also take place in the absence of Cdc2 kinase activity. However, a typical mitotic spindle was not formed and the chromosomes failed to align, and additional microtubule nucleation centers were occasionally observed. These results are in agreement with those of Izumi and Maller (1995), who showed that a protein phosphatase inhibitor can cause chromosome condensation and nuclear envelope

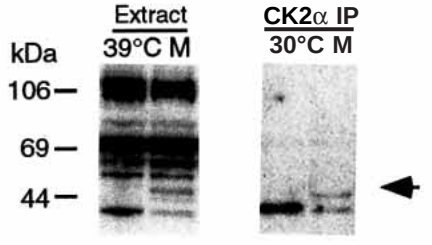

Fig. 9. Identification of the 44-kDa kinase as hyperphosphorylated CK2α. In-gel kinase assays were carried out on extracts from cells arrested at 39°C or in mitosis (left panel), or on CK2α immunoprecipitates (IP) obtained from these extracts (right panel). The arrow indicates the position of the 44-kDa and 45-kDa kinases. The positions of marker proteins are shown.

breakdown in vitro in *Xenopus* extracts without Cdc2 kinase, and underline the importance of kinases other than Cdc2 in the structural changes the nucleus undergoes at mitosis.

Chromosome condensation at mitosis is accompanied by an increase in histone H1 phosphorylation by Cdc2 kinase and it was originally thought that this increase in histone H1 phosphorylation caused chromosome condensation (Bradbury et al., 1973; Gurley et al., 1978). However, condensation triggered by okadaic acid occurs without increased histone H1 phosphorylation (Guo et al., 1995), but is consistently correlated with an increase in histone H3 phosphorylation (Guo et al., 1995; Ajiro et al., 1996b). The mitotic phosphorylation site on histone H3 is not a recognition site for proline-directed kinases, indicating that it is not phosphorylated by a cyclin-dependent kinase. Therefore, chromosome condensation is controlled by a protein kinase that is not Cdc2 kinase and which can be activated independently of Cdc2 kinase.

Nuclear lamina depolymerization at mitosis is controlled by phosphorylation of lamins at sites which can be phosphorylated by Cdc2 kinase (Peter et al., 1990). However, the β_{II} form of PKC can also phosphorylate lamins at Ser395 and Ser405 and induce lamina depolymerization in vitro (Hocevar et al., 1993). PKCβ_{II} is activated in the nucleus at the G_2/M transition (Goss et al., 1994) and its activity is required for nuclear lamina depolymerization and entry into mitosis (Thompson and Fields, 1996). This indicates that lamina depolymerization, like chromosome condensation, may be triggered by kinases other than Cdc2 kinase.

The regulation of centrosome separation and spindle formation by protein kinases at mitosis is complex and only partly understood. Cdc2 kinase may participate directly in some aspects of this process by phosphorylating centrosomal proteins (Nigg et al., 1996). Polo-like kinases have been implicated in centrosome maturation (Nigg et al., 1996) and spindle pole organization (Ohkura et al., 1995; Sunkel and Glover, 1988) and Nek2 kinase and members of the Ipl-1/Aurora kinase family associate with centrosomes and mitotic spindle poles (Fry et al., 1998; Gopalan et al., 1997; Roghi et al., 1998). It is possible that okadaic acid activates some of these kinases without a requirement for Cdc2 kinase. The abnormalities in mitotic spindle formation observed in this study may be due to a requirement for Cdc2 kinase or protein phosphatase activity (Mayer-Jaekel et al., 1993; Ohkura et al., 1989).

Our results also show that okadaic acid treatment of cells lacking Cdc2 kinase activity stimulates Cdc25C activity to a level intermediate between that of G_2 cells and mitotic cells. Furthermore, the activity of a tyrosine phosphatase, possibly Cdc25C itself, is required for okadaic acid to induce mitotic entry. In *Xenopus* extracts (Izumi and Maller, 1995), microcystin can activate Cdc25 in the absence of Cdc2 kinase activity, but it was not determined whether Cdc25 activity was required for chromosome condensation and nuclear envelope breakdown. tsTM13 cells arrest in mitosis at the restrictive temperature with high Cdc2 kinase activity and condensed chromosomes. Treatment of these cells with vanadate, at the concentrations used in our study, causes a decrease in Cdc2 kinase activity and an increase in the Tyr phosphorylation of p34^{cdc2} (Ajiro et al., 1996a), providing evidence that vanadate indeed inhibits Cdc25C in mitotic cells. This indicates that the effects of the tyrosine phosphatase inhibitors observed in the present study were mediated by the inhibition of Cdc25C, suggesting that Cdc25C plays a central role in the control of chromosome condensation, nuclear lamina depolymerization and centrosome separation, independently of its activation of Cdc2 kinase.

It is clear from experiments with staurosporine that the induction of mitotic events by okadaic acid requires not only tyrosine phosphatase activity but also protein kinase activity. We demonstrate using in-gel kinase assays that some kinases normally active only at mitosis can be activated by okadaic acid treatment of cells lacking Cdc2 kinase. This shows that a subset of the protein kinases normally active at mitosis can be activated independently of Cdc2 kinase. The 44-kDa kinase was identified as a phosphorylated form of CK2α, described previously in mitotic cells (Bosc et al., 1995; Litchfield et al., 1992). This kinase is not activated specifically at mitosis, but rather is active throughout the cell cycle and migrates as a distinct band at mitosis because of its phosphorylation state. The 45-kDa kinase displays two properties of CK2: ability to use ATP or GTP as a phosphate donor, and inhibition by heparin. However, unlike the 44-kDa kinase, it was not immunoprecipitated by the CK2α antiserum and therefore appears to be a distinct kinase. The 45-kDa kinase showed no activity when histone H3 was used as a substrate (our unpublished data) and is therefore not the mitotic histone H3 kinase.

The 45-kDa kinase is not only activated during a normal mitosis but also remains active when mitotic cells are treated with the Cdc2 kinase inhibitor olomoucine, providing independent evidence that it does not require Cdc2 kinase for its activity at mitosis. Experiments with protein kinase inhibitors show that only staurosporine inhibited its activity. However, it is not clear whether staurosporine inhibits the 45-kDa kinase directly or indirectly. Experiments using tyrosine phosphatase inhibitors showed that the activity of the 45-kDa kinase at mitosis requires a tyrosine phosphatase, possibly Cdc25C itself. The activation and inhibition patterns of this kinase follow closely those of the mitotic events triggered by okadaic acid, suggesting that it may play important roles in mitosis.

The results presented here suggest the following model for the induction of entry into mitosis by okadaic acid. Cdc25C activity in G_2 phase is negatively regulated by protein phosphatase 2A and positively regulated by protein kinases, including Plx1. Inhibition of protein phosphatase 2A by okadaic acid or fostriecin causes the activation of Cdc25C, which activates Cdc2 kinase, the histone H3 kinase, the 45-kDa kinase and probably others. In cells lacking Cdc2 kinase, these other kinases can be activated nevertheless and trigger early mitotic events. Verification of this model will require the identification and characterization of these mitotic protein kinases.

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