Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9896–9900, October 1994
Biochemistry

A role for cAMP-dependent protein kinase in early embryonic divisions

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Communicated by Ira Pastan, June 13, 1994

ABSTRACT **The cAMP-dependent protein kinase (PKA) pathway affects cell cycle progression in "cycling" *Xenopus* egg extracts. The concentration of free PKA catalytic subunit oscillates during the cell cycle with a peak at the mitosis–interphase transition and a minimum at the onset of mitosis. Inhibition of endogenous PKA in interphase hastens the onset of mitosis. Stimulation of PKA induces interphase arrest, preventing the activation of the M-phase-promoting factor. PKA does not block the accumulation of cyclin or its binding to $p34^{cdc2}$, but the resultant complex lacks kinase activity and $p34^{cdc2}$ remains tyrosine-phosphorylated. PKA appears to stimulate an okadaic acid-sensitive serine/threonine phosphatase that acts upon cdc25. In this way PKA could downregulate the $p34^{cdc2}$ tyrosine phosphatase activity of cdc25 and consequently block the activation of the M-phase-promoting factor.**

The progression through M and S phases in the early *Xenopus* embryo is regulated by cycles of activation and inactivation of the M-phase-promoting factor (MPF), a protein kinase composed of two subunits, cyclin B and $p34^{cdc2}$ (1). During the cell cycle the concentration of the catalytic subunit ($p34^{cdc2}$) remains constant while cyclin accumulates from interphase to mitosis and is degraded at each metaphase–anaphase transition (2). In interphase, association with cyclin induces phosphorylation of $p34^{cdc2}$ at key tyrosine and threonine residues. In this state the complex is enzymatically inactive (preMPF; refs. 3 and 4). At mitosis, the complex is dephosphorylated and activated by the *Xenopus* homologue of the *Schizosaccharomyces pombe* cdc25 protein (5–7). cdc25 undergoes serine/threonine phosphorylation at mitosis, which enhances its phosphatase activity (8–10). Since MPF can phosphorylate cdc25, an autoregulatory loop has been proposed in which, at mitosis, MPF phosphorylates and activates cdc25, which in turn dephosphorylates and further activates preMPF (10). In interphase, cdc25 is inactivated by an okadaic acid-sensitive phosphatase (8–10).

MPF activation is affected by the cAMP-dependent protein kinase (PKA) in meiotic maturation. *Xenopus* oocytes are physiologically arrested at the G_2/M border of the first meiotic prophase. The natural inducer of meiotic maturation, progesterone, initiates MPF activation by downregulating the cAMP–PKA pathway (11). The concentration of cAMP decreases during oocyte maturation (11, 12). Inhibition of the cAMP–PKA pathway induces and PKA activation blocks meiotic maturation in many species (11, 13).

In this paper we explore the role of PKA in the mitotic cell cycle, using cycling extracts (2) from activated *Xenopus* eggs. Our evidence suggests that oscillations in PKA activity regulate MPF activation via the cdc25 phosphatase pathway and help set the timing and progression of the embryonic cell cycle.

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MATERIALS AND METHODS

Extract Preparation and PKA and Histone H1 Kinase Assays. Cycling, cytostatic factor-arrested (CSF), and interphase extracts were prepared from *Xenopus* eggs essentially as described (2, 3). Histone H1 kinase was assayed in EB buffer (2). The kinase reaction mixture included 20 μM PKA inhibitor peptide (PKI; Sigma). Free PKA catalytic subunit activity was assayed by addition of 8 μl of a mixture containing 200 μM Kemptide (Sigma), 100 μM ATP, and 2.5 μCi of [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol; 1 Ci = 37 GBq) to 4 μl of diluted sample (1:50 in EB buffer) and incubation for 10 min at 30°C. Where indicated, 20 μM PKI was added to the reaction mixture. Total PKA (holoenzyme plus free catalytic subunit) activity was assayed with the same mixture plus 200 μM 8-Br-cAMP. Kemptide was separated in an SDS/18% polyacrylamide gel and radioactivity was quantified with a Betascope analyzer (Betagen).

p13-Sepharose Isolation of $p34^{cdc2}$–Cyclin Complex. Sea urchin cyclin B was synthesized in a reticulocyte lysate (Stratagene) containing sea urchin cyclin B mRNA at 0.1 mg/ml and [^{35}S]methionine (Amersham, >1000 Ci/mmol) at 1 mCi/ml. For cyclin–p34 binding experiments, portions of a reticulocyte lysate containing [^{35}S]methionine-labeled sea urchin cyclin B were added to portions of extract (1/20th of extract volume) arrested in interphase by cycloheximide (3). After incubation, samples (50 μl) were put on ice and 25 μl of packed p13-Sepharose beads was added. Washes were performed as described (3).

$p34^{cdc2}$ Immunoblot. $p34^{cdc2}$ was affinity purified from 100 μl of extract with p13-Sepharose beads. Samples were electrophoresed in an SDS/12% polyacrylamide gel and blotted onto a nitrocellulose filter (Amersham). The filter was then probed with an anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology), and the reaction was visualized with an alkaline phosphatase-conjugated secondary antibody (Promega, manufacturer's protocol). Equal amounts of $p34^{cdc2}$ antigen were detected in parallel filters by using an anti-*S. pombe* cdc2 polyclonal antibody (gift of D. Beach, Cold Spring Harbor Laboratories; data not shown).

cdc25 Phosphatase Assay. A recombinant glutathione-*S*-transferase–cdc25 fusion protein, GST-hcdc25-C, was prepared as described (10). Approximately 4 μg of protein bound to glutathione-Sepharose beads was incubated for 1 hr at 23°C, in constant rotation, with 60 μl of EB buffer, 40 μl of either CSF- or interphase-arrested extract, and 1 mM ATP. The immunoblot was probed with an anti-C terminus human cdc25 antibody produced as described (10). ^{32}P-labeled cdc25 for use as phosphatase substrate was obtained as follows. GST-hcdc25-C (10 μg) bound to glutathione-Sepharose beads was incubated for 30 min at 23°C with 60 μl of EB buffer, 40 μl of CSF-arrested extract, 3 μM okadaic acid, and 100 μCi

Abbreviations: CSF, cytostatic factor; MPF, M-phase-promoting factor; PKA, cAMP-dependent protein kinase; PKI, PKA inhibitor peptide.

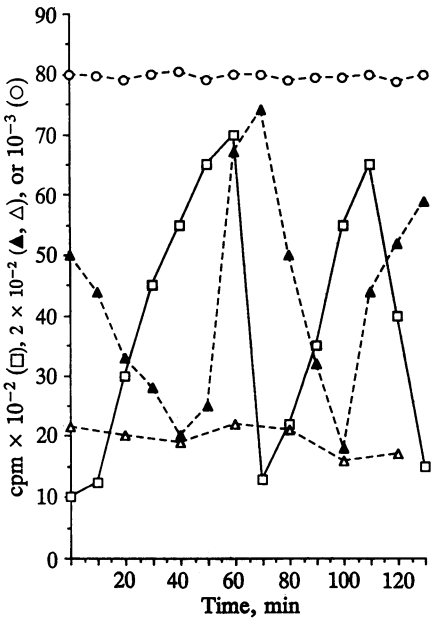

FIG. 1. Free PKA catalytic subunit concentration oscillates during the cell cycle. Kinase activity as a function of time of incubation of a cycling extract at 23°C. □, Histone H1 kinase; ▲, free PKA catalytic subunit; ○, total PKA (holoenzyme plus free catalytic subunit); △, free PKA catalytic subunit assayed in the presence of PKI. The experiment shown is representative of six experiments done with six independent extracts. The patterns of PKA and MPF oscillations were essentially reproducible, although the precise time of appearance of the two peaks was extract-dependent.

of [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol). 1 mM ATP was then added and incubation continued for 30 min. The beads were washed with EB buffer. The bound material was eluted with Laemmli buffer. After SDS/PAGE, the 97-kDa hyperphosphorylated form of cdc25 was eluted from the gel slice in 50 mM Tris, pH 7.8/50 mM NaCl. The protein was renatured with four cycles of dilution–concentration with a Centricon 30 (Amicon) cartridge and brought to a final volume of 40 μl with 50 mM Tris, pH 7.8/50 mM NaCl. Samples of a cyclin-depleted interphase extract (30 μl) were added with either water or 40 μM 8-Br-cAMP or 40 μM 8-Br-cAMP/1.3 μM okadaic acid (Boehringer Mannheim) incubated for 15 min at 23°C, and then mixed with 6 μl of [^{32}P]cdc25 and further incubated at 23°C. Aliquots (4 μl) were taken at the indicated times and mixed with equal volumes of 2× Laemmli buffer to stop the reaction. After SDS/10% PAGE, the samples were autoradiographed and scanned with a Betascope analyzer (Betagen, Waltham, MA). No bands of lower molecular weight appeared during incubation, thus excluding significant proteolysis.

RESULTS

PKA and the Onset of Mitosis. Murray and Kirschner (2) described an *in vitro* system, derived from activated *Xenopus* egg extracts, that passes through multiple cell cycles (2). Oscillations between interphase and mitosis can be monitored by assaying histone H1 kinase activity of MPF during incubation at 23°C. MPF kinase peaks when the extract reaches metaphase, abruptly declines at telophase, remains low during interphase, and rises again at entry into the next mitosis. To determine whether PKA activity oscillates during the cell cycle, we measured MPF kinase and PKA activity in an incubated *Xenopus* egg extract (Fig. 1). PKA activity was measured by phosphorylation of a PKA substrate peptide. The activity of free PKA catalytic subunit oscillated during the cell cycle. These oscillations were inhibited by adding PKI (14) to the kinase reaction mixture. Activity was high during interphase, fell at the time of mitosis onset, and rose again at the following mitosis–interphase transition. The total concentration of PKA, determined by adding an excess of

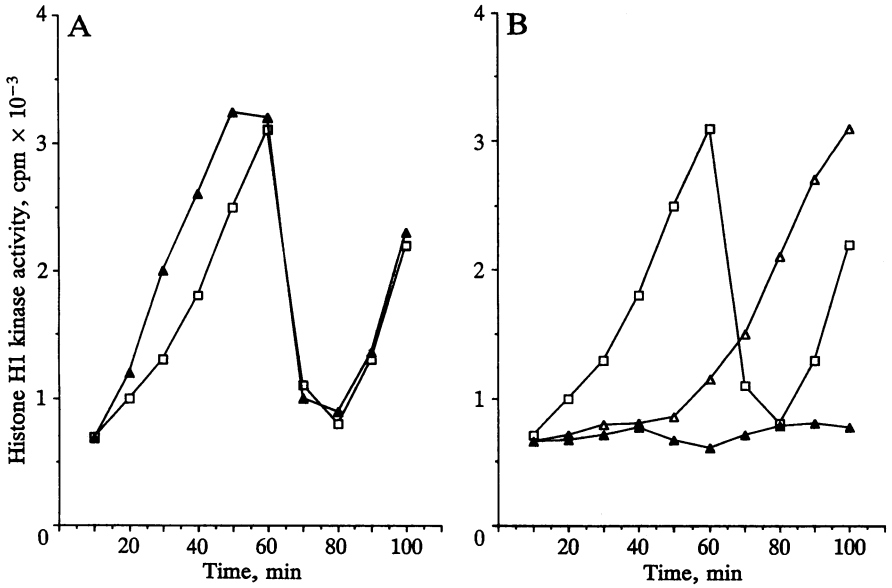

FIG. 2. PKA inhibition activates MPF prematurely, whereas PKA stimulation prevents MPF activation, in cycling extracts. Histone H1 kinase activity is shown as a function of time of incubation at 23°C. (*A*) □, Control sample; ▲, sample of the same extract containing 200 μM PKI. (*B*) □, Control sample; △, sample of the same extract containing 4 μM 8-Br-cAMP; ▲, sample of the same extract containing 40 μM 8-Br-cAMP. Extract samples were diluted with 0.1 volume of water (control), PKI, or 8-Br-cAMP. In two of the eight extracts tested, PKI accelerated the appearance of active MPF by 20 min rather than 10 min. Similarly, the prolongation of MPF peak activity induced by PKI in two extracts lasted 40 min. In contrast, the fall in MPF activity in control extracts without PKI always followed the peak by 10 min. Premature activation and sustained high levels of MPF were also obtained with a protein fusion of glutathione-*S*-transferase with the rat PKA regulatory subunit type II (data not shown). Results comparable to those found with the addition of 40 μM 8-Br-cAMP were obtained with PKA catalytic subunit (1 unit/μl of extract), the SP isomer of cyclic adenosine 3′,5′-monophosphothioate (40 μM), or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) (data not shown).

8-Br-cAMP to the kinase reaction mixture, remained essentially constant.

To determine whether inhibition of endogenous PKA affects cell cycle progression, we added PKI to a cycling extract. MPF kinase activity was followed as a function of time of incubation at 23°C. Treatment with PKI significantly altered cycle progression (Fig. 2*A*). MPF activity in a control extract peaked at 60 min and fell to basal levels at 70 min. PKI, added prior to incubation, advanced the peak of MPF activity to 50 min. MPF activity remained high in the PKA-inhibited extract for the following 10 min and fell to basal levels at 70 min. The second mitosis proceeded with kinetics similar to the control. We believe this was due to the degradation of the PKI peptide, since acceleration of the second MPF peak could be induced by adding PKI at the beginning of the second interphase (data not shown).

Whereas inhibiting PKA reduced the length of interphase and accelerated mitosis onset, sustaining PKA activity had the opposite effect on cycle progression. Addition of 8-Br-cAMP delayed the time of MPF activation in a dose-dependent fashion (Fig. 2*B*). At a high concentration of 8-Br-cAMP, extracts were arrested in interphase. At a low dose, extracts eventually entered mitosis after a prolonged interphase. The subsequent exit from mitosis and the duration of the second interphase were unaffected (data not shown).

PKA Inhibits p34^{cdc2} Tyrosine Dephosphorylation and Its Effects Are Reversed by Okadaic Acid. The accumulation of cyclin is necessary for MPF activation and entry into mitosis, and its degradation is required for transition to anaphase (2). Because of the pivotal role of cyclin, we asked whether PKA influences the cell cycle by altering cyclin synthesis or stability. We monitored the appearance and disappearance of cyclin in an extract arrested in interphase with 40 μM 8-Br-cAMP as described in Fig. 2*B*. Cyclin, marked by continuous labeling of the extract with [^{35}S]methionine, accumulated in a control extract (Fig. 3*A Left*) until metaphase (70 min) and disappeared 10 min later (80 min). In the sample treated with 8-Br-cAMP, in which no H1 kinase activation was detected (data not shown), cyclin accumulated throughout the course of the experiment (Fig. 3*A Right*). These results show that PKA acts downstream of cyclin synthesis to prevent the appearance of active MPF. This was confirmed by adding purified recombinant sea urchin cyclin B to an extract blocked in interphase by cycloheximide (2, 3). In a control extract, cyclin addition induced MPF activation (Fig. 3*B*, lanes 3–6); simultaneous addition of 8-Br-cAMP completely inhibited cyclin-induced MPF activation (lanes 7–10).

We next asked whether PKA controls MPF activation by preventing the association of cyclin with p34^{cdc2}. To follow the fate of cyclin, we added [^{35}S]methionine-labeled sea urchin cyclin B to a cycloheximide-arrested extract. After a 20-min incubation p34^{cdc2} was isolated by affinity chromatography on p13-Sepharose, and the amount of bound cyclin determined. Cyclin associated with p34^{cdc2} in the absence (Fig. 3*C*, lane 2) or in the presence of 8-Br-cAMP (lane 3). Thus, PKA does not prevent the association of cyclin with p34^{cdc2}, but the resultant complex lacks kinase activity.

Okadaic acid, a serine/threonine-phosphatase inhibitor, prematurely activates MPF in *Xenopus* egg extracts (3, 6). Given the similarity between the effects of okadaic acid and PKI, we considered the possibility that PKA controls MPF activation through an okadaic acid-sensitive step. In a control extract, MPF kinase peaked at 40 min, rapidly decayed, and then rose again at the approach of the next mitosis (Fig. 4*A*). As previously described (3), okadaic acid induced the rapid activation of MPF (20 min, Fig. 4*B*), and high kinase levels persisted for the duration of the experiment. The inhibition of MPF kinase induced by 8-Br-cAMP (Fig. 4*C*) was entirely reversed by addition of okadaic acid at 40 min (Fig. 4*D*) or at later times during interphase arrest (data not shown). Since okadaic acid did not decrease the concentration of free PKA catalytic subunit in the extracts (data not shown), these results imply that PKA stimulates one or more serine/threonine protein phosphatases that negatively regulate MPF.

MPF activation is associated with dephosphorylation of the Tyr-15 residue of p34^{cdc2} (3, 4). The inactive MPF that accumulates in the presence of 8-Br-cAMP retained the Tyr-15 phosphate (Fig. 4*E*; see Fig. 4*C* at indicated times for H1 kinase activity). The restoration of MPF activity by okadaic acid coincided with the removal of p34^{cdc2} tyrosine phosphate (Fig. 4*F*; see Fig. 4*D*).

PKA Stimulates Dephosphorylation of cdc25. These results indicate that PKA, through an okadaic acid-sensitive phosphatase, either stimulates the p34^{cdc2} tyrosine kinase pathway, inhibits the p34^{cdc2} tyrosine phosphatase pathway, or

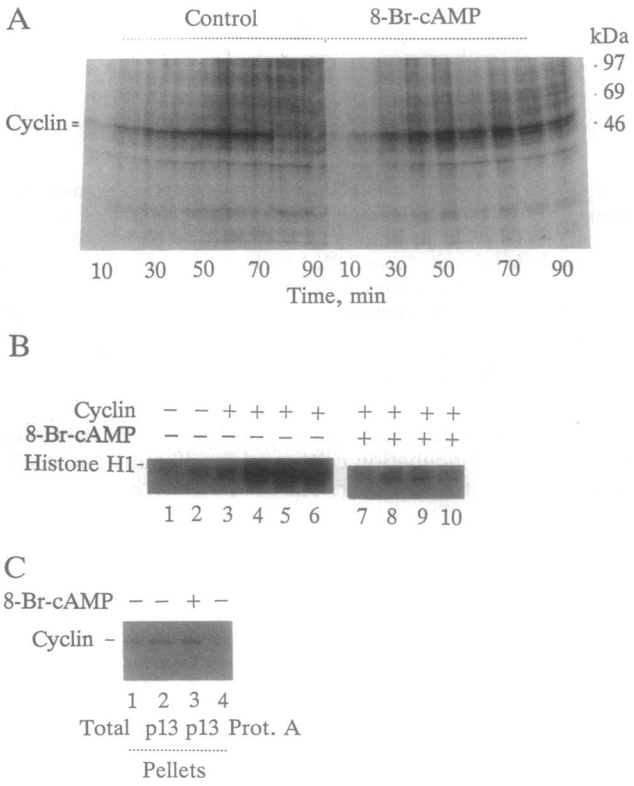

FIG. 3. (*A*) Cyclin accumulation in 8-Br-cAMP-treated extracts, shown by autoradiography following SDS/12% PAGE of proteins synthesized in an incubated extract. [^{35}S]Methionine (0.4 mCi/ml of extract) was added at time 0. (*Left*) Control extract. Cyclin accumulated until metaphase (70 min) and disappeared at telophase (80 min). (*Right*) Portion of the same extract to which 40 μM 8-Br-cAMP was added. Cyclin accumulated continuously for the duration of the experiment (90 min). (*B*) PKA inhibits MPF in the absence of *de novo* protein synthesis. $CaCl_2$ (0.4 mM) and cycloheximide (100 μg/ml) were added to a CSF-arrested extract to advance the extract from metaphase to interphase and to block cyclin synthesis. The extract was preincubated 40 min at 23°C. Histone H1 kinase was then determined. Autoradiographs of ^{32}P-labeled histone are shown. Lanes 1 and 2, with 0 or 40 min of additional incubation; lanes 3–6, after 0, 20, 40, or 60 min with recombinant sea urchin cyclin B protein (200 nM); lanes 7–10, after 0, 20, 40, or 60 min with cyclin and 8-Br-cAMP (40 μM). (*C*) Cyclin binds p34^{cdc2} in the presence of 8-Br-cAMP. A CSF-arrested extract was treated with $CaCl_2$ and cycloheximide (see *B*). After 40 min of preincubation, a portion of reticulocyte lysate containing [^{35}S]methionine-labeled sea urchin cyclin B was added to extract samples. Lane 1, total input cyclin; lane 2, control sample; lane 3, plus 8-Br-cAMP (40 μM). The samples in lane 2 and 3 were incubated for 20 min, followed by p13-Sepharose affinity purification of p34^{cdc2}. Lane 4, as in lane 2 except for a mock purification with protein A-Sepharose.

does both. We show below that PKA appears to exert a negative control on the activity of cdc25, the tyrosine phosphatase that specifically dephosphorylates and activates $p34^{cdc2}$ (6, 7). The tyrosine phosphatase activity of cdc25 positively correlates with its degree of serine/threonine phosphorylation (8–10). We asked whether treatment of interphase extracts with 8-Br-cAMP would stimulate the dephosphorylation of cdc25. To obtain the phosphorylated cdc25 substrate, recombinant GST-hcdc25-C fusion protein was isolated and phosphorylated with a metaphase-arrested extract. These extracts contain active MPF kinase and have lower cdc25 phosphatase activity than interphase extracts (8–10). The reaction was monitored by Western blot analysis of the treated protein (Fig. 5*A*). The cdc25 fusion protein was extensively phosphorylated, as indicated by an increase in molecular weight (compare lanes 1 and 2). cdc25 incubated with a control interphase extract was not phosphorylated (lane 3). ^{32}P-labeled cdc25, prepared as in Fig. 5*A*, lane 2, was incubated for various times with an interphase extract (Fig. 5*B*). Pretreatment of the extract for 15 min with 8-Br-cAMP significantly stimulated the initial rate of cdc25 dephosphorylation. An extract pretreated with 8-Br-cAMP and okadaic acid showed little dephosphorylation of cdc25 (see Fig. 5*C* for quantitation).

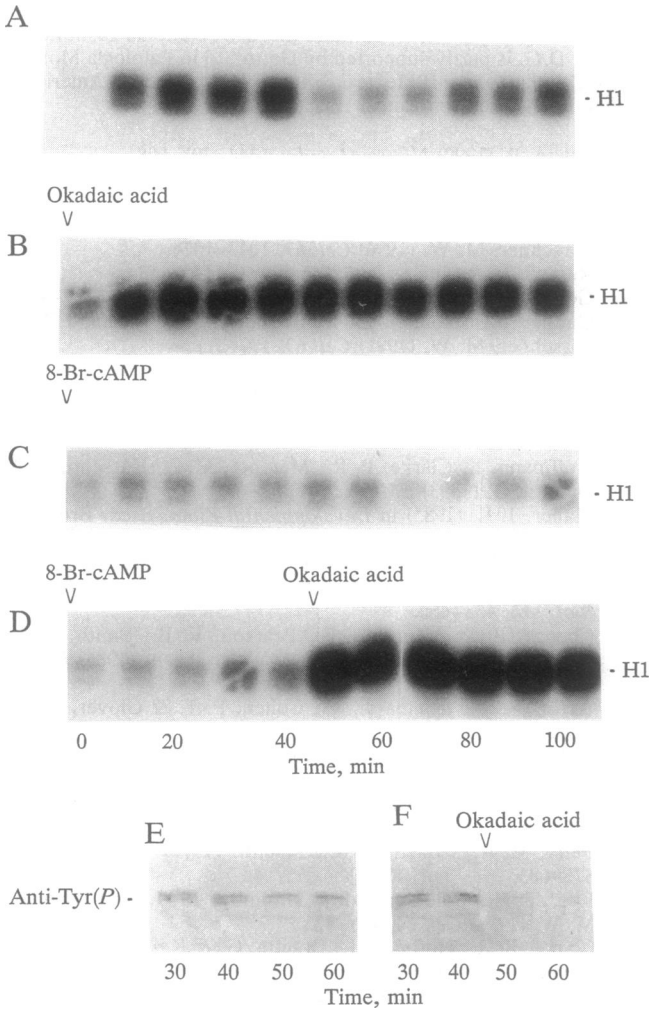

FIG. 4. (*A–D*) Okadaic acid reactivates 8-Br-cAMP-inhibited MPF and removes $p34^{cdc2}$ tyrosine phosphate. Histone H1 kinase activity was measured in samples of a cycling extract incubated at 23°C with no additions (*A*), 1.3 μM okadaic acid (*B*), 40 μM 8-Br-cAMP (*C*), or 8-Br-cAMP with okadaic acid added at 40 min (*D*). The induction of MPF kinase activity by okadaic acid was also confirmed by assaying p13-Sepharose precipitates from these samples (data not shown). (*E* and *F*) Phosphotyrosine content of $p34^{cdc2}$ (see *Materials and Methods*) was assessed in a sample inhibited with 8-Br-cAMP (*E*) and in a sample inhibited with 8-Br-cAMP to which okadaic acid was added at 40 min. The appearance of a doublet signal is presumably due to the different degree of serine/threonine phosphorylation of tyrosine-phosphorylated $p34^{cdc2}$ (see ref. 3).

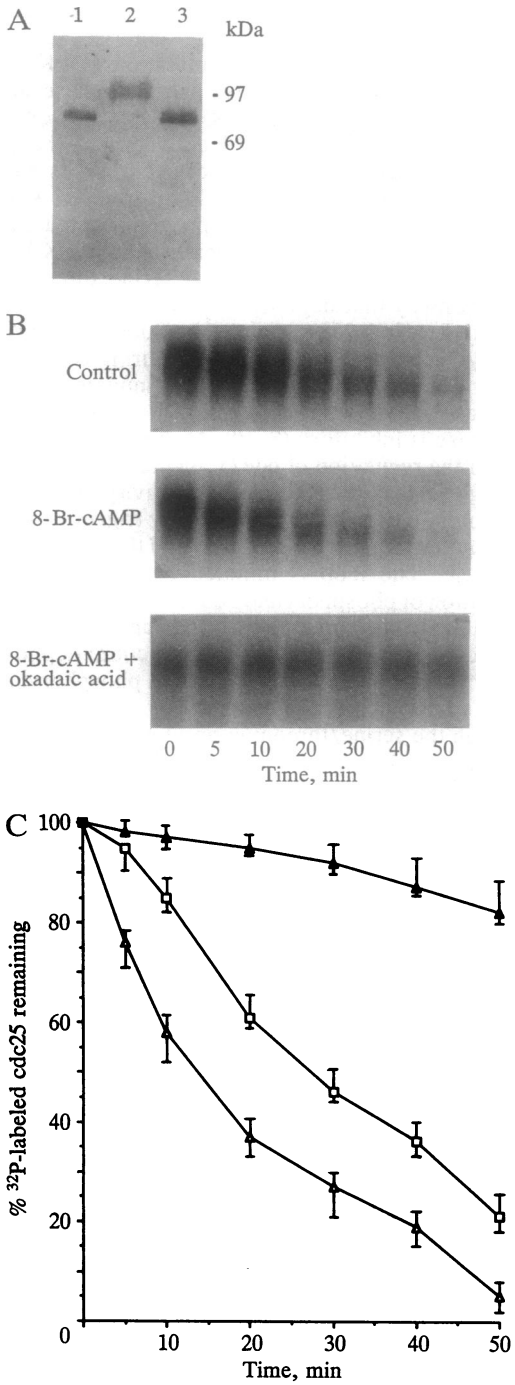

FIG. 5. PKA activation stimulates cdc25 serine/threonine dephosphorylation. (*A*) Western blot probed with an antibody against the C terminus of human cdc25 of recombinant fusion protein GST-hcdc25-C (lane 1), the protein after 1 hr of incubation with a CSF-arrested metaphase extract (lane 2), and after 1 hr of incubation with an interphase extract (lane 3). (*B*) Dephosphorylation of ^{32}P-labeled cdc25 during incubation with a control interphase extract and with the same extract pretreated with 8-Br-cAMP or with 8-Br-cAMP and okadaic acid. (*C*) Quantitation of the experiment described in *B*. □, Control sample; △, plus 8-Br-cAMP; ▲, plus 8-Br-cAMP and okadaic acid. Error bars indicate the range of values within four independent experiments.

DISCUSSION

These experiments indicate an active role of the cAMP–PKA pathway for cell cycle progression in *Xenopus* embryos. Measurements of PKA activity in cycling egg extracts show that PKA holoenzyme undergoes cycles of association and dissociation during the cell cycle. The levels of free PKA catalytic subunit are high during interphase, decrease to a minimum at the onset of mitosis, and rise again at the metaphase–anaphase transition. Inhibition of PKA prematurely activates MPF and delays the metaphase–anaphase transition. Sustained activation of PKA by 8-Br-cAMP prolongs interphase by blocking MPF activation. Although PKA does not affect the accumulation of cyclin or its binding to $p34^{cdc2}$, the resultant complex lacks histone H1 kinase activity. The inactive MPF remains phosphorylated on Tyr-15 of $p34^{cdc2}$, and the cyclin component is stable. PKA appears to stimulate an okadaic acid-sensitive serine/threonine phosphatase pathway that dephosphorylates cdc25.

The activity of cdc25 is regulated by fluctuations in its level of serine/threonine phosphorylation during the cell cycle. Phosphorylation of cdc25 at mitosis stimulates its tyrosine phosphatase activity and, conversely, its dephosphorylation at interphase is inhibitory (8–10). Multiple factors may participate in the regulation of cdc25, including MPF itself (10).

We suggest that the fall in PKA activity at the onset of mitosis could initiate MPF activation by triggering a positive autoregulatory loop involving cdc25 and the PKA-stimulated phosphatase. The loop is initiated by an increase in the concentration of phosphorylated cdc25 due to the decrease in PKA and a resulting reduction of the phosphatase activity acting upon cdc25. The phosphorylated cdc25 dephosphorylates $p34^{cdc2}$, activating MPF. MPF then phosphorylates and activates additional cdc25 (10).

Although the serine/threonine protein phosphatase that is the target of PKA is not yet defined, two okadaic acid-sensitive phosphatases, PP-1 and PP-2A, have been implicated in the regulation of mitosis. PP-1 appears to be required for exit from mitosis (15, 16). On the other hand, PP-2A appears to suppress entry into mitosis, since inactivation of one PP-2A gene results in premature mitosis in *S. pombe*. That *S. pombe* PP-2A interacts with cdc25 is suggested by genetic evidence (17). In *Xenopus* egg extracts, inhibition of PP-2A induces premature activation of MPF (18). INH, which blocks MPF activation, has been found to be a catalytic subunit of PP-2A (19). Finally, inhibition of PP-2A by okadaic acid completely blocks cdc25 dephosphorylation, whereas specific inhibition of PP-1 has no effect (20). PP-2A, therefore, is a reasonable candidate for the phosphatase activity stimulated by PKA; it is sensitive to okadaic acid, it dephosphorylates cdc25, and it prevents entry into mitosis.

A role for PKA in the control of MPF activation via other phosphorylation–dephosphorylation systems has not been excluded. These include the $p34^{cdc2}$ tyrosine kinase pathway, which is regulated by serine/threonine phosphorylation (21) and the phosphorylation of $p34^{cdc2}$ Thr-161 (22).

We suggest that the onset of mitosis is critically dependent on the concentrations of active PKA and assembled preMPF. Cycles of PKA activity may regulate cell cycle progression in the early embryonic divisions, a stage at which external stimuli are not required for the cell cycle. Whether these oscillations reflect changes in the rate of cAMP synthesis and/or hydrolysis is not known. Preliminary results indicate that the extracts can both synthesize and degrade cAMP. The increase in PKA activity observed at the transition between mitosis and interphase raises the interesting possibility that the high level of MPF activity reached at metaphase may stimulate the cAMP–PKA pathway, creating an interdependence between the two oscillating activities.

Oscillations of the cAMP–PKA pathway also mark the somatic cell cycle (23), and downregulation of PKA induces chromatin condensation in cultured fibroblasts (24). Finally, the presence of high concentrations of unreplicated DNA in *Xenopus* extracts inhibits the onset of mitosis and $p34^{cdc2}$ tyrosine dephosphorylation (25, 26). The signal indicating completion of DNA replication thus might be transmitted to MPF via the cAMP–PKA pathway.

We thank Dr. G. Salvatore (II Medical School, University of Naples). Special thanks go to Dr. M. Pagano, Dr. I. Hoffmann, and Dr. G. Draetta (Differentiation Program, European Molecular Biology Laboratory, Heidelberg); Dr. I. Daar and Dr. G. Vande Woude (National Cancer Institute, Frederick, MD); and Dr. S. Obici (Columbia University, New York) for reagents provided and helpful discussion. This work was supported by Associazione Italiana per la Ricerca sul Cancro, Consiglio Nazionale delle Ricerche (Progetti Finalizzatti Oncologia, Applicazioni Cliniche delle Ricerche Oncologica, e Ingegneria Genetica), and the U.S. National Institutes of Health. D.G. is partly supported by Dottorato in Patologia Molecolare e Cellulare of the II Medical School, Naples, and the American–Italian Foundation for Cancer Research, New York.

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