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Cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cell cycle: evidence from the effects of okadaic acid

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In *Xenopus* embryos, the cell cycle is abbreviated to a rapid alternation between interphase and mitosis. The onset of each M phase is induced by the periodic activation of the cdc2 kinase which is triggered by a threshold level of cyclins and apparently involves dephosphorylation of $p34^{cdc2}$. We have prepared post-ribosomal supernatants from eggs sampled during interphase (interphase extracts) and just before the first mitosis of the early embryonic cell cycle (prophase extracts). In 'interphase extracts', the cdc2 kinase never activates spontaneously upon incubation at room temperature whereas in 'prophase extracts' it does. We show here that in 'interphase extracts', specific inhibition of type 2A phosphatase by okadaic acid induces cdc2 kinase activation. This requires a subthreshold level of cyclin and the presence of a particulate factor in the extract. Inhibition of type 1 phosphatases by inhibitor 1 and inhibitor 2 never results in cdc2 kinase activation. These results demonstrate that during the period of cyclin accumulation, cdc2 kinase activation is inhibited by a type 2A phosphatase. In 'prophase extracts', spontaneous activation of the cdc2 kinase is inhibited by β-glycerophosphate and NaF, but not by okadaic acid, inhibitor 1 and inhibitor 2 or divalent cation chelation. This demonstrates that when enough cyclin has accumulated, cdc2 kinase activation involves a protein phosphatase which must be distinct from the type 1 and 2A phosphatases, and from the calcium-dependent (type 2B) and magnesium-dependent (type 2C) phosphatases.

Key words: cdc2 kinase/okadaic acid/phosphatase 2A/*Xenopus* egg extracts

Introduction

Cdc2 kinase is a key component of the cell cycle control pathway in eukaryotic cells (Nurse and Bisset, 1981; Beach *et al.*, 1982; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbé *et al.*, 1988a). Although the level of the cdc2 protein is constant throughout the cell cycle (Simanis and Nurse, 1986; Draetta and Beach, 1988; Labbé *et al.*, 1988a; Brizuela *et al.*, 1989), its kinase activity assayed on histones H1 is activated specifically during the M phase (Picard *et al.*, 1985; Arion *et al.*, 1988; Labbé *et al.*, 1988b). Activation of this enzyme at the G2-M transition requires the accumulation of cyclin molecules to a threshold level (Evans *et al.*, 1983; Pines and Hunt, 1987; Minshull *et al.*, 1989; Murray and Kirschner, 1989) as well as a cascade of reactions that proceed independently of protein synthesis (Félix *et al.*, 1989a). The mitotic kinase activity of cdc2 is switched off abruptly at the metaphase-anaphase transition as a result of rapid cyclin degradation (Murray *et al.*, 1989), which is itself triggered by the mitotic kinase activity of the cdc2 protein (Félix *et al.*, unpublished). This suggests the existence of a feedback loop between cdc2 kinase activity and the destruction of cyclin that could underlie the basic early embryonic cell cycle oscillator in *Xenopus* (Félix *et al.*, unpublished). Cyclin interacts physically with the cdc2 protein and the active mitotic kinase complex is formed of one molecule of $p34^{cdc2}$ and one molecule of cyclin (Booher *et al.*, 1989; Meijer *et al.*, 1989; Labbé *et al.*, 1989a; Pines and Hunter, 1989). Although cyclin is necessary for cdc2 kinase activation, its association with $p34^{cdc2}$ is not sufficient (Booher *et al.*, 1989; Moreno *et al.*, 1989; Pines and Hunter, 1989). The nature of the post-translational events actually required for activation of the mitotic kinase activity of the $p34^{cdc2}$-cyclin complex is still unclear. The dephosphorylation of $p34^{cdc2}$ on tyrosine and/or threonine residues observed at the G2-M transition in several species certainly plays an important role in the activation of the mitotic kinase (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Labbé *et al.*, 1989b; Morla *et al.*, 1989). The cyclin subunit also undergoes post-translational modifications (Pines and Hunt, 1987; Labbé *et al.*, 1989a; Booher *et al.*, 1989; Meijer *et al.*, 1989).

We have recently developed a cell-free system from activated *Xenopus* eggs in which the protein synthesis-independent phase of the cdc2 kinase activation can be studied (Félix *et al.*, 1989a,b). *Xenopus* eggs are lysed 60 min after egg activation (shortly before their first entry into M phase and the first cdc2 kinase activation) and concentrated post-ribosomal supernatants are prepared at 4°C. Upon incubation of these extracts at room temperature, the cdc2 kinase is activated and inactivated once. The proteins phosphorylated in the extract at the peak of cdc2 kinase activity are similar to those that are phosphorylated *in vivo* during mitosis and cdc2 kinase inactivation coincides temporally with destruction of added exogenous cyclin. The whole oscillation occurs in the absence of protein synthesis with approximately the same kinetics as *in vivo*. This shows that the system reproduces faithfully the biochemical reactions that take place during the protein synthesis-independent period preceding the onset of mitosis *in vivo*.

Given the evidence for a role of $p34^{cdc2}$ dephosphorylation in activation of its mitotic kinase activity, it was essential to examine the nature of the protein phosphatases involved in the oscillation of cdc2 kinase activity in these extracts. Many phosphatase inhibitors have been described (for reviews, see Ballou and Fischer, 1986; Cohen, 1989). β-glycerophosphate and sodium fluoride inhibit all serine/threonine phosphatases. Type 1 phosphatases are specifically inhibited by the heat-stable inhibitors 1 and 2, and a potent and specific inhibitor of type 1 and 2A phosphatases, okadaic

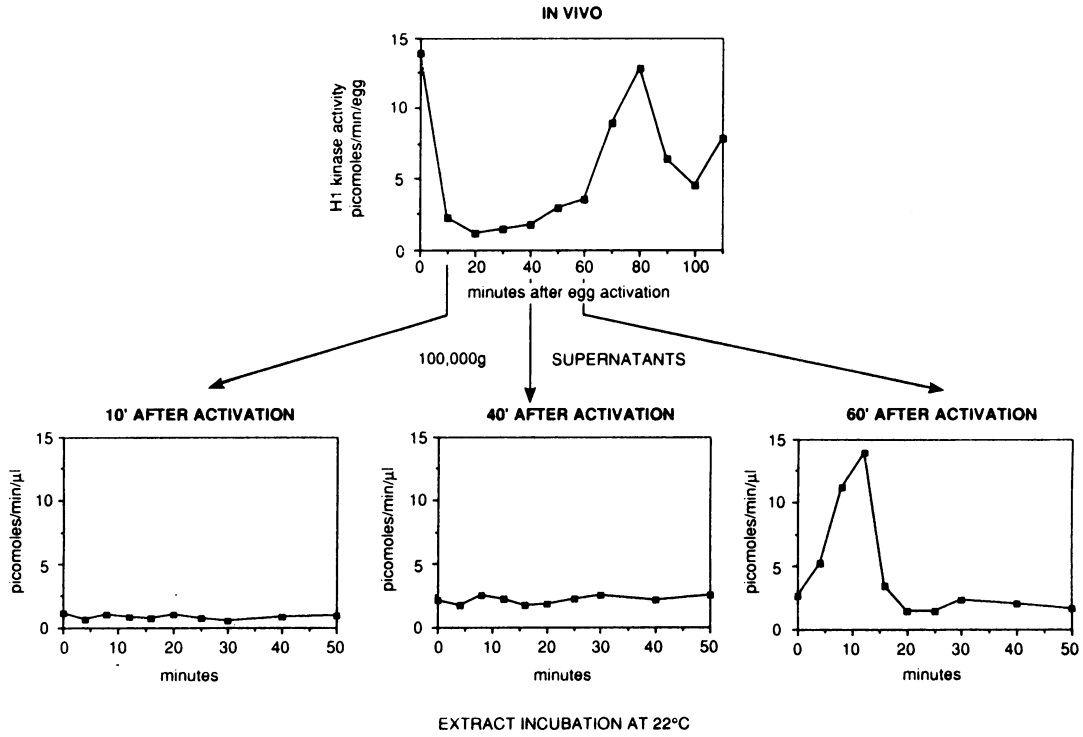

Fig. 1. Oscillation of H1 kinase activity *in vivo* (**upper panel**) and in 100 000 *g* supernatants of *Xenopus* eggs (**lower panel**). Upper panel: at the indicated times after activation, two eggs were crushed directly in 50 µl of extraction buffer and the yolk pelleted by a 5 min centrifugation at 10 000 *g* in an Eppendorf centrifuge. Exogenous histone H1 phosphorylation was assayed in the supernatant. Lower panel: concentrated cell-free extracts were prepared from eggs collected at different times in the first cell cycle (10, 40 and 60 min after egg activation) by centrifugation at 100 000 *g*. The extracts were then incubated at 22°C and samples diluted at different time-points in histone H1 kinase assay buffer. The kinase activity was then assayed on exogenous histone H1.

acid, has recently been described (Bialojan and Takai, 1988; Cohen *et al.*, 1989; Haystead *et al.*, 1989) and reported to induce maturation when injected into *Xenopus* oocytes (Goris *et al.*, 1989). Using these inhibitors, we present evidence that post-translational activation of the $p34^{cdc2}$–cyclin complex requires phosphatases that are distinct from the well characterized type 1, 2A, 2B and 2C phosphatases. By contrast, we show that type 2A phosphatase regulates cdc2 kinase activation negatively during the phase of cyclin accumulation. This suggests that during the early embryonic cell cycle in *Xenopus*, the timing of cdc2 kinase activation is tightly controlled by specific phosphatases acting on different substrates and having opposite effects.

Results

Effect of okadaic acid on cdc2 kinase activity in egg extracts prepared at different phases of the first embryonic cell cycle

Xenopus laevis eggs are laid arrested in metaphase of the second meiotic division. Upon fertilization or artificial activation, meiosis is completed and, after an 'interphase' of 60 min (which comprises an S and a short G2-like phase), the embryo enters a series of rapid synchronous cell cycles, alternating between S and M phases every 30 min. The cdc2 kinase is activated at each mitosis (Figure 1, top panel, measured on exogenous histones H1). Concentrated 100 000 *g* post-ribosomal supernatants were prepared from eggs collected at different times in the first cell cycle (Figure 1, lower panels). No protein synthesis was detected in these supernatants incubated at 22°C (Félix *et al.*, 1989a,b). In supernatants prepared from eggs sampled during the protein synthesis-dependent period of the first cell cycle (10', 40') the histone H1 kinase activity was low. It did not increase spontaneously upon incubation of the extracts at 22°C (Figure 1, lower panel). However, in extracts prepared 60 min after egg activation (60'), the histone H1 kinase was successively activated and inactivated with the same kinetics and amplitude as in living eggs. These latter extracts contained enough cyclin to irreversibly commit the system to full activation of the histone H1 kinase since they were prepared at a time in the cell cycle when protein synthesis is no longer required for the onset of mitosis (Karsenti *et al.*, 1987).

Addition of 1 µM okadaic acid, a specific inhibitor of type 2A and type 1 phosphatases (Bialojan and Takai, 1988; Haystead *et al.*, 1989), to extracts prepared 10 min after egg activation induced a slow increase of their H1 kinase activity. It reached an activity of 6 pmol phosphate/min/µl extract after 50 min of incubation at 22°C (Figure 2A). Addition of the same amount of okadaic acid to extracts prepared 40 min after egg activation resulted in a rapid activation of the H1 kinase that peaked to a mitotic level 10 min after addition of the drug (Figure 2B; compare with Figure 1). The effect of okadaic acid was clear at 330 nM and maximal at 1 µM. H1 kinase activity then decreased relatively slowly and plateaued after 20 min at a relatively high level (7 pmol/min/µl for 1 µM okadaic acid). In 'cycling' extracts prepared 60 min after egg activation, addition of 1 µM okadaic acid accelerated reproducibly the rate of H1 kinase activation and decreased the rate and the extent of its inactivation (Figure 2C). The poor effect of okadaic acid on

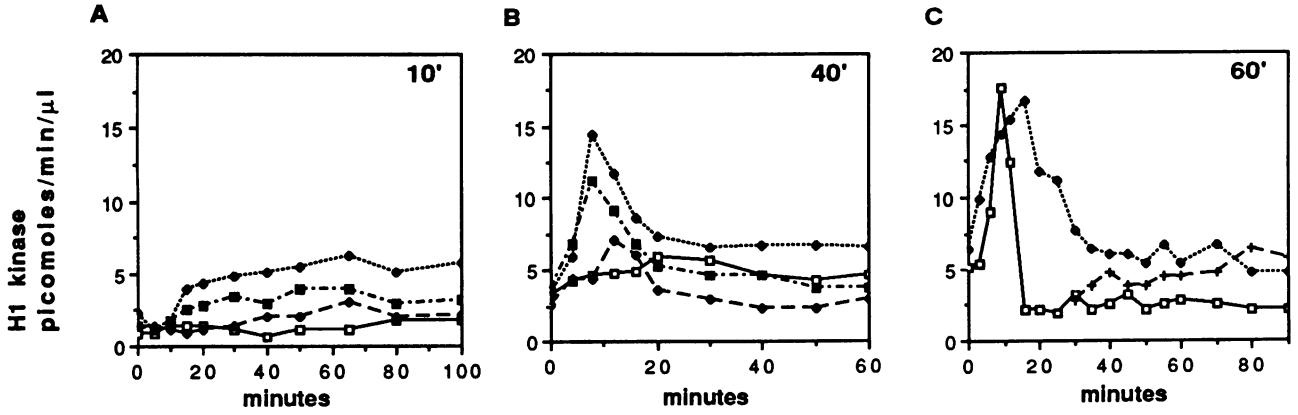

Fig. 2. Effect of okadaic acid on the H1 kinase activity in 100 000 *g* supernatants of *Xenopus* eggs. The 100 000 *g* supernatants prepared 10 (**A**), 40 (**B**) or 60 (**C**) min after egg activation were incubated at 22°C with different concentrations of okadaic acid: 0 nM (open squares), 100 nM (open circles), 330 nM (filled squares), 1 µM (filled circles). The histone H1 kinase activity was assayed on samples taken at different time-points during incubation of the extract at 22°C. In (C), 1 µM okadaic acid was added before (0′, filled circles), or after (30′, crosses) preincubation of the extract at 22°C.

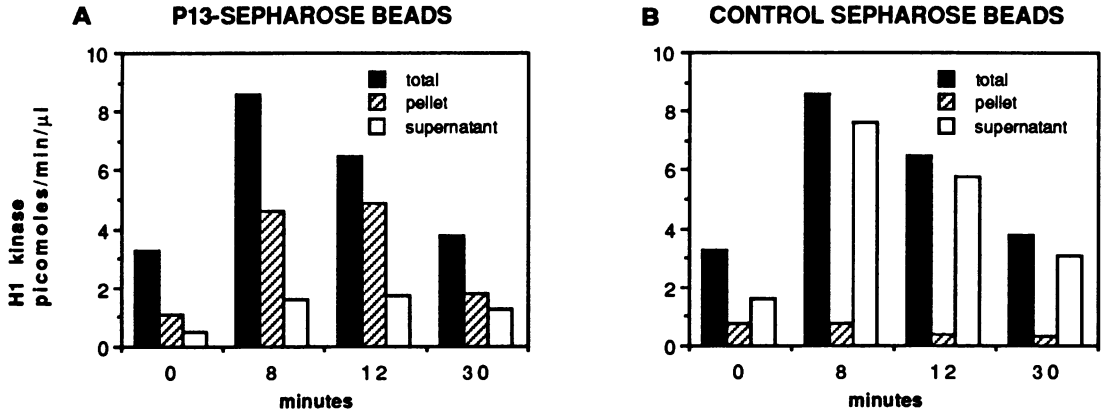

Fig. 3. The H1 kinase induced by okadaic acid in extracts prepared 40 min after egg activation binds to p13^{suc1+}-Sepharose beads. A 100 000 *g* supernatant prepared 40 min after egg activation was incubated at 22°C and aliquots sampled at the times indicated in abscissa were added to p13^{suc1+}-Sepharose beads (**A**) or control-Sepharose beads (**B**) as described in Félix *et al.* (1989a). Histone H1 kinase activity was tested in the total extract (filled bars), in the pellet (dashed bars) and in the supernatant (open bars). The activities were measured in duplicates and expressed in pmol/min/µl of the non-diluted extract (ordinate).

histone H1 kinase activation in extracts prepared 10 min after egg activation compared to the strong effect observed in the 40 min extracts suggested that an essential component, like the cyclins, had to accumulate in the eggs before preparation of the extract. In order to test this possibility, a cycling extract was incubated for 30 min at room temperature. During this time, the H1 kinase was activated and inactivated once (Figure 2C) and the endogenous cyclin degraded (not shown). Then, okadaic acid (1 µM) was added and histone H1 kinase assayed during a further 1 h incubation of the extract at 22°C. As shown in Figure 2C, under these conditions the histone H1 kinase was hardly activated. A possible interpretation of these results is that the H1 kinase activation induced by okadaic acid required the presence of some level of cyclin protein.

We have shown previously (Felix *et al.*, 1989a) that most of the histone H1 kinase measured in *Xenopus* egg extracts can be attributed to the homologue of the yeast *cdc2* gene product by virtue of its affinity for p13^{suc1}, a fission yeast protein known to interact specifically with the cdc2-kinase (Brizuela *et al.*, 1987; Arion *et al.*, 1988; Dunphy *et al.*, 1988). We have verified that the histone H1 kinase activated by okadaic acid was due to the same cdc2 protein. As shown in Figure 3, most of the histone H1 kinase activated by 1 µM okadaic acid in 40 min extracts was removed by p13^{suc1}-Sepharose beads, and >50% of the activity was recovered in the pelleted beads. Similar depletion and recovery were obtained when the cdc2 kinase purified from starfish eggs was adsorbed to these beads (not shown).

Cyclin is normally degraded at each cell cycle at the end of the M phase. In extracts prepared 60 min after egg activation, sea urchin cyclin added to the extract was specifically degraded after the rise in cdc2 kinase activity, at the time of its inactivation (Félix *et al.*, 1989a). In fact, induction of cyclin degradation in the extract requires a threshold level of cdc2 kinase activity (Félix *et al.*, unpublished). In extracts prepared 40 min after egg activation, the cdc2 kinase activity remained low and, therefore, sea urchin cyclin was not degraded (Figure 4, left). Addition of 1 µM okadaic acid induced cdc2 kinase activation (Figure 2B) and triggered cyclin degradation (Figure 4, right).

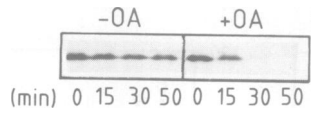

Fig. 4. Okadaic acid induces the degradation of exogenously added sea urchin cyclin in extracts prepared 40 min after egg activation. *Arbacia punctulata* cyclin translated *in vitro* in the presence of [^{35}S]methionine was added to a 100 000 *g* supernatant prepared 40 min after egg activation in the absence (**left panel**) or in the presence (**right panel**) of 1.0 μM okadaic acid. Aliquots were diluted at different time-points in gel sample buffer and analysed by SDS−PAGE and autoradiography.

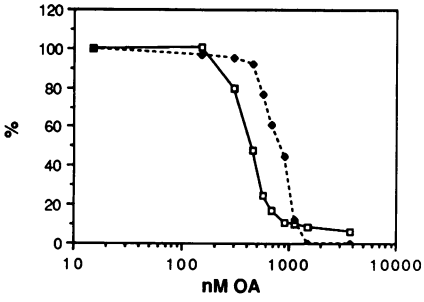

Fig. 5. Type 2A phosphatase activity is more sensitive to okadaic acid than type 1 phosphatase activity in concentrated extracts of *Xenopus* eggs. Concentrated extracts (35 mg/ml of proteins) of eggs homogenized 40 min after activation were prepared by centrifugation at 100 000 *g* for 1 h. Phosphatase assays were carried out at 22°C in 50% diluted extracts. Type 2A phosphatase activity was measured using casein as a substrate (open squares). Type 1 phosphatase activity was measured using phosphorylase kinase as a substrate, and subtracting the activity measured in the presence of 0.25 μM inhibitor 1 plus 0.25 μM inhibitor 2 (filled symbols). The phosphatase activities at different okadaic acid concentrations (abscissa) were expressed as a percentage of the control activity in the absence of okadaic acid (ordinate). The phosphorylase kinase substrate contained 0.34 mol phosphate/β subunit and 0.11 mol phosphate/α subunit. The phosphatase activities were determined by measuring the TCA-soluble counts released after 30 s of incubation of the ^{32}P-labelled substrate in the extract. Under these conditions, the extent of substrate dephosphorylation never exceeded 20%, and dephosphorylation was linear over the incubation time. The okadaic acid concentrations were expressed per unit volume of extract.

Cdc2 kinase is negatively regulated by a type 2A phosphatase and not by a type 1 phosphatase

Okadaic acid is a more potent inhibitor of the purified catalytic subunit of phosphatase 2A (I_{50} = 0.1 nM) than of phosphatase 1 (I_{50} = 10 nM). However, since the concentration of okadaic acid required for inhibition of phosphatase 2A is similar to that of the phosphatase (Cohen *et al.*, 1989), it was necessary to determine the concentration of okadaic acid required to inhibit both phosphatase 1 and 2A, as well for activation of cdc2 kinase, in the concentrated egg extracts.

Endogenous phosphatase activities were measured by adding ^{32}P-labeled substrates specific for type 1 and type 2A phosphatases to the extracts. Phosphocasein and the α-subunit of phosphorylase kinase (Stewart *et al.*, 1981; McGowan and Cohen, 1988) were used as specific substrates of type 2A phosphatases. The β-subunit of phosphorylase kinase was used as a specific substrate of type 1 phosphatases. The dephosphorylation of these substrates was monitored by the release of TCA-soluble counts. The α- and β-subunits of the phosphorylase kinase were separated by SDS −PAGE.

Type 1 and 2A phosphatase activities were first measured on phosphorylase kinase and casein respectively. All assays were made at a 50% dilution of the extract at 22°C. The phosphatase activities remained approximately constant during a 60 min incubation of the extracts at 22°C (data not shown). A 50% inhibition of type 2A phosphatase activity (assayed on phosphocasein) was obtained by adding 375 nM okadaic acid to the extract, whereas a 50% inhibition of type 1 phosphatase activity (assayed on phosphorylase kinase) required the addition of 900 nM okadaic acid. Therefore, type 2A phosphatase was more sensitive to okadaic acid than type 1 phosphatase in these concentrated *Xenopus* egg extracts (Figure 5).

Since type 1 phosphatase activity on phosphorylase kinase was not significantly inhibited by 375 nM okadaic acid (<10%) we chose this concentration to assay the activity of type 1 and 2A phosphatases on phosphorylase kinase labeled on its α- and β-subunits. As shown in Figure 6A, the type 2A phosphatase activity measured on the α-subunit was strongly inhibited (circles) whereas type 1 phosphatase activity measured on the β-subunit was not (squares). Addition of 375 nM okadaic acid to the same 40 min extract under similar conditions induced cdc2 kinase activation (Figure 6B). Thus, specific inhibition of type 2A phosphatase by 375 nM okadaic acid was sufficient to activate the cdc2 kinase in this extract. Cdc2 kinase did not activate to a maximum level, probably because type 2A phosphatase was not fully inhibited at this concentration (Figures 5 and 6A). However, it was also possible that maximal activation of the cdc2 kinase required inhibition of both type 1 and 2A phosphatases.

In order to examine this possibility we tested the effect of inhibitors 1 and 2, the specific inhibitors of type 1 phosphatases (Cohen, 1989), on cdc2 kinase activation in the extracts. These inhibitors specifically blocked the dephosphorylation of the β-subunit of the phosphorylase kinase (Figure 6C) but did not activate the cdc2 kinase (Figure 6D, open squares). Adding inhibitors 1 and 2 together with 375 nM okadaic acid to the same extract did not increase the level of kinase activation (Figure 6D). Therefore, type 1 phosphatase did not seem to play a significant role in the okadaic acid induced activation of cdc2 kinase. Addition of inhibitors 1 and 2 also did not induce cyclin degradation (data not shown).

In conclusion, the okadaic acid induced activation of cdc2 kinase in *Xenopus* interphase extracts is probably mediated by the inhibition of a type 2A phosphatase. It is logical to think that a substrate(s) of this type 2A phosphatase is(are) involved in cdc2 kinase activation when it (they) becomes hyperphosphorylated since inhibition of their dephosphorylation leads to cdc2 kinase activation.

Activation of cdc2-kinase by okadaic acid requires a particulate component

'Spontaneous' activation of the cdc2 kinase in 'cycling' extracts prepared 60 min after egg activation required the participation of some insoluble material (Félix *et al.*, 1989a,b). Our 100 000 *g* egg supernatants contained much insoluble material because of their high protein concentration. Removal of all insoluble material from these extracts required centrifugation in high gravity fields (Félix *et al.*, 1989b). As shown in Figure 7A, elimination of the insoluble components from the 40 min extracts also prevented the

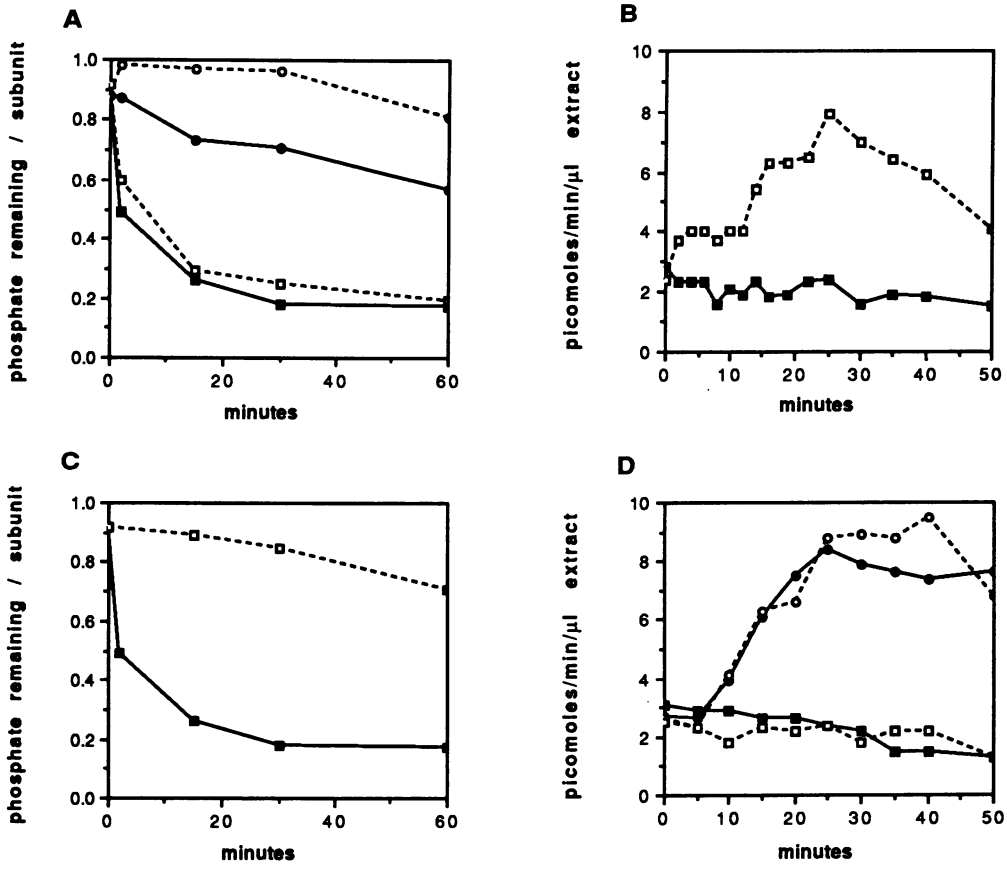

Fig. 6. Inhibition of type 2A phosphatases, but not of type 1 phosphatases, induces cdc2 kinase activation. (**A**) Dephosphorylation of the α-subunit (by type 2A phosphatase, circles) and β-subunit (by type 1 phosphatase, squares) of phosphorylase kinase in the *Xenopus* egg extract. Experiments were carried out at a 50% dilution of the extract, in the absence (filled symbols) or in the presence of 375 nM okadaic acid (open symbols). The phosphorylase kinase substrate contained 0.92 mol phosphate/β subunit and 0.88 mol phosphate/α subunit. (**B**) Cdc2 kinase activation in the *Xenopus* egg extract. Experiments were carried out at a 50% dilution of the extract, in the absence (filled squares) or in the presence (open squares) of 375 nM okadaic acid. H1 kinase assays were performed on exogenous histones as described in Félix *et al.* (1989a). (**C**) Dephosphorylation of the β-subunit of phosphorylase kinase (by type 1 phosphatase) by the *Xenopus* egg extract. Experiments were carried out at a 50% dilution of the extract, in the absence (filled squares) or in the presence of inhibitors 1 and 2 (open squares). Extracts were pre-incubated for 10 min with 0.25 µM of the active thiophosphorylated peptide of inhibitor 1 (residues 9–41) and 0.25 µM inhibitor 2. 0.1 µM of the inhibitor 1 peptide was added every 10 min during the incubation, in case it was degraded or dephosphorylated by the extract. Dephosphorylation of the α-subunit of phosphorylase kinase (by type 2A phosphatase) was not inhibited at all by inhibitor 1 and inhibitor 2 (not shown). (**D**) Cdc2 H1 kinase activation in the *Xenopus* egg extract. Experiments were carried out at a 50% dilution of the extract, in the absence or in the presence of 375 nM okadaic acid and/or inhibitors 1 and 2: control (filled squares), 0.25 µM inhibitor 1 plus 0.25 µM inhibitor 2 (open squares), 375 nM okadaic acid (filled circles), 375 nM okadaic acid plus 0.25 µM inhibitor 1 plus 0.25 µM inhibitor 2 (open circles).

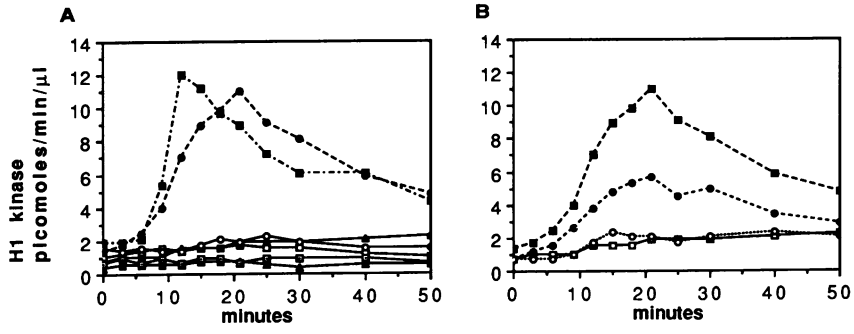

Fig. 7. Okadaic acid induced cdc2 kinase activation requires an insoluble component. Extracts of eggs crushed by centrifugation 40 min after activation were incubated at 22°C in the absence or in the presence of 1 µM okadaic acid. Samples were taken at different time-points in extraction buffer and assayed for histone H1 kinase activity. (**A**) 200 µl of a 100 000 *g* extract (squares) were centrifuged in a Beckman TL100 ultracentrifuge at 4°C for 30 min at 150 000 *g*. The supernatant was collected and the pellet resuspended in 100 µl acetate buffer. The supernatant and the resuspended pellet were mixed 1:1 with acetate buffer or together, in the absence (open symbols) or in the presence (filled symbols) of 1 µM okadaic acid. Supernatant plus buffer: triangles; supernatant plus pellet: circles; buffer plus pellet plus okadaic acid: dotted squares. (**B**) Various dilutions of the pellet in acetate buffer were added to the supernatant together with 1 µM okadaic acid. Pellet (prepared as in A), filled squares; diluted twice, filled circles; diluted four times, open circles; buffer, open squares.

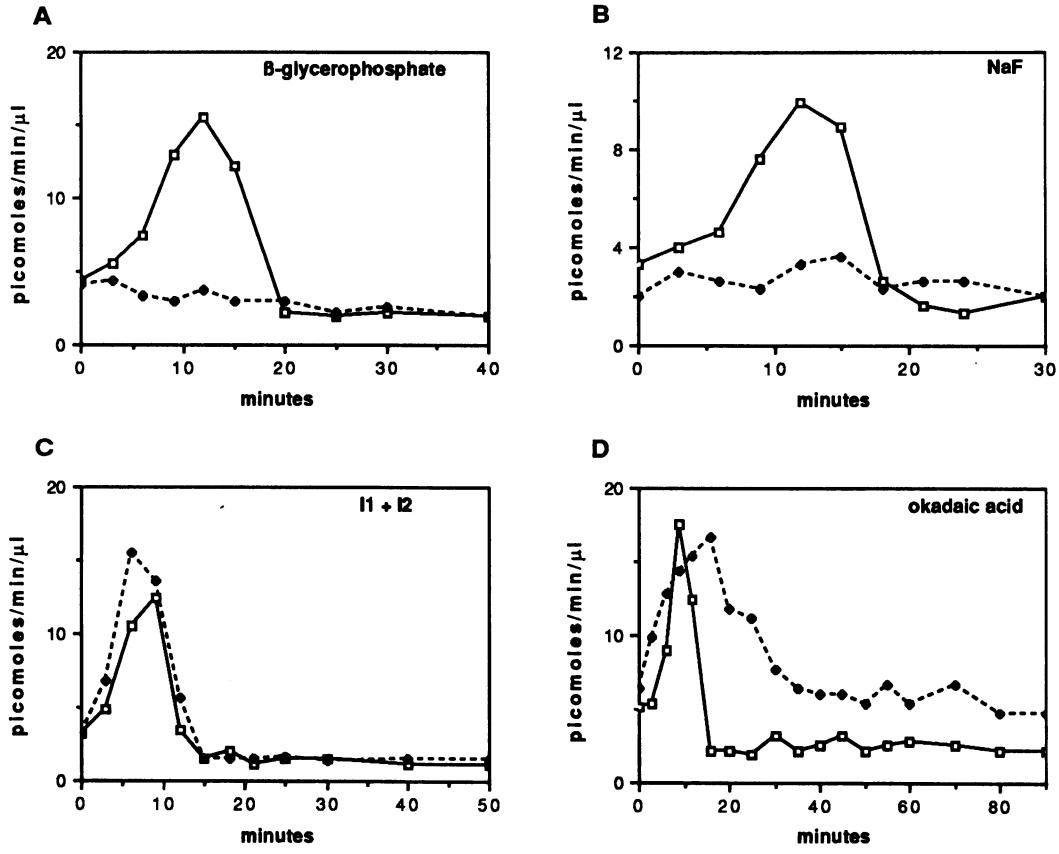

Fig. 8. Effect of phosphatase inhibitors on cdc2 H1 kinase activation in 100 000 *g* supernatant prepared 60 min after egg activation. Histone H1 kinase activity was followed in 100 000 *g* supernatants prepared 60 min after egg activation and incubated at 22°C in the absence (open symbols) or in the presence (filled symobls) of various phosphatase inhibitors. (**A**) 20 mM Na β-glycerophosphate; (**B**) 100 mM NaF; (**C**) 0.25 µM inhibitor 1 plus 0.25 µM inhibitor 2; (**D**) 1 µM okadaic acid.

okadaic acid dependent cdc2 kinase activation in the soluble supernatant but adding the pellet back to the supernatant restored the activation of the cdc2 kinase. The level of H1 kinase activation was strongly dependent on the amount of pellet added (Figure 7B). We have been able to solubilize this activator from the particulate material by extraction with 0.4 M NaCl but not by 0.2% Triton X-100 (data not shown).

Activation of cdc2-kinase requires phosphatases distinct from the known type 1 and 2 phosphatases

In order to examine whether another (okadaic acid insensitive) phosphatase was involved in activating the cdc2 kinase, we tested the effect of different phosphatase inhibitors on the spontaneous activation of cdc2 kinase in 'cycling' extracts. General Ser/Thr phosphatase inhibitors like β-glycerophosphate (20 mM) and sodium fluoride (50−100 mM) completely abolished cdc2 kinase activation (Figure 8A and B). Known Ser/Thr phosphatases (reviewed in Cohen, 1989) are type 1 phosphatases which are specifically inhibited by inhibitors 1 and 2, type 2A phosphatases which are specifically inhibited by okadaic acid, type 2B phosphatases which require Ca^{2+} ions and type 2C phosphatases which are Mg^{2+} dependent. Inhibition of type 1 and 2A phosphatases by inhibitors 1 and 2 or by okadaic acid did not inhibit cdc2 kinase activation in 'cycling' extracts (Figure 8C and D). Since the extracts contained 10−20 mM EGTA it was also impossible that phosphatase 2B could function. Apparently, spontaneous activation of the cdc2 kinase can also occur when Mg^{2+} ions are chelated by 5 mM EDTA (Félix *et al.*, 1989a), suggesting that a type 2C phosphatase is not involved either. Therefore, it seems that an unidentified Ser/Thr phosphatase participates in the final activation of cdc2 kinase (our results), as well as a tyrosine phosphatase (Dunphy and Newport, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989).

Discussion

Negative regulation of cdc2 kinase by a type 2A phosphatase and positive regulation by unknown phosphatases

This study clearly shows that different phosphatases act at various levels in the regulation of cdc2 mitotic kinase activity during the cell cycle. Cdc2 kinase activation is repressed by an okadaic acid sensitive phosphatase until enough cyclin molecules have been accumulated. At this stage, another phosphatase(s) is(are) positively involved in the final activation of cdc2 kinase. It is noteworthy that an analogous cascade of *kinases* was characterized genetically in fission yeast (Russel and Nurse, 1987), where nim1+ kinase activates cdc2+ kinase through the inhibition of the wee1+ kinase.

Okadaic acid induces cdc2 kinase activation by inhibiting a protein phosphatase: addition of the purified catalytic subunit of phosphatase 2A (final concentration 300 mU/ml) completely prevents cdc2 kinase activation in 'cycling' extracts (not shown). This is consistent with previous reports showing that microinjection of protein phosphatases 1 and

2A in starfish oocytes inhibits their maturation (Meijer *et al.*, 1986). In our system, okadaic acid induces cdc2 kinase activation at a concentration that inhibits type 2A phosphatases specifically but does not affect type 1 phosphatases. Moreover, inhibition of type 1 phosphatases by their specific inhibitors 1 and 2 has no effect on cdc2 kinase activation. Although we cannot rule out that an okadaic acid sensitive phosphatase distinct from the well characterized type 2A phosphatase is involved, we will call the enzyme type 2A for the time being. We should simply bear in mind that isozymes of these proteins have been cloned (da Cruz et Silva and Cohen, 1987), as well as a novel phosphatase 'PP−X' which is more closely related to phosphatase 2A than phosphatase 1 (da Cruz et Silva *et al.*, 1988). The specificity and regulation of the last mentioned enzyme is unknown.

Apparently, final activation of cdc2 kinase requires the participation of phosphatases that are distinct from the classes of phosphatases that have already been characterized. Final activation of the cdc2 kinase is probably achieved by the dephosphorylation of specific Thr/Tyr residues of the cdc2 kinase subunit (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Labbé *et al.*, 1989b; Morla *et al.*, 1989). We had already reported that final cdc2 kinase activation requires a particulate activator. Here, we find again that activation of the cdc2 kinase by addition of okadaic acid to interphase extracts requires the participation of insoluble components. It is possible that the particulate activator is a 'mitotic' phosphatase involved in $p34^{cdc2}$ dephosphorylation, or a protein that regulates its activity. Treatment of the pellet with irreversible Ser/Thr phosphatase inhibitors (NaF/pyrophosphate) did not inhibit its ability to activate cdc2 kinase, but rather preserved its stimulatory action (not shown). Thus the particulate activator does not appear to be a NaF/pyrophosphate sensitive Ser/Thr phosphatase. Nevertheless, it could still be a tyrosine phosphatase.

Negative regulation of cdc2 kinase by a type 2A phosphatase during cyclin accumulation

During the first cell cycle in fertilized *Xenopus* eggs, cdc2 kinase activation only starts 60 min after fertilization. Yet, the protein synthesis required for this activation is already completed by 45−50 min (Karsenti *et al.*, 1987). It has recently been demonstrated that cyclins are the only proteins that need to be synthesized and that a threshold level of these proteins is required to trigger cdc2 kinase activation (Murray and Kirschner, 1989). Therefore, by 50 min after fertilization, enough cyclin must have accumulated in the egg to trigger cdc2 kinase activation. Yet, there is still a lag of 10−15 min before the onset of cdc2 kinase activation which is slow and lasts for 10 min or more.

Here, we show that okadaic acid activates the cdc2 kinase poorly in extracts prepared at 10 min in the first cell cycle whereas the same toxin triggers full cdc2 kinase activation in extracts prepared from eggs sampled at 40 min, when almost enough cyclin has been synthesized to trigger spontaneous cdc2 kinase activation. This activation is relatively slow (10−20 min). In 60 min extracts, that have already gone through one round of spontaneous cdc2 kinase activation−inactivation *in vitro*, okadaic acid has no effect (Figure 2C). In these extracts endogenous cyclin has been degraded concomitantly with kinase inactivation (Félix *et al.*, 1989a, and unpublished). Okadaic acid also does not stimulate cdc2 kinase in extracts prepared from activated eggs incubated for 2 h in the presence of 100 μg/ml cycloheximide, another condition where the extracts are depleted of cyclin. Overall, these results suggest that inhibition of phosphatase 2A bypasses the requirement for a cyclin threshold to initiate cdc2 kinase activation, although some cyclin level seems to be required. Whether and how a cyclin threshold can initiate the cascade of phosphorylation−dephosphorylation that leads to cdc2 kinase activation under normal conditions is still unclear.

A phosphorylated cdc2 kinase activator?

If inhibition of a type 2A phosphatase activates cdc2 kinase, then a phosphorylation step must be required in cdc2 kinase activation. Since $p34^{cdc2}$ is activated by dephosphorylation, it is possible that the $p34^{cdc2}$ phosphatase is activated by phosphorylation and dephosphorylated (and inhibited) by phosphatase 2A (see also Dorée *et al.*, 1989). However, more work is required to determine exactly at which level the phosphatase 2A sensitive phosphorylation event is located in the cascade leading to cdc2 kinase activation. For example, it is unknown if the particulate activator is the cdc2 phosphatase itself or another protein required for its activity.

Phosphatase 2A and cdc2 kinase inactivation

During the cell cycle *in vivo*, the cdc2 kinase is rapidly inactivated at the metaphase−anaphase transition. This requires cyclin degradation (Murray *et al.*, 1989). Okadaic acid stimulates cyclin degradation (Figure 4) in the extracts while it decreases the rate and extent of inactivation of cdc2 kinase (Figure 2). This suggests that the inactivation mechanism of cdc2 kinase at the metaphase−anaphase transition involves a type 2A phosphatase acting downstream of the cyclin degradation event. This could be the dephosphorylation and inactivation of the same cdc2 kinase activator discussed above (e.g. the $p34^{cdc2}$ phosphatase) Picard *et al.* (1989) also found that okadaic acid injected into starfish oocytes completely protected cdc2-kinase from inactivation. However, in contrast to our results, they reported that okadaic acid inhibited cyclin degradation. The reason for the apparent discrepancy with our results is unclear, but it could be due to species differences. In fact, Picard *et al.* (1989) report that inhibition of type 1 phosphatase is sufficient to activate MPF in sea cucumber, but not in starfish eggs. Alternatively, different conditions may prevail in the meiotic cell cycle.

Stimulation of cyclin degradation by okadaic acid in the 40 min extracts (Figure 4) may occur through the activation of cdc2 kinase. Cyclin destruction in the extract in the absence of okadaic acid is actually induced by a threshold level of cdc2 kinase activity (Félix *et al.*, unpublished). Inhibition of phosphatase 2A could also act specifically on the cyclin destruction pathway. For instance it could induce the hyperphosphorylation of cyclin (or of another protein), thereby rendering cyclin more susceptible to degradation.

Involvement of a type 1 phosphatase in cell cycle regulation

Several lines of evidence indicate that protein phosphatase 1 is required to complete mitosis (for review, see Cohen and Cohen, 1989). In *Drosophila*, mutations in a gene coding for protein phosphatase 1α causes death of the embryo at a late larval stage. In these mutant embryos, mitotic cells

show excessive chromosome condensation and hyperploid cells are also frequent. This suggests that the phosphatase 1α gene is essential for proper chromosome separation (Axton *et al.*, unpublished). In *Aspergillus nidulans*, a temperature-sensitive cell cycle mutant is unable to complete the anaphase separation of daughter nuclei, and the wild type gene encodes a homologue of a mammalian phosphatase 1α (Doonan and Morris, 1989). However, in this mutant, other aspects of the cell cycle are not arrested since cycles of chromatin condensation and decondensation persist. A similar mutation also exists in *Schizosaccharomyces pombe* (Ohkura *et al.*, 1989). The above mentioned studies show that protein phosphatase 1 plays an essential role in chromosome separation. This is different from the results reported here where we have examined the role of protein phosphatases in cdc2 kinase activation and cyclin degradation. However, Booher and Beach have recently reported that a plasmid-borne sequence, $bws1^+$, that causes the $cdc25^{ts}-wee1^-$ double mutant to revert to a temperature-sensitive cdc phenotype, is highly homologous to mammalian protein phosphatase 1α (*bws1*+ is allelic to the *dis2*+ gene described by Ohkura *et al.*, 1989). Since *cdc25*+ and *wee1*+ genes are involved in the temporal regulation of the cdc2 kinase activity, these authors have suggested that the *bws1*+ gene and, by extension protein phosphatase 1α, could be involved in the regulation of cdc2 kinase activity (Booher and Beach, 1989). This is not necessarily the case. An excess of phosphatase 1α might block cdc2 kinase activation without being the phosphatase that normally regulates this kinase *in vivo*. In fact, in the present paper, we have shown that specific inhibition of phosphatase 1 in 40 min extracts does not activate cdc2 kinase. However, addition of a 10-fold excess of purified phosphatase 1 catalytic subunit to 60 min 'cycling' extracts inhibits the spontaneous activation of cdc2 kinase (not shown). One should bear in mind that protein phosphatases have broad substrate specificities. Their specificity of action may be conferred by accessory proteins, as has been demonstrated for the type 1 phosphatases associated with the glycogen particles and myofibrils of skeletal muscles (reviewed in Cohen and Cohen, 1989). This is a case where the use of specific inhibitors under carefully controlled conditions, or *mutations* in the phosphatase genes may be required to establish which phosphatase is actually involved in specific events of mitosis.

Finally, inhibition of phosphatase 1 by the injection of 15 μM phosphoinhibitor 1 (Huchon *et al.*, 1981) or 4 μM inhibitor 2 (Foulkes and Maller, 1982) into *Xenopus* oocytes was reported to block progesterone-induced maturation. Cyert and Kirschner (1988) found that 3 μM phosphoinhibitor 1 inhibited activation of the mitosis promoting factor (MPF) in *Xenopus* oocyte extracts. These observations are also not necessarily in contradiction with our results. Oocytes are arrested in the G2 phase of meiosis and this arrest requires a phosphorylated factor. This factor may act upstream of the basic cell cycle oscillator and its dephosphorylation, perhaps by phosphatase 1, seems to be required to allow reinitiation of meiosis (Maller, 1985).

We conclude that, at least in *Xenopus* early embryos, phosphatase 1 is not required for the oscillation of cdc2 kinase, but of course may be important in many other aspects of the cell cycle like chromosome separation and the coupling of the basic oscillator with cell cycle arrest mechanisms.

Materials and methods

Materials

Okadaic acid was dissolved in dimethyl sulphoxide to give a 5 mM solution, and further diluted in aqueous buffers before use. 'Inhibitor 1' was an active thiophosphorylated peptide of inhibitor 1 (residues 9–41). Inhibitor 2 was purified from rabbit skeletal muscle (Cohen *et al.*, 1988). $P13^{suc1}$+ was purified and used as in Félix *et al.* (1989a).

Preparation of extracts

Extracts were prepared as described in Félix *et al.* (1989a). The eggs were dejellied with 2% cysteine–HCl (pH 7.8) and then washed extensively with modified Ringer's MMR/4 (25 mM NaCl, 0.4 mM KCl, 0.25 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.25 mM Hepes, 25 μM EDTA, pH 7.2). To prepare extracts, the eggs were first activated by an electric shock (Karsenti *et al.*, 1984), incubated at 20°C in MMR/4 for 10, 40 or 60 min and then transferred to SW 50.1 or SW 60 tubes filled with ice-cold acetate buffer [100 mM K-acetate, 2.5 mM Mg-acetate, 60 mM EGTA, 5 μg/ml cytochalasin D (Sigma), 1 mM DTT, pH 7.2]. Excess acetate buffer was removed prior to centrifugation. The eggs were crushed by centrifugation at 10 000 *g* for 10 min at 4°C in an L5-65 Beckman centrifuge with maximum acceleration rate. The cytoplasmic material between the upper lipid layer and the yolk pellet was collected and an ATP regenerating system was added to a final concentration of 10 mM creatine phosphate (Boehringer, 500 mM stock in H_2O), 80 μg/ml creatine phosphokinase (Boehringer, 4 mg/ml stock in 50% glycerol in H_2O), 1 mM ATP (disodium salt, Boehringer, 50 mM Stock in H_2O, pH 7). The 100 000 *g* extracts were obtained by further centrifugation at 100 000 *g* for 60 min at 4°C in the SW50.1 or SW60 rotors with adaptors for 0.6 ml tubes. The supernatant was collected, frozen and kept as 50–100 μl aliquots in liquid nitrogen. These supernatants contained 30–40 mg/ml of proteins and much insoluble material. No protein synthesis occurred in such supernatants.

Histone H1 kinase assay

Histone H1 kinase activity was determined as described in Félix *et al.* (1989a), using exogenous H1 histones as a substrate (H III-S from calf thymus, Sigma). The labeled histones were precipitated on P81 phosphocellulose paper and counted dry on the tritium channel.

Assay for cyclin degradation in the extract

Arbacia punctulata cyclin mRNA was transcribed from a full length clone of *cyc4* in pGEM1 (described in Pines and Hunt, 1987) with T7 RNA polymerase and was translated in the presence of [^{35}S]methionine in a rabbit reticulocyte lysate. The total translation mix containing [^{35}S]cyclin was added to the extract in a proportion of 1 vol. per 10 vol. of extract. The extract was incubated at 22°C and 4 μl of it was transferred at different time-points into 16 μl of SDS gel sample buffer. The samples were run on a 10% gel. The gel was fixed in 45% methanol, 7% acetic acid for 3 × 30 min and processed using the Intensify (DuPont) procedure.

Phosphatase assays

^{32}P-labelled rabbit skeletal muscle phosphorylase kinase (Stewart *et al.*, 1981) and bovine casein (64 nmol phosphate/mg) (McGowan and Cohen, 1988) were prepared by phosphorylation with cAMP-dependent protein kinase. The specific activity of each substrate was ~10^6 dpm/nmol. The reactions were performed at a 50% dilution of the extract in 50 mM Tris–Cl, pH 7.0, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, using 2 μM phosphorylase kinase and 6 μM [^{32}P]casein. The phosphatase activities were assayed either by measuring the TCA-soluble counts released after 30 s of incubation of the labelled substrate in the extract (Cohen *et al.*, 1988), or by separating the α- and β-subunits of phosphorylase kinase by SDS–PAGE (Stewart *et al.*, 1981) and measuring the counts remaining on each subunit after cutting them out of the gel. When inhibitor 1 and inhibitor 2 were included, the extracts were preincubated with these proteins for 10 min prior to initiating the reactions with substrates.

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