Activation of M-phase-specific histone H1 kinase by modification of the phosphorylation of its p34^{cdc2} and cyclin components

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An M-phase-specific histone H1 kinase (H1K) has been described in a wide variety of eukaryotic cell types undergoing the G_2/M transition in the cell division cycle. We have used p13^{suc1}–Sepharose affinity chromatography to purify H1K to near homogeneity from matured starfish oocytes. A yield of 67% was obtained. Active H1K behaves as a 90- to 100-kD protein and appears to be constituted of equimolar amounts of cyclin and p34^{cdc2}. The p34^{cdc2} subunit becomes tyrosine-dephosphorylated as the H1K is activated during entry of the oocytes into M phase, whereas the cyclin subunit is reciprocally phosphorylated. Acid phosphatase treatment of inactive p34^{cdc2}/cyclin complex induces p34^{cdc2} dephosphorylation and three- to eightfold stimulation of the enzyme activity. These results suggest that active M-phase-specific H1K is constituted of both dephosphorylated p34^{cdc2} and phosphorylated cyclin.

[*Key Words*: p34^{cdc2}; histone H1 kinase; cyclin]

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All eukaryotic cells that have been investigated display a calcium- and cyclic nucleotide-independent histone H1 kinase (H1K), referred to either as the M-phase specific or growth-associated H1K (for review, see Wu et al. 1986; Meijer and Pondaven 1988). We have studied this enzyme in the oocytes of starfish, because large amounts of cells can be obtained at defined stages of the cell cycle. The oocyte is naturally arrested in late G_2 of the first meiotic prophase and can be caused to enter the first M phase within minutes of exposure to the natural hormone 1-methyladenine (1-MeAde; Kanatani et al. 1969; for review, see Meijer and Guerrier 1984). Following oocyte maturation, the H1K becomes stimulated 10- to 30-fold (Sano 1985; Meijer et al. 1987; Pelech et al. 1987; Arion et al. 1988; Labbé et al. 1988).

Recently, it has been established that one subunit of the starfish H1K is the homolog of the yeast *cdc2/CDC28* protein kinase (Arion et al. 1988; Labbé et al. 1988, 1989) and that a p34^{cdc2}-like protein in frog eggs is also a component of the M-phase promoting factor (MPF; Dunphy et al. 1988; Gautier et al. 1988; for review, see Lohka 1989). Therefore, H1K and MPF may be the same entity (Arion et al. 1988; Labbé et al. 1989). Furthermore, in a variety of cell types, including fission yeast (Booher et al. 1989), clams (Draetta et al. 1989; Westendorf et al. 1989), sea urchins (Meijer et al. 1989), and human cells (Draetta and Beach 1988; Giordano et al. 1989; Pines and Hunter 1989), a fraction of the p34^{cdc2} protein kinase has been found in physical association with a class of proteins known as mitotic cyclins. Cyclins display the unusual feature of being subjected to abrupt and quantitative proteolytic degradation at the metaphase/anaphase transition of each cell division (Evans et al. 1983; Standart et al. 1986). The synthesis of this class of proteins is an absolute requirement of the G_2/M transition (Swenson et al. 1986; Pines and Hunt 1987; Murray and Kirschner 1989; Murray et al. 1989; Westendorf et al. 1989), and cyclin appears to be a second subunit of H1K (Meijer et al. 1989; Pines and Hunter 1989).

Activation of H1K at the G_2/M transition of the starfish first meiotic division is not expected to be determined simply by cyclin accumulation, because H1K can be activated in response to hormone in the absence of protein synthesis (Pelech et al. 1987). In this paper we report the investigation of the subunit composition of the M-phase-specific H1K in starfish oocytes and the mechanism of its activation. H1K appears to be constituted of an equimolar p34^{cdc2}/cyclin complex, and its activation at the G_2/M transition can be attributed to two post-translational modifications of its subunits: dephosphorylation of p34^{cdc2} and phosphorylation of cyclin.

Results

Purification of H1K by p13–Sepharose chromatography

We have used p13–Sepharose affinity chromatography to purify the starfish H1K. p13 is the product of the fission yeast *suc1*$^+$ gene that was isolated by virtue of its

ability to suppress certain temperature-sensitive *cdc2* mutants on a high copy vector (Hayles et al. 1986). p13 is a subunit of the *cdc2* protein kinase in yeast and human cells (Brizuela et al. 1987; Draetta and Beach 1988; Hadwiger et al. 1989). This protein, expressed in *Escherichia coli* and covalently attached to Sepharose, retains a high specificity for $p34^{cdc2}$, which makes it a useful reagent for studying the properties of the protein kinase in frog eggs (Dunphy et al. 1988; Dunphy and Newport 1989), starfish oocytes (Arion et al. 1988), clam (Draetta et al. 1989) and sea urchin eggs (Meijer et al. 1989), human cells (Brizuela et al. 1989; Gioridano et al. 1989), and fission yeast, itself (Booher et al. 1989).

An 8-ml p13–Sepharose column was prepared and batch-loaded with a lysate from 40 ml of hormone-activated M-phase starfish oocytes (see Experimental procedures). After extensive washing, the column was eluted with a 1 mg/ml solution of p13, which displaced the H1K activity (Fig. 1A). This single step resulted in 1200-fold purification of the enzyme with 67% recovery. After concentration of the p13–Sepharose eluate on a 1-ml hydroxyapatite column, components of the enzyme were resolved by SDS-PAGE and visualized by Coomassie Blue staining. Only two prominent polypeptides, of ~34 and 54 kD, were visible (Fig. 2B). Western immunoblotting confirmed that these two proteins were the starfish $p34^{cdc2}$ and cyclin (Fig. 2C).

The similar intensity of Coomassie Blue staining of the $p34^{cdc2}$ and cyclin subunits of the kinase suggests that the two proteins are present in stoichiometric amounts. To investigate the apparent molecular weight of the native kinase complex, we subjected the p13–Sepharose-purified material to gel filtration chromatography. In a Sephacryl S-200 column, active H1K eluted with an apparent molecular mass of 90–100 kD (Fig. 1B). This is consistent with a subunit structure consisting of a single $p34^{cdc2}$ and cyclin molecule (see Discussion). Because the enzyme was eluted from the p13–Sepharose column with soluble p13, it may also contain one p13 subunit. Immunoblotting with anti-$p34^{cdc2}$ serum allowed the detection of $p34^{cdc2}$ only in the 90- to 100-kD fractions containing active H1K (Fig. 1C).

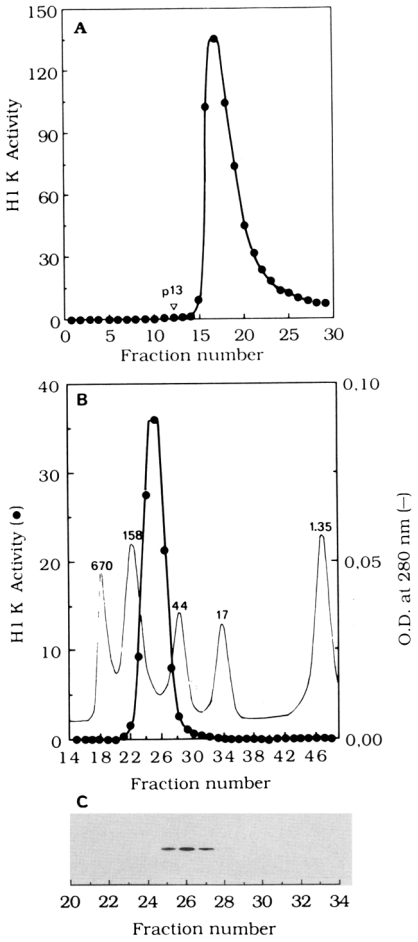

Figure 1. Affinity-purification of H1K. (*A*) M-phase oocyte extract (40 ml) was batch-loaded on p13–Sepharose, and, after washing, the column was eluted with 1 mg/ml p13 (indicated by open arrowhead). H1K activity was assayed in 1 μl of each 4-ml fraction (representative of four independent experiments). (*B*) p13–Sepharose-purified H1K was loaded on a 400-ml Sephacryl S-200 column, and activity was assayed in 10 μl of each 10-ml fraction. The column was calibrated with the indicated markers of 670, 158, 44, 17, and 1.35 kD (representative of two independent experiments). (*C*) Immunoblotting of Sephacryl S-200 fractions. Each fraction was incubated with p13–Sepharose beads, and the bound proteins were resolved by SDS-PAGE. $p34^{cdc2}$ was detected by immunoblotting.

Dephosphorylation of $p34^{cdc2}$ and phosphorylation of cyclin

The starfish oocyte provides a particularly attractive opportunity to study the mechanism of activation of the H1K. After exposure to 1-MeAde, the enzyme is stimulated within minutes, even in the absence of protein synthesis (Pelech et al. 1987). Extracts of control and hormone-treated oocytes were loaded onto p13–Sepharose, and the bound material was analyzed by SDS-PAGE. Two major proteins were found in silver-stained gels at all time intervals (Fig. 3A). One was identified as $p34^{cdc2}$ by comigration with authentic $p34^{cdc2}$ (data not shown) and by cross-reactivity with anti-$p34^{cdc2}$ serum (Fig. 3B). The other was identified as cyclin by comigration with purified sea urchin cyclin (data not shown), by cross-reactivity with anti-cyclin serum (Fig. 4A) and, as in sea urchin eggs (Meijer et al. 1989), as the major synthesized protein binding to p13–Sepharose (Fig. 4C, D). Even in unstimulated oocytes that do not display active H1K, the $p34^{cdc2}$ and cyclin subunits bind to p13–Sepharose and appear to be present in stoichiometric amounts (Fig. 3A). This suggests that hormonal activation of the enzyme is likely to be accomplished by post-translational modification of the preformed $p34^{cdc2}$/cyclin complex.

During hormone activation of the oocyte, the mobility of $p34^{cdc2}$ in SDS-PAGE increased (Fig. 3A, B), whereas

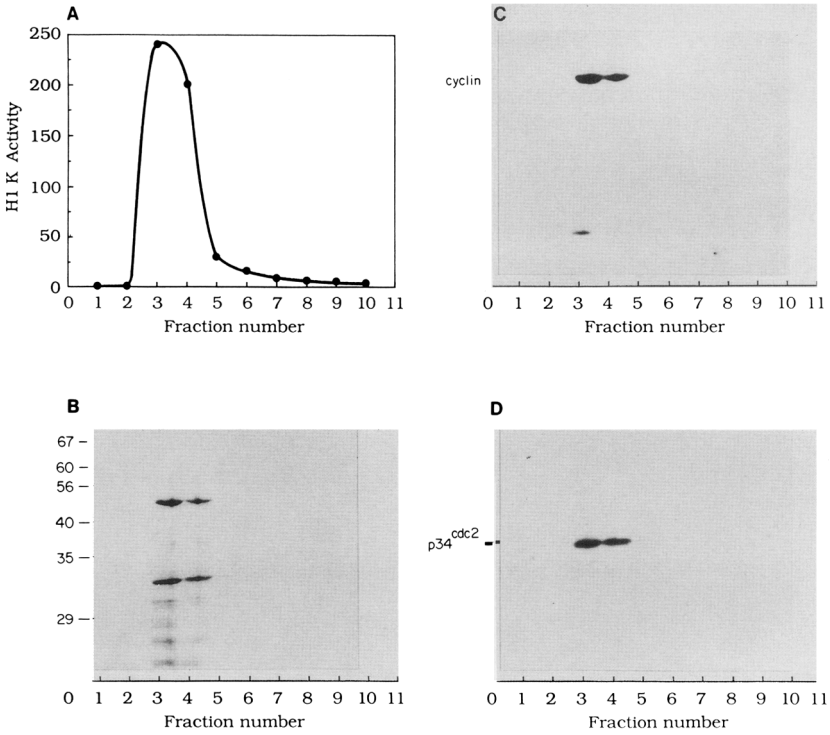

Figure 2. Subunit composition of H1K. (*A*) p13–Sepharose-purified H1K was concentrated by loading on a 1-ml hydroxyapatite column. H1K was eluted in 2-ml fractions with 0.5 M phosphate buffer and 0.1 μl was assayed (representative of three independent experiments). (*B*) Coomassie Blue-stained gel of 20-μl samples of fractions eluted from the hydroxyapatite column (representative of three independent experiments). (*C*) Anti-cyclin immunoblot of the same fractions. (*D*) Anti-p34^{cdc2} immunoblot of the same fractions.

that of cyclin decreased (Figs. 3A and 4). In other cell types, both the p34^{cdc2} and cyclin proteins are phosphorylated in a cell-cycle-dependent manner. p34^{cdc2} becomes dephosphorylated during entry into mitosis (Draetta et al. 1988; Dunphy et al. 1989; Gautier et al. 1989; Labbé et al. 1989; Morla et al. 1989), whereas cyclin becomes phosphorylated (Standart et al. 1987; Meijer et al. 1989). Therefore, we tested whether the altered electrophoretic mobility of p34^{cdc2} and cyclin during activation of H1K may be attributable to changes in their state of phosphorylation.

Oocytes were prelabeled with γ-$^{32}PO_4$ and stimulated with 1-MeAde. Extracts were prepared at intervals and loaded onto p13–Sepharose. The material eluted by sample buffer was visualized by autoradiography after SDS-PAGE. During oocyte maturation, p34^{cdc2} became dephosphorylated and cyclin became reciprocally phosphorylated (Fig. 5A). To quantify these effects, the labeled bands were excised from the gel and counted in a liquid scintillation counter. The activation of H1K followed p34^{cdc2} dephosphorylation and cyclin phosphorylation precisely (Fig. 5B). The low level of [^{32}P]phosphate incorporation in the two proteins precluded a direct phosphoamino acid analysis. However, immunoblotting with an anti-phosphotyrosine antibody showed that p34^{cdc2} dephosphorylation occurred on tyrosine, at least, but that cyclin was apparently not phosphorylated on tyrosine (Fig. 6).

In a further test of the potential significance of these events, we monitored the phosphorylation state of p34^{cdc2} and cyclin under two different circumstances. First, oocytes were exposed to differing concentrations of 1-MeAde. The activation of H1K, extent of germinal vesicle breakdown (GVBD), and the phosphorylation state of p34^{cdc2} and cyclin were monitored. Only at doses of the hormone that allowed activation of the enzyme and GVBD (>10^{-8} M) did p34^{cdc2} become dephosphorylated (Fig. 7A) and did cyclin become phosphorylated (data not shown). Second, we exposed oocytes to an activating concentration of the hormone for brief intervals, followed by its removal. Exposure for at least 4 min [corresponding to the hormone-dependent period (see Meijer and Guerrier 1984)] was required to commit the oocytes to maturation and H1K activation (Fig. 7B). Under these circumstances, maturation was invariably associated with dephosphorylation of p34^{cdc2} (Fig. 7B) and with cyclin phosphorylation (data not shown).

In vitro activation of p34^{cdc2}/cyclin

The preceding experiments point to the possibility that dephosphorylation of p34^{cdc2} may be a necessary step in the activation of H1K. We tested this directly by loading p13–Sepharose beads with inactive p34^{cdc2}/cyclin complex prepared from unstimulated oocytes. The beads were washed and exposed to varying amounts of potato acid phosphatase (Fig. 8A). After further washing, histone was added for assay of kinase activity. Phosphatase treatment of inactive *cdc2*/cyclin attached to p13–Sepharose stimulated the enzyme three- to eightfold, to a level close to that of fully activated H1K from hormone-treated cells (Fig. 8B). No such effect was obtained with purified phosphoprotein phosphatases 1 and 2A.

Exposure of the fully active M-phase H1K to phosphatase failed to either further activate or inhibit the enzyme. In vitro dephosphorylation increased the mobility

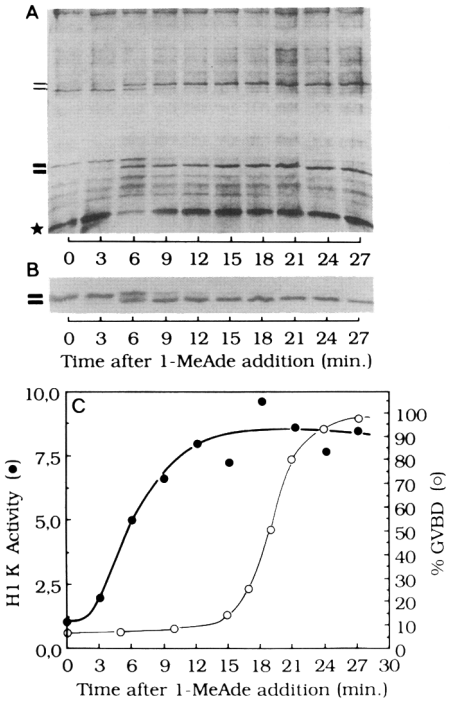

Figure 3. Modification of p34^{cdc2} during oocyte maturation. (*A*) Oocytes were treated with 1 μM 1-MeAde at time 0. At intervals thereafter, cell extracts were prepared and loaded onto p13–Sepharose. The bound proteins were resolved on SDS-PAGE and visualized by silver staining. The two thin markers indicate the position of cyclin, and the two thick markers indicate the position of p34^{cdc}. The star indicates p13 that became uncoupled from the Sepharose beads. (*B*) Anti-p34^{cdc2} immunoblot of the same p13–Sepharose eluates. (*C*) Time course of H1K activation and germinal vesicle breakdown (GVBD) after hormonal stimulation (representative of four independent experiments).

of p34^{cdc2} on SDS-PAGE to a level between that of the unactivated and the activated oocyte (Fig. 8B, top).

Immunoblotting of material eluted from p13–Sepharose with anti-phosphotyrosine antibodies revealed that p34^{cdc2} was tyrosine phosphorylated in the unactivated, but not the activated, starfish oocyte (Figs. 6 and 8C). Furthermore, during in vitro activation of the oocyte enzyme by exposure to potato phosphatase, phosphate was removed from tyrosine residues (Fig. 8C). During in vitro activation of H1K by acid phosphatase, no phosphorylation-induced shift of cyclin was observed (Fig. 8B).

Discussion

We have studied the properties of the starfish H1K during the rapid activation of oocytes in response to 1-MeAde. Both the active and inactive enzyme appear to consist of stoichiometric amounts of p34^{cdc2} and cyclin. At least one step in the activation of the enzyme is directly shown to involve dephosphorylation of the p34^{cdc2} subunit.

In this study the native molecular mass of H1K was estimated to be 100 kD. This is consistent with previous studies of the enzyme in the starfish using Sephacryl chromatography (Pelech et al. 1987); however, both starfish and mammalian enzymes have been estimated at 220 kD by means of Superose chromatography (Arion et al. 1988; Draetta and Beach 1988; Brizuela et al. 1989). There is clearly a difference in the apparent molecular mass of the enzyme, using these two reagents. It thus remains a possibility that H1K consists of a dimeric structure of paired p34^{cdc2}/cyclin.

The present results are in contrast to those indicating that starfish H1K and MPF consist exclusively of monomeric p34^{cdc2} (Labbé et al. 1989). The reasons for this discrepancy are not completely clear; however, the present results are entirely consistent with the model suggesting that one step in activation of p34 as H1K and MPF involves complex formation with cyclin (Brizuela et al. 1989; Draetta et al. 1989; Meijer et al. 1989; Pines and Hunter 1989). In human HeLa cells, only the fraction of p34^{cdc2} that is complexed with cyclin (p62) displays H1K activity, even though monomeric p34^{cdc2} can phosphorylate the artificial substrate casein. The present observations make it equally clear that the complex between p34^{cdc2} and cyclin, which is fully formed in the starfish oocyte, is insufficient to activate p34^{cdc2} as a

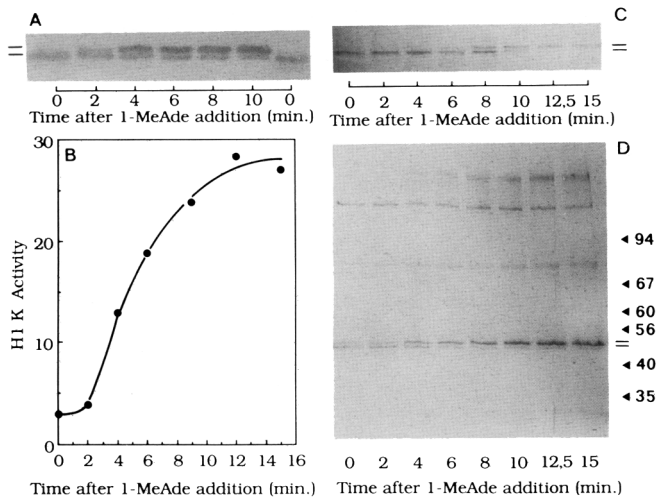

Figure 4. Modification of cyclin during oocyte maturation. (*A*) Oocytes were treated as in Fig. 3; the p13–Sepharose-bound proteins were resolved by SDS-PAGE and immunoblotted with anti-cyclin serum. The thin markers indicate the position of cyclin. (*B*) Time course of H1K activation in the same experiment (representative of three independent experiments). (*C*) Oocytes were labeled with [^{35}S]methionine and treated as above. Thin markers indicate silver staining of the p13-bound cyclin. (*D*) Autoradiograph of ^{35}S-labeled, p13-bound proteins of the same experiment (thin markers indicate the position of cyclin; representative of two independent experiments).

histone kinase. Association with cyclin gives the enzyme the potential to display M-phase specificity, but this is not realized until the catalytic subunit is dephosphorylated and, possibly, the cyclin subunit is phosphorylated.

Several studies have investigated that phosphorylation of p34^{cdc2} during the cell cycle (Simanis and Nurse 1986; Draetta and Beach 1988; Draetta et al. 1988; Potashkin and Beach 1988; Dunphy and Newport 1989; Gautier et al. 1989; Labbé et al. 1989; Morla et al. 1989). Although p34^{cdc2} can be phosphorylated, it appears that the entry into metaphase is consistently associated with dephosphorylation of p34^{cdc2}. The p34^{cdc2} protein is heavily tyrosine-phosphorylated (Draetta et al. 1988), and tyrosine dephosphorylation of p34^{cdc2} has been observed during entry into M phase. Indeed, in frog eggs, this process (either directly or indirectly) is inhibited by addition of excess p13, which also prevents activation of H1K (Dunphy et al. 1988; Dunphy and Newport 1989). Also, in mouse 3T3 cells, p34^{cdc2} becomes tyrosine dephosphorylated upon entry into M phase, but specific tyrosine dephosphorylation in vitro is insufficient to activate the enzyme. This may be because mouse p34^{cdc2} is also heavily threonine phosphorylated, and threonine dephosphorylation occurs during entry into M phase (Morla et al. 1989). Exactly the same situation exists in the fission yeast, in which tyrosine dephosphorylation of p34^{cdc2} depends on the activity of the *cdc25*$^{+}$ gene product (Gould and Nurse 1989). The present results, obtained using the broad spectrum potato acid phosphatase, provide the first unequivocal proof that dephosphorylation of p34^{cdc2} contributes to activation of the p34^{cdc2}/cyclin complex. This phosphatase removes tyrosine phosphate from p34^{cdc2} (Fig. 8) but, presumably, also removes threonine phosphate.

Several studies have demonstrated the importance of cyclin in the activation of H1K (Booher et al. 1989; Draetta et al. 1989; Meijer et al. 1989; Murray and Kirschner 1989; Westendorf et al. 1989). Cyclin is transiently phosphorylated after its synthesis and before its destruction (Standart et al. 1987; Meijer et al. 1989). We have shown that the appearance of the phosphorylated form of cyclin corresponds exactly to the time of H1K

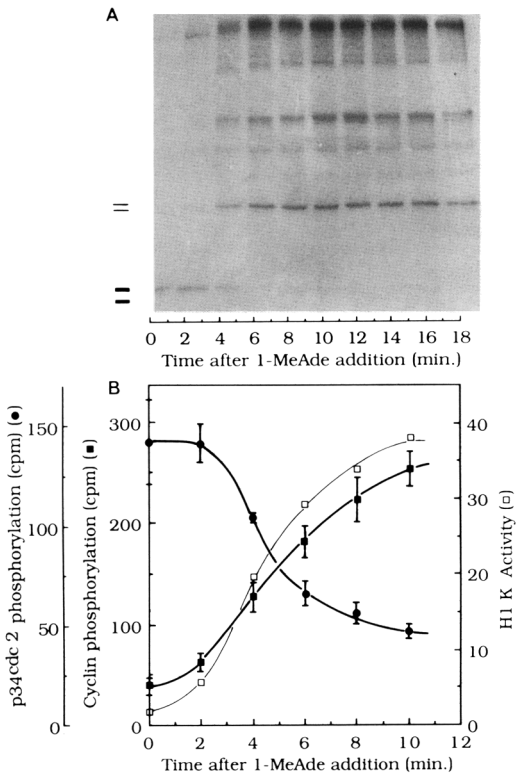

Figure 5. Dephosphorylation of p34^{cdc2} and phosphorylation of cyclin. (*A*) Extracts were prepared at the indicated times after hormonal stimulation of [^{32}P]phosphate-labeled oocytes. p13-bound proteins were analyzed by SDS-PAGE and autoradiography. The autoradiograph shows the phosphorylation of cyclin and its position on the silver-stained gel (thin markers) and the dephosphorylation of p34^{cdc2}; thick markers indicate the position of p34^{cdc2} (representative of two independent experiments). (*B*) In another experiment, the cyclin and p34^{cdc2} bands were excised from the polyacrylamide gel and counted (average plus S. D. of four determinations); H1K activation was monitored in the same oocyte batch.

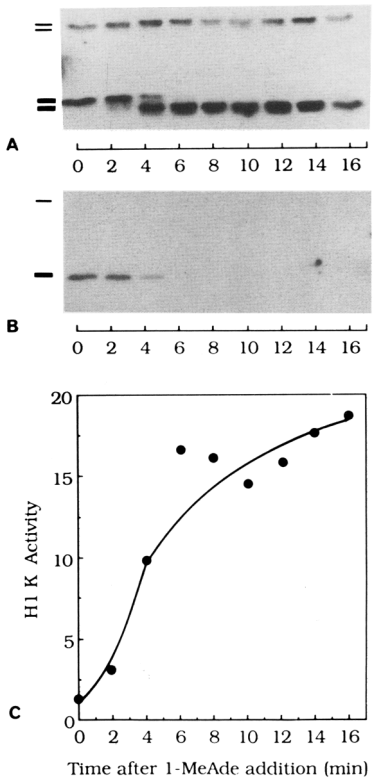

Figure 6. Tyrosine dephosphorylation of p34^{cdc2} during oocyte maturation. Oocytes were treated as described in Fig. 3, and the p13-bound proteins resolved by SDS-PAGE. (*A*) Immunoblot with anti-p34^{cdc2} and anti-cyclin. Thin markers indicate position of cyclin; thick markers indicate position of p34^{cdc2}. (*B*) Immunoblot with anti-phosphotyrosine. Thin marker indicates cyclin; thick marker indicates p34^{cdc2}. (*C*) Time course of H1K activation in the same experiment (representative of two independent experiments).

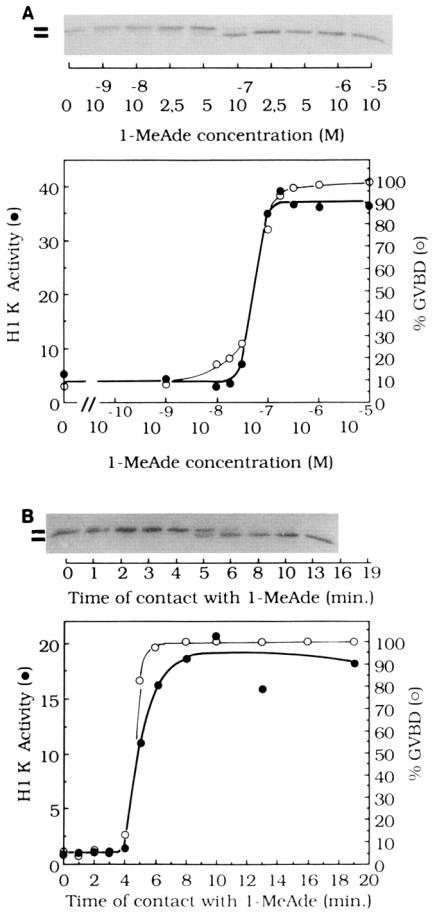

Figure 7. p34^{cdc2} modification after subthreshold hormonal stimulation. (*A*) Oocytes were treated with various concentrations of 1-MeAde. After 30 min, samples were prepared for SDS-PAGE analysis of p13–Sepharose-bound proteins. (*Top*) Anti-p34^{cdc2} immunoblot of the gel; H1K activity and extent of GVBD reached at each concentration (representative of two independent experiments). (*B*) Oocytes were treated for various times with 0.1 μM 1-MeAde, after which the hormone was washed away. After 30 min, samples were prepared for anti-p34^{cdc2} immunoblotting (*top*) or determination of H1K activity and percent GVBD (*bottom*) (representative of two independent experiments).

activation and that anti-cyclin antibodies immunoprecipitate H1K (Meijer et al. 1989). In the present study we show that cyclin phosphorylation is linked to H1K activation in starfish oocytes entering the M phase.

In contrast to previously described purification schemes for H1K, we have used p13–Sepharose as a preparative affinity column rather than for purely analytical purposes (Brizuela et al. 1989). The advantages of this reagent are clear. In a single step, the enzyme is enriched >1000-fold, with a yield of ~70%. Thus, from activated starfish oocytes, which are highly synchronized in M phase, it has been possible to purify milligram, rather than submicrogram, amounts of the enzyme.

Previously, p13–Sepharose was used as an affinity reagent to bind frog egg *cdc2* and its associated MPF activity (Dunphy et al. 1988). It was not possible to elute active MPF from the column because p13 is inhibitory to MPF. The apparent discrepancy with the present results is attributable to the different ways in which p34^{cdc2} is assayed. p13 is not inhibitory to the enzymatic activity of H1K assayed in vitro (Arion et al. 1988; Booher et al. 1989; Draetta et al. 1989; Dunphy and Newport 1989), but it does inhibit MPF activity either assayed in a cell-free lysate or following microinjection into frog oocytes (Dunphy et al. 1988). p13 has been shown to inhibit MPF by preventing the autoactivation of endogenous interphase *cdc2* by the small amount of fully active MPF that is added to the cell-free lysate in the usual MPF assay (Dunphy and Newport 1989). It is not known exactly how p13 inhibits the autoactivation reaction, during which p34^{cdc2} becomes tyrosin dephosphorylated. In light of the present experiments, in which p34/cyclin bound to p13–Sepharose could be activated by the particular phosphatase used, it may be that p13 inhibits p34^{cdc2} activation by interfering with a step other than the dephosphorylation event itself.

Experimental procedures

Preparation of oocytes

The starfish *Marthasterias glacialis* was collected in northern Brittany and kept in running seawater until use. The gonads were dissected out of the animals, gently torn open in ice-cold calcium-free artificial seawater (CaFASW), and filtered through cheesecloth. Oocytes were then washed three or four times in CaFASW to remove the 1-MeAde-producing follicle cells. They were resuspended, as a 20% (vol/vol) suspension in Millipore-filtered natural seawater (NSW) until use. Oocyte maturation was triggered by the addition of 1-MeAde to a final concentration of 1 μM (for further experimental details on oocyte preparation and induction of maturation, see Meijer et al. 1985).

Buffers

Homogenization buffer Homogenization buffer consists of 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM sodium vanadate, 0.1 mM NaF, 10 μM/ml leupeptin, 10 μg/ml aprotinin, 10 μ/ml soybean trypsin inhibitor, and 100 μM benzamidine. This buffer had previously been shown to stabilize the starfish meiotic oocyte M-phase-specific histone H1K (Pelech et al. 1987).

Buffer C Buffer C consists of homogenization buffer, but with 5 mM EGTA, no NaF, and no protease inhibitors.

Buffer D Buffer D consists of 12.5 mM MOPS (pH 7.2), 1 mM EGTA, 7.5 mM $MgCl_2$, 2 mM DTT, and 0.1 mM NaF.

Phosphatase buffer Phosphatase buffer consists of 50 mM PIPES (pH 6.0), 0.1% β-mercaptoethanol, 0.1 mM PMSF, 0.1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml leupeptin.

KII KII consists of 25 mM β-glycerophosphate, 25 mM MOPS (pH 7.2), 1 mM EGTA, 15 mM $MgCl_2$, 2 mM DTT, 0.1 mM NaF.

KIV KIV consists of KII diluted 1 : 1 with distilled water.

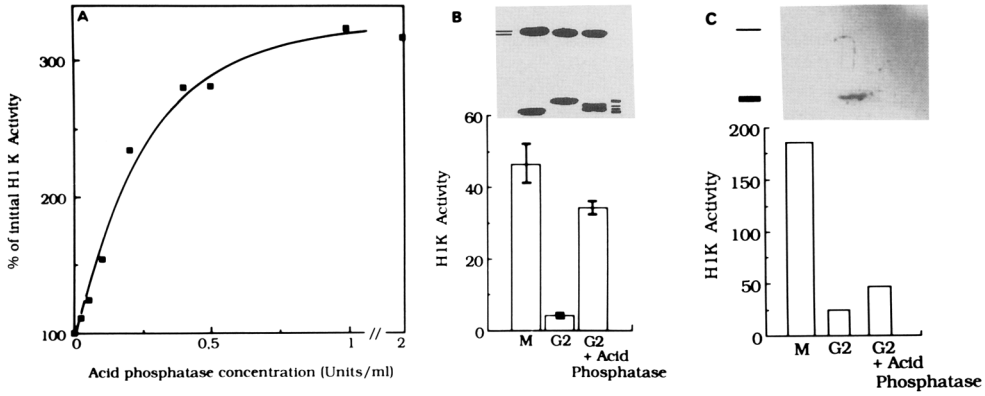

Figure 8. In vitro activation of H1K with phosphatase. (*A*) Extracts from unactivated oocytes were loaded onto p13–Sepharose beads. After several washes, the beads were exposed for 10 min to the indicated concentration of potato acid phosphatase. After further washes, the p13–Sepharose-bound H1K activity was determined (data from four independent experiments). (*B*) In a parallel experiment, extracts were prepared from either unactivated (G_2) or hormone-stimulated (M) oocytes and loaded onto p13–Sepharose. H1K activity was determined in each; the G_2 material was determined after exposure to phosphatase (*bottom*) (measurements are averaged from four separate experiments). (*Top*) A combined anti-cyclin and anti-$p34^{cdc2}$ immunoblot of proteins eluted from p13–Sepharose under each condition (representative of two independent experiments). (*C*) In a similar experiment, the p13-bound proteins in each condition were immunoblotted with anti-phosphotyrosine (*top*). (*Bottom*) H1K activity in the corresponding conditions (representative of two independent experiments).

KV KV consists of 0.1% Brij 35 in KIV.

KVI KVI consists of 200 mM NaCl in KV.

Bead buffer Bead buffer consists of 50 mM Tris (pH 7.4), 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 100 μM benzamidine.

Transfer buffer Transfer buffer consists of 39 mM glycine, 48 mM Tris, 0.37% SDS, and 20% methanol.

Net gel Net gel consists of 150 mM NaCl, 5 mM EDTA, 2.5 gm/liter gelatin, 0.01% NP-40, and 50 mM Tris (pH 7.4).

Preparation of cell extracts

At various times after the addition of 1-MeAde, 2-ml aliquots of the oocyte/egg suspension were rapidly centrifuged (5 sec full speed in an Eppendorf centrifuge). The supernatant was removed by aspiration, and 400 μl of homogenization buffer was added to the cell pellet. The tube was frozen immediately in liquid nitrogen and kept at −20°C until further processing (within 24 hr). Oocytes were homogenized by a 10-sec sonication. After centrifugation for 10 min at 14,000*g* at 4°C, the supernatant was recovered and either frozen in liquid nitrogen and stored at −20°C until the kinase assay or immediately loaded on p13–Sepharose beads.

Purification of H1K

At ~30 min after 1-MeAde addition, 40 ml of oocytes was pelleted by brief centrifugation (1 min at 1000*g*) (nuclear envelope breakdown typically occurs ~20 min posthormone addition), and the seawater was removed. The cells were homogenized with 3 or 4 volumes of homogenization buffer in a glass homogenizer. After centrifugation at 100,000*g* for 45 min at 4°C, the supernatant was immediately batch-loaded on 8 ml of p13–Sepharose equilibrated with bead buffer. After a 60-min incubation at 4°C under constant agitation, the p13–Sepharose beads were washed five times with bead buffer and packed into a chromatographic column. After further washing with bead buffer, the H1K was eluted from the column with 1 mg/ml p13. The presence of H1K was detected by assaying 1 μl of each fraction. The pooled active fractions were then loaded on a 1-ml hydroxyapatite column equilibrated with buffer D. After washing with buffer D, H1K was eluted with 0.5 M phosphate buffer (pH 7.4).

To determine the molecular weight of H1K, active fractions of the p13–Sepharose column were diluted with 1 volume of 0.2% Brij 35 in KIV and loaded on an 8-ml reactive yellow 86 agarose column equilibrated with KV. After washing with KV, H1K was eluted with KVI. Five milliliters of the most active fraction was loaded on a 400-ml Sephacryl S-200 column equilibrated with KVI. Molecular weight markers from Bio-Rad, dissolved in KVI, were used to calibrate the column. To determine H1K activity, 10 μl of each fraction was used. For immunoblotting of the fractions, each was then incubated for 1 hr at 4°C with 40 μl of p13–Sepharose beads. The beads were then washed with 1 ml bead buffer and treated with 150 μl 2× Laemmli sample buffer (Laemmli 1970) before analysis on SDS-PAGE.

H1K assay

All kinase assays contained 50 nM TTYADFIASGRTGRRNAIHD [a synthetic peptide with a structure that corresponds to the inhibitory site of cAMP-dependent protein kinase inhibitor (Scott et al. 1986)], 1 mg/ml histone H1 (Sigma, type III-S), 15 μM [^{32}P]ATP at ~800 cpm/pmole, 5 μl of diluted extract or column fraction, and buffer C, in a final volume of 25 μl. Kinase reactions were started by the addition of radioactive ATP and were of a 10-min duration at 30°C. Assays were terminated by spotting 20-μl aliquots onto 2.5 × 3-cm pieces of Whatman P81 phosphocellulose paper, and, after 20 sec, the filters were washed five times (for at least 5 min each time) in a solution of 10 ml of phosphoric acid per liter of water. The wet filters were transferred into 6-ml plastic scintillation vials, 5 ml of ACS (Amersham) scintillation fluid was added, and the samples

were counted in a Packard counter. The kinase activity was expressed in picomoles of phosphate incorporated in 5 μg histone H1 per fraction or extract volume at 10 min of incubation.

To assay H1 kinase activity on material bound to p13–Sepharose beads, the bead pellet was resuspended in 30 μl of a mixture containing 1 mg/ml H1, 50 nM of the PKI-synthetic peptide and 15 μM [^{32}P]ATP. After 10 min of incubation at 30°C, the p13–Sepharose beads were pelleted, and 20 μl of supernatant was removed and spotted on Whatman P81 phosphocellulose papers, which were further processed as described above.

Labeling of oocytes

To measure cyclin synthesis, starfish oocytes were labeled with [^{35}S]methionine (Amersham; final concentration, 25 μCi/ml) added 2 hr before addition of 1-MeAde. Oocytes were then processed as described above, and the [^{35}S]methionine-labeled proteins bound to p13–Sepharose were analyzed by SDS-PAGE and autoradiography.

To measure phosphate incorporation into cyclin and p34^{cdc2}, starfish oocytes were prelabeled with [^{32}P]phosphate (Amersham; final concentration, 82 μCi/ml) added 3.5 hr before addition of 1-MeAde. Oocytes were then processed as described above, and the [^{32}P]phosphate-labeled protein bound to p13–Sepharose was analyzed by SDS-PAGE and autoradiography. In some experiments the bands corresponding to p34^{cdc2} and cyclin were excised from the gel and counted in a Packard counter.

Acid phosphatase treatment

Starfish oocyte extracts were loaded on p13–Sepharose beads. The beads were washed twice with bead buffer and three to five times with phosphatase buffer. Then the beads were exposed at 30°C for 10 min to various dilutions of potato acid phosphatase (Boehringer grade II). The p13–Sepharose beads were then washed twice with bead buffer and assayed for H1K activity or processed for SDS-PAGE and immunoblotting.

Preparation and use of p13–Sepharose

p13 was purified from an overproducing strain of *E. coli* by gel filtration on Sepharose CL-6B (Pharmacia), as described previously (Brizuela et al. 1987) with a further step of Mono Q (Pharmacia) ion-exchange chromatography. It was conjugated to CnBr-activated Sepharose 4B (Sigma), according to the instructions of the manufacturer. Unreacted groups on the resin were quenched with 1 M ethanolamine (pH 8.0). The concentration of coupled p13 was 5 mg/ml of gel. The p13 beads used in this study are essentially the same as those described by Dunphy et al. (1988). The p13–Sepharose beads were kept at 4°C as a 20% (vol/vol) suspension in bead buffer. Just before use, 10 μl of packed p13 beads was washed with 1 ml of bead buffer and resuspended in 400 μl of bead buffer. The egg extract supernatant (390 μl) was added to the beads, and the tubes were kept under constant rotation at 4°C for 30 min. After a brief centriguation at 10,000*g*, and removal of the supernatant, the beads were washed three times with 1 ml of bead buffer. The beads were then either resuspended in 60 μl of PAGE sample buffer and boiled for 3 min or used immediately for H1 kinase activity determination.

Electrophoresis and Western blotting

Proteins bound to p13 beads were recovered with Laemmli sample buffer (Laemmli 1970). Typically, samples were run in 10% polyacrylamide gels and stained with the Bio-Rad silver staining kit. [^{35}S]methionine- or [^{32}P]phosphate-labeled-gels were dried prior to overnight exposure to β-Max X-ray films (Amersham). For immunoblotting, experimental samples were run in 10% SDS-polyacrylamide gels; proteins were transferred to 0.1-μm nitrocellulose paper (Schleicher and Schuell) in a Milliblot-SDE system (Millipore) for 30–40 min at 2.5 mA/cm^2 in transfer buffer. Subsequently, the filters were blocked with PBS containing 1% ovalbumin for 60–90 min at 37°C. The filters were then incubated overnight, at room temperature, with a 1 : 1000 dilution of the G_1 serum against p34^{cdc2} (kindly provided by G. Draetta), a 1 : 500 dilution of the serum against sea urchin egg cyclin (kindly provided by T. Hunt), a mixture of both, or 0.6 μg/ml of affinity-purified polyclonal antibodies against phosphotyrosine (kindly provided by A. Morla and J.Y.J. Wang). After four washes of 15 min each with net gel, the filters were exposed to hyperfilm MP or β-Max X-ray film overnight.

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