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Activation of human CDC2 protein as a histone H1 kinase is associated with complex formation with the p62 subunit

(protein phosphorylation/HeLa cell division cycle/p34 kinase)

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ABSTRACT **p34 kinase, the product of the *CDC2* gene, is a cell-cycle regulated protein kinase that is most active during mitosis. In HeLa cells, p34 kinase has previously been shown to exist in both a low- and a high-molecular-mass form, the latter of which is only found in cells in the G_2/M phase of the cell cycle and contains a 62-kDa subunit. Here we show that although each form of the kinase phosphorylates casein *in vitro*, only the high-molecular-mass form uses histone H1 as substrate. The high-molecular-mass form of p34 kinase from nocodazole-treated HeLa cells was purified 6700-fold. The apparent molecular mass of the mitotic *CDC2*-encoded protein kinase complex was 220 kDa. The purified enzyme phosphorylated not only its endogenous 62-kDa subunit but also phosphorylated histone H1 with a K_m of 3 μM and used ATP 40 times more efficiently than GTP (K_m 54 μM and 2 mM, respectively). The enzyme activity was unaffected by cAMP, calcium/calmodulin, or by the heat-stable inhibitor of cAMP-dependent protein kinase. These characteristics are typical of growth-associated histone H1 kinase from different organisms. These results suggest that CDC2 protein may be activated as an M-phase-specific protein kinase in part by its association with the p62 subunit.**

Several genes coding for putative protein kinases that are involved in the control of mitotic initiation have been isolated in fission yeast, namely *cdc2*$^+$ (1, 2), *wee1*$^+$ (3), and *nim1*$^+$ (4). Among these, *cdc2*$^+$ has been the object of extensive genetic and biochemical analysis because of its dual regulatory role, in G_1 before the initiation of DNA synthesis and in G_2 before mitosis (1, 5). *cdc2*$^+$ is the homolog of the *CDC28* "start" gene of budding yeast (6) and encodes a 34-kDa protein with kinase activity (2). This protein has been shown to be associated with the 13-kDa product of the *suc1*$^+$ gene of fission yeast (7) and also with the 63-kDa yeast cyclin, which is encoded by the *cdc13*$^+$ gene (R. Booher, C. Alfa, J. Hyams, and D.B., unpublished data). A CDC2 protein homolog has also been identified in human cells (9) in association with the 13-kDa SUC1 protein homolog. The human *CDC2* gene has been isolated by complementation of a yeast *cdc2* temperature-sensitive mutant (10).

Entry of interphase cells into mitosis is regulated by the M-phase-promoting factor (MPF), a non-species-specific mitotic inducer that was first described in amphibian oocytes (11, 12). The cdc2 protein kinase (p34) has been shown to be a component of the frog MPF (13, 14) in addition to being a component of the M-phase-specific histone H1 kinase of starfish oocytes (15, 16).

Mammalian cells have a CDC2 protein kinase (9), a "growth-associated" histone H1 kinase (17), and assayable MPF activity during mitosis (18). However, none of these activities have yet been directly interconnected in mammalian cells. The properties of p34 kinase in HeLa cells are consistent with its involvement in initiating mitosis. The protein kinase is activated in a series of steps during cell-cycle progression and is maximally active during mitosis (19). Activation involves phosphorylation of p34 kinase and association with a 62-kDa protein, which also acts as substrate of p34 kinase *in vitro*. A possible regulatory role of the phosphotyrosine content of p34 kinase on its mitotic activity has also been recently suggested (20). In this paper we have characterized the CDC2 mitotic complex and show that it is a high-affinity histone H1 kinase.

MATERIALS AND METHODS

Cell Culture and Preparation of Extracts. For the purification of the mitotic form of p34 kinase, HeLa cells were grown in suspension at 37°C in Joklik-modified minimal essential medium supplemented with 5% calf serum (GIBCO) to a final density of 5×10^5 cells per ml. Nocodazole (Jannsen Pharmaceutica) was then added to a final concentration of 1 μg/ml. Cells were harvested after 4 hr of drug treatment and washed with ice-cold phosphate-buffered saline. Cells obtained from 32 liters of culture were lysed in 100 ml of hypotonic buffer (10 mM sodium phosphate, pH 7.0/10 mM NaF/5 mM $MgCl_2$/1 mM EDTA/10 mM β-glycerophosphate) using a Dounce homogenizer, in the presence of a mixture of protease inhibitors (19). The cell lysate was centrifuged at 100,000 × *g* for 45 min, and the supernatant was collected. For the Superose 12 fractionation experiments, extract preparation and chromatographic procedure were done exactly as described (19).

Kinase Assays. Anti-p34 kinase immunocomplexes and p13–Sepharose precipitates, prepared as described previously (16, 19), or purified enzyme were preincubated for 1.5 min at 30°C in a final volume of 45 μl of buffer K (50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM dithiothreitol. Five microliters of ATP was then added (to a final concentration of 5 μM and 5 μCi per reaction; 1 Ci = 37 GBq). The reaction mixtures were incubated for 10 min after adding ATP. Calf histone H1 (Boehringer Mannheim) or dephosphorylated casein (Sigma) were included at a final concentration of 50 μg/ml and 1 mg/ml, respectively. The reaction was stopped by adding 30 μl of electrophoresis sample buffer (21). Samples were then analyzed by SDS/PAGE (7.5–15% gels). Subsequently, the proteins were transferred to 0.1 μm nitrocellulose filters, followed by autoradiography. To estimate the incorporation of ^{32}P into protein, a fraction of the stopped reaction was trichloroacetic acid-precipitated and counted.

Purification of the High-Molecular-Mass Form of the CDC2 Kinase. The assay used to detect the high-molecular-mass CDC2 kinase complex during the purification procedure made use of the autophosphorylation of the p62 subunit in p13–Sepharose precipitates. The protocol for protein purification was as follows: 25–45% $(NH_4)_2SO_4$ precipitate was obtained from the 100,000 × *g* supernatant of 32 liters of HeLa

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Abbreviation: MPF, M-phase-promoting factor.

cells and resuspended in 100 ml of buffer 1A (50 mM Tris·HCl, pH 7.0/5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/10 mM β-glycerophosphate). The protein solution was dialyzed for 4 hr against 1–2 liters of buffer 1A, and the conductivity of the solution was adjusted to that of buffer 1B (buffer 1A contained 80 mM NaCl). The protein solution was loaded onto a 10-ml DE-52 column (Whatman) equilibrated with buffer 1B. The flow-through and a 10-ml wash were pooled and concentrated down to 5 ml, followed by overnight dialysis against 1000 ml of buffer 2 (50 mM Hepes, pH 7.0/5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/10% glycerol/100 mM NaCl). The sample was then loaded onto a Mono S 10/10 column (Pharmacia) equilibrated in the same buffer, and protein was eluted with 125 ml of linear NaCl gradient (from 100 to 500 mM). The fractions with kinase activity were pooled and diluted with buffer 1A until they reached the same conductivity value of buffer 1B. The sample was then loaded onto a 5-ml casein–Sepharose column (5 mg of casein per ml) equilibrated in buffer 1B and eluted with a 70-ml NaCl gradient (80–500 mM gradient). One-milliliter fractions were collected. Active fractions were pooled and diluted with buffer 1A to the conductivity value of buffer 1B and loaded onto a Mono Q 5/5 column (Pharmacia). A 20-ml NaCl gradient was applied (from 80 to 500 mM), and 0.4-ml fractions were collected. The fractions with activity were pooled and concentrated to 0.5 ml, which was then loaded onto a Superose 12 column (Pharmacia) equilibrated with buffer S, and 0.25-ml fractions were collected. Protein concentration of the different fractions was determined using the colorimetric assay described by Bradford (22) by using IgG (Bio-Rad) as standard. In addition absorbance at 280 nm was used to estimate protein concentrations in later steps of purification.

Calculation of Kinetic Parameters. Characterization of the CDC2 kinase activity was done by incubating 5 μl of the Mono Q pool for 3 min as described under kinase assays. All reagents used were obtained from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) and [γ-^{32}P]GTP (650 Ci/mmol) were obtained from NEN and ICN, respectively.

RESULTS

Histone H1 Kinase Activity of CDC2 Protein Complex. We have previously found that in exponentially growing HeLa cells, p34 kinase exists in at least two different forms, depending on the state of association with other cellular proteins (19). The two forms could be dissociated by gel-filtration chromatography into fractions with apparent molecular masses of ≈30 and 200 kDa. The high-molecular-mass form, which increases in abundance during progression through interphase, contains a 62-kDa subunit that acts as a substrate of the CDC2 kinase *in vitro*. Activity with respect to p62 as substrate is enhanced in cells arrested in mitosis by nocodazole. With the same chromatographic procedure used previously (19) we have fractionated 100,000 × *g* supernatants of HeLa cells with or without prior exposure to nocodazole. A portion of each fraction was precipitated with p13–Sepharose (Fig. 1), a reagent that has been used to assay cdc2 kinase activity in starfish and clam oocytes (16, 23). The precipitates were incubated with [γ-^{32}P]ATP and either casein (Fig. 1 *Upper*) or histone H1 (Fig. 1 *Lower*).

In common with previous experiments in which anti-p34 kinase serum was used to precipitate the protein kinase from column fractions (19), the casein kinase activity of CDC2 protein resolved into two distinct peaks of very different molecular mobility. p62 is only present in the high-molecular fraction (Fig. 1; ref. 19). Only the higher-molecular-mass form was activated after exposure of cells to nocodazole. Also the preference of the kinase for each of the two forms of casein (α and β) was different in the two fractions. This apparent change in specificity was much more marked using histone H1 as substrate. Histone H1 was phosphorylated exclusively by the high-molecular-mass protein kinase complex, and the histone H1 kinase activity was enhanced by exposure to nocodazole (Fig. 1 *Lower*). Also histone H1 conspicuously competed with phosphorylation of p62 in the immune complex, whereas casein did not. Precisely the same observations were made using anti-p34 kinase antibodies to immunoprecipitate and assay the column fractions (data not shown).

Purification of CDC2 Protein Kinase Complex. Purification of the mitotic complex was followed by analyzing the phosphorylation of p62 in p13–Sepharose precipitates of column fractions. The design of the strategy is 3-fold. (*i*) p13–Sepharose is used to assay p34 kinase-dependent kinase activity because it displays the same specificity as anti-p34 antibodies (Fig. 1; ref. 19). Use of this reagent both accelerates and simplifies the assay procedure compared with immunoprecipitation. (*ii*) By assaying autophosphorylation of p62, only the high-molecular-mass CDC2 complex is purified. (*iii*) To enrich for the activity of the p34 kinase mitotic complex

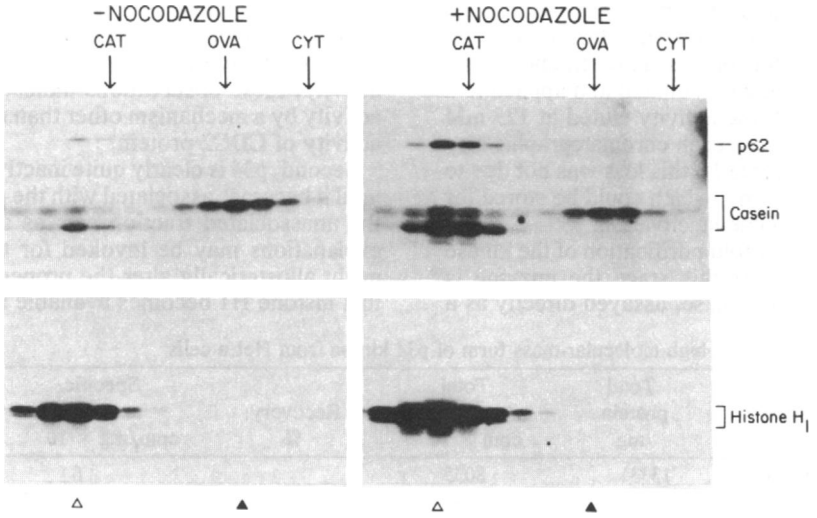

FIG. 1. Changes in substrate specificity of p34 protein. Protein extract (1.5 mg) from HeLa cells treated for 4 hr with nocodazole at 1 μg/ml (*Right*) or equivalent amount of protein from control cells (*Left*) were fractionated by gel filtration on a Superose 12 column. p13–Sepharose precipitates of each fraction were assayed for kinase activity using either casein at 1 mg/ml or histone H1 at 50 μg/ml as substrates (*Upper* and *Lower*, respectively) in the presence of [γ-^{32}P]ATP. The elution positions of catalase (CAT), cytochrome *c* (CYT), and ovalbumin (OVA) from the Superose 12 column are indicated. △ and ▲ represent the elution position of the high- and low-molecular-mass forms of p34, respectively.

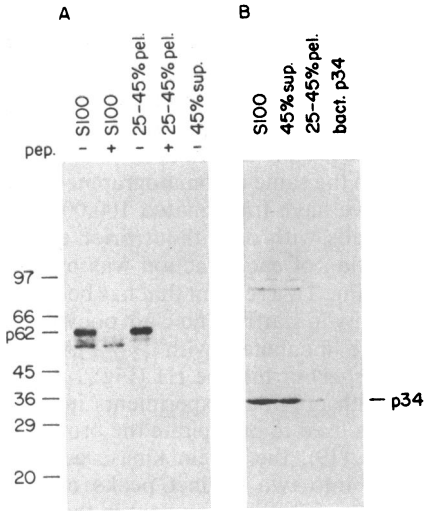

FIG. 2. High-molecular-mass complex contains only a minor fraction of p34 kinase. (*A*) Anti-p34 immunoprecipitates of equivalent volumes of HeLa 100,000 × *g* supernatant (S100) and $(NH_4)_2SO_4$ fractions were prepared without and with antigenic peptide (− and + pep) and assayed for kinase activity in the absence of externally added substrates. Molecular mass of standard proteins is given in kDa. (*B*) Immunoblot of equivalent volumes of 100,000 × *g* supernatant and relevant $(NH_4)_2SO_4$ fractions using 1:1000 dilution of anti-p34 immune serum. Thirty nanograms of bacterial (bact.) p34 kinase was used as marker. sup, Supernatant; pel, pellet.

in the starting material, HeLa cells were incubated for 4 hr with nocodazole before harvesting.

The first step of purification was ammonium sulfate precipitation of the 100,000 × *g* supernatant of the cell extract. The CDC2 protein kinase activity precipitated 25–45% salt saturation (Fig. 2*A*), but the great majority of p34 kinase, detected by immunoblotting (Fig. 2*B*), remained soluble at 45% salt saturation.

After ammonium sulfate precipitation, the CDC2 protein kinase complex was recovered in the flow-through and wash of the DE-52 column equilibrated with a buffer at pH 7.0 containing 80 mM NaCl. Most proteins were retained by the column under these conditions (Table 1). The active material was loaded onto a Mono S column to which it bound in low salt and could be eluted at 260 mM NaCl (Fig. 3*A*). The next step of purification took advantage of the ability of p34 kinase to bind to a casein–agarose matrix (20), from which it eluted at 250 mM NaCl (Fig. 3*B*). The pool of most active fractions from casein–agarose chromatography was diluted and applied to a Mono Q column, from which the activity eluted at 125 mM NaCl (data not shown). Although each chromatographic step lost protein kinase activity (Table 1), this loss was not due to inherent instability of the enzyme, which could be stored for weeks at 4°C without appreciable inactivation.

After the Mono Q step, 3600-fold purification of the kinase activity had been achieved. At this stage the enzyme is substantially pure as a protein kinase, assayed directly as a p62, casein, or histone H1 kinase, without prior immunoprecipitation or incubation with p13–Sepharose (Fig. 4). At 6700-fold purification, achieved by Superose 12 chromatography of the Mono Q fractions, silver staining of column fractions resolved by SDS/PAGE revealed the p34 and p62 polypeptides (data not shown) but did not allow an unequivocal determination of the total subunit composition of the kinase due to the presence of less abundant, but detectable, high-molecular-mass polypeptides. This has been a problem in other efforts to purify complexes containing CDC2 protein (16, 24). We could not unequivocally establish whether the p13 subunit was present in the most purified fractions due to the lack of crossreactivity of the anti-p13 serum with the mammalian p13 immobilized in nitrocellulose and also because of the poor reactivity of this protein in silver staining. The apparent molecular mass of the CDC2 kinase complex was 220 kDa, as determined by gel filtration (Fig. 3*C*).

Kinetic characterization of the purified (post-mono Q) CDC2 protein kinase complex was performed by following the activity of the enzyme in solution. Incorporation of ^{32}P into histone H1 was linear during the first 3 min of reaction at 30°C (data not shown). Table 2 summarizes several kinetic properties of the kinase. K_m values for ATP (54 μM), GTP (2 mM), and histone H1 (3 μM) were determined. No effect on the kinase activity was seen upon incubation of the enzyme with 10 μM cAMP, 0.6 μM heat-stable protein kinase A inhibitor, or 1 μM bovine testis calmodulin. Purified p13 was also added to kinase reactions at concentrations up to 20 μM. No inhibition of the enzyme was noted during the standard 10-min reactions and, indeed, at prolonged intervals of incubation there was a slight stabilization of enzymatic activity (data not shown). Optimal activity for the phosphorylation of histone H1 was achieved in a wide pH range between 8 and 9 at a $MgCl_2$ concentration of 10 mM, which could be substituted by $MnCl_2$. No ability of the kinase to phosphorylate histone H3 was found.

DISCUSSION

The most striking observations of the present experiments are 2-fold. First, p13–Sepharose can be used interchangeably with anti-p34 serum as a reagent to assay both the complexed and low-molecular-mass forms of human CDC2 protein, even though much of the p34 kinase is complexed with p13 *in vivo* (9, 19). This observation and a similar one with respect to the histone H1 kinase activity of starfish (16) are surprising because exogenous p13 inhibits frog MPF activity in a cell-free assay at concentrations as low as 2 μM (20). In our experiments, p13 at 20 μM did not inhibit histone H1 kinase activity; these observations indicate that p13 inhibits MPF activity by a mechanism other than inhibition of the catalytic activity of CDC2 protein.

Second, p34 is clearly quite inactive as a histone H1 kinase until it becomes associated with the p62 subunit, even though the unassociated fraction acts as a casein kinase. Several explanations may be invoked for this observation: (*i*) p62 might allosterically alter the properties of p34 kinase, such that histone H1 becomes available as a substrate. (*ii*) Alter-

Table 1. Purification of the high-molecular-mass form of p34 kinase from HeLa cells

Step	Total protein, mg	Total activity, cpm × 10^{-3}	Recovery, %	Specific activity, cpm/mg × 10^{-3}	Purification, -fold
Supernatant (100,000 × *g*)	1333	8055		6	
Ammonium sulfate	350	5140	64	15	2.5
DE-52	56	5000	62	90	15
Mono S 10/10	4	2432	30	608	100
Casein–agarose	0.15	950	11.8	6,100	1016
Mono Q 5/5	0.02	520	6.5	22,000	3600
Superose 12 10/30	0.008	320	4	40,000	6666

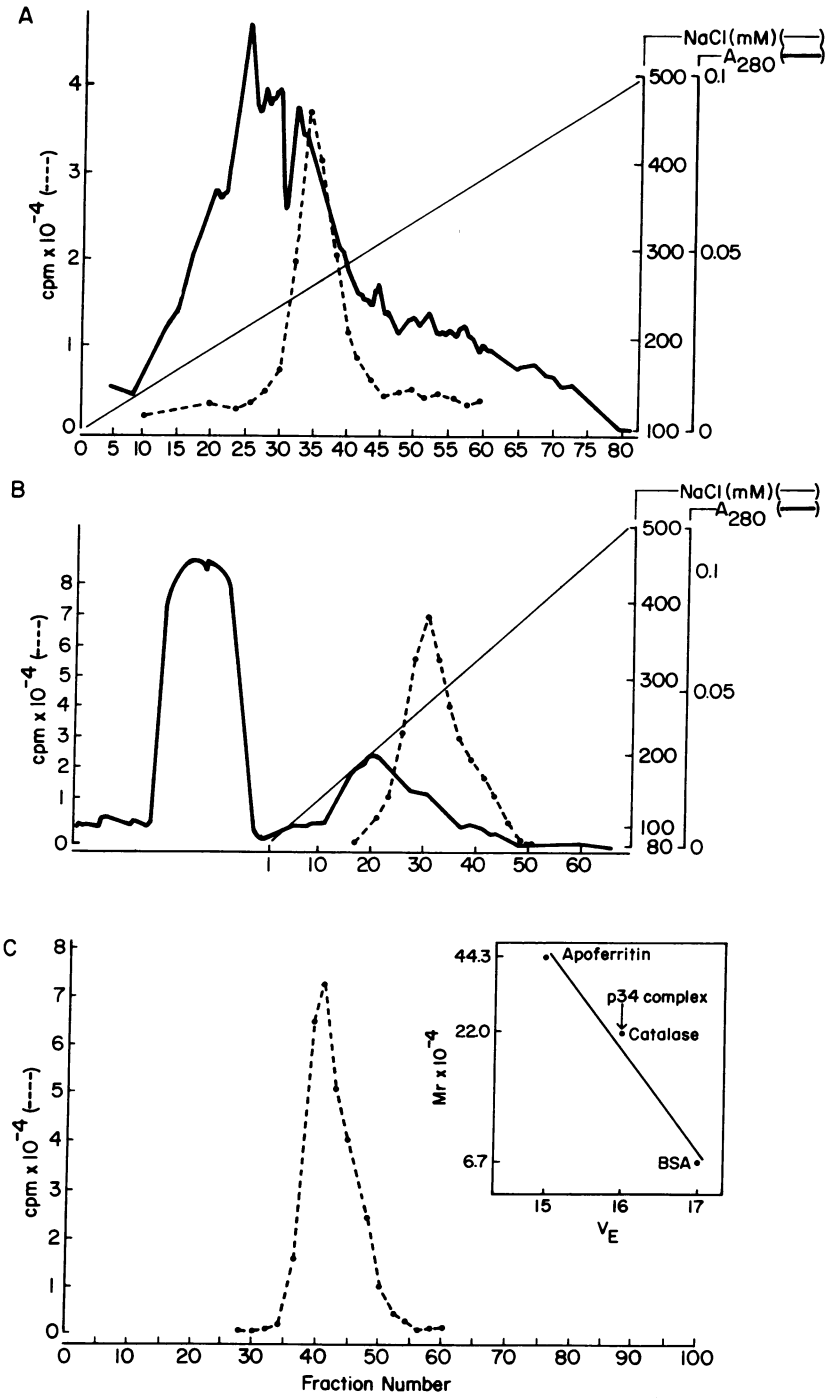

FIG. 3. Chromatography of CDC2 protein kinase complex. (*A*) Mono S chromatography of DE-52 eluate. Flow-through and wash of the DE-52 column were pooled and loaded onto a Mono S column and the bound proteins were eluted with 125-ml NaCl gradient (thin line). Absorbance at 280 nm represents the protein profile (thick line). Migration of the kinase activity was followed by ^{32}P incorporation into p62 (split line). (*B*) Chromatography of Mono S pool on casein–agarose: fractions 33–40 from the Mono S chromatography were pooled and diluted to be loaded onto a 5-ml casein–agarose column. The bound proteins were eluted with 70 ml of NaCl gradient. The same symbols as in *A* were used to represent the salt gradient, the protein, and the activity profile. (*C*) Superose 6 chromatography of Mono Q pool. Fractions containing activity from the Mono Q column were pooled and concentrated to 0.5 ml before being loaded onto a Superose 6 column. Split lines show the migration of the kinase activity, and the inset graphically represents the elution volumes (V_E) of apoferritin, catalase, bovine serum albumin (BSA), and the p34 kinase complex in relation to the molecular weights of the standards.

natively, complex formation with p62 might facilitate a secondary event, such as a phosphorylation of p34 kinase at a particular site, which alters its substrate specificity. (*iii*) An explanation that is not formally excluded by our experiments is that p34 kinase is not the catalytic subunit of the histone H1 kinase, but rather some other CDC2-associated protein present in the mitotic complex.

We think that p62 itself is probably not a protein kinase but rather a mitotic cyclin. In fission yeast, a 63-kDa cdc2 protein-associated endogenous substrate is the product of *cdc13*$^+$ (8), which is homologous to cyclins of multicellular organisms (25). Furthermore, this complex shows a histone H1 kinase activity *in vitro* of which p34 kinase has been shown to be the catalytic subunit. The *cdc2/cdc13* complex

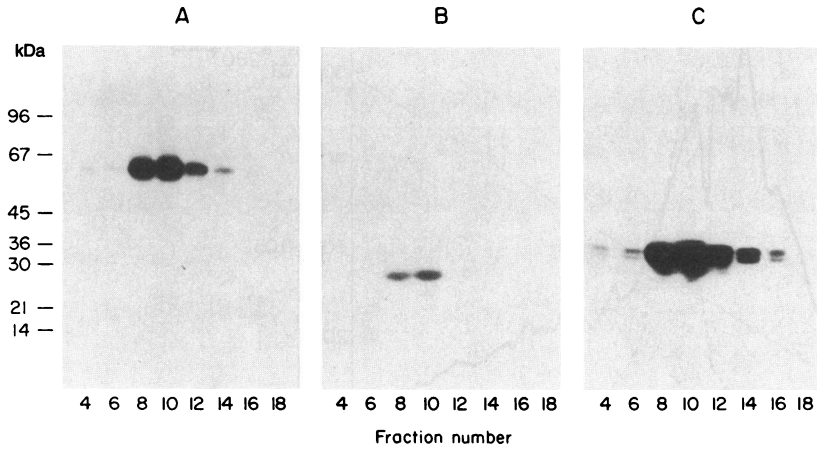

FIG. 4. Kinase activity of CDC2 protein kinase complex. (*A*) Ten-microliter aliquots from the Mono Q fractions were directly assayed for kinase activity by addition of [γ-^{32}P]ATP. (*B* and *C*) Casein at 1 mg/ml and histone H1 at 50 μg/ml, respectively, were added as substrates. Autoradiogram in *A* represents a 4-hr exposure of the blotted proteins, whereas *B* and *C* correspond to 10-min exposures.

exhibits thermolabile histone H1 kinase activity in extracts prepared from temperature-sensitive *cdc2* mutants (R. Booher, C. Alfa, J. Hyams, and D.B., unpublished data). Thus, it is likely that in yeast and presumably also in HeLa cells CDC2 protein is the catalytic subunit of the p34/p62 histone H1 kinase. The role of the cyclin appears, at least in part, to confer on the CDC2 protein kinase "M-phase specificity." Degradation of the cyclin at the metaphase/anaphase transition would cause loss of such specificity and, thus, loss of MPF activity (23).

The properties of the histone H1 kinase described here are similar to those of the "growth-associated" histone H1 kinase partially purified from other mammalian cell types in addition to representatives of other phyla (26, 27). This kinase activity has been found in actively proliferating cells. Furthermore, each of these kinase activities is independent of cAMP, Ca^{2+}, or calmodulin (28, 29) and has similar K_m values for histone H1 (29, 30). The mitotic complex characterized here uses ATP as a phosphoryl donor with a K_m similar to that reported for Chinese hamster fibroblasts (29) and the starfish histone H1 kinase (30). On the other hand, only the starfish histone H1 kinase and the H1 kinase reported here use GTP, but with very different K_m values (10 μM and 2 mM, respectively).

Phosphorylation of histone H1 has been repeatedly postulated but never proven to have a causal role in chromatin condensation and initiation of mitosis (8, 31). The protein kinase purified in this study may be used to investigate the significance of histone phosphorylation *in vitro* and also to find other potentially more important mitotic substrates of the kinase.

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Table 2. K_m and IC_{50} for different compounds

Compound	K_m	IC_{50}
ATP	54 μM	
GTP	2 mM	
Histone H1	3 μM	
β-Glycerophosphate		40 mM
NaCl		250 mM
PKI		No effect to 0.6 μM
cAMP		No effect to 10 μM
Calmodulin*		No effect to 1 μM
p13		No effect to 20 μM

IC_{50} was calculated in the presence of 6 μM histone H1 and 200 μM ATP. PKI, heat-stable inhibitor of the cAMP-dependent protein kinase.
*Calmodulin was measured in the presence of 1 mM $CaCl_2$.

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