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Inactivation of cdc2 kinase during mitosis requires regulated and constitutive proteins in a cell-free system

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SUMMARY

Inactivation of the cyclin-p34^{cdc2} protein kinase complex is a major requirement for anaphase onset and exit from mitosis. To facilitate identification of specific molecules that regulate this event in mammalian cells, I have developed a cell-free assay in which cdc2 kinase associated with a chromosomal fraction from metaphase tissue culture cells is inactivated by a cell-cycle-regulated cytosolic system. In vitro kinase inactivation requires ATP, Mg^{2+} and the dephosphorylation of one or more sites in the chromosomal fraction by protein phosphatase 1 and/or 2A. Cyclin B is destroyed during inactivation, while the level of p34^{cdc2} remains constant. Ammonium sulfate fractionation resolves the cytosolic inactivating system into at least two distinct protein components that are both required for inactivation and are differentially regulated during mitosis.

Key words: cdc2 kinase, mitosis, cell-free system

INTRODUCTION

The transition from interphase to mitosis is triggered universally in eukaryotic cells by the 34 kDa cdc2 protein kinase (Nurse, 1990). cdc2 kinase activity toward mitotic substrates increases dramatically late in interphase, following association of p34^{cdc2} with mitotic cyclins (Draetta et al., 1989; Labbe et al., 1989; Meijer et al., 1989) and subsequent modification of the complex by phosphorylation and dephosphorylation at multiple sites (Dunphy and Newport, 1989; Gould and Nurse, 1989; Morla et al., 1989; Solomon et al., 1990, 1992). The cyclin-p34^{cdc2} complex in turn phosphorylates numerous substrates, including histone H1 (Arion et al., 1988; Labbe et al., 1988; Brizuela et al., 1989), lamins (Peter et al., 1990; Ward and Kirschner, 1990) and vimentin (Chou et al., 1990); thereby promoting chromosome condensation (Gurley et al., 1978; Hanks et al., 1983), disassembly of the nuclear lamina (Gerace and Blobel, 1980; Heald and McKeon, 1990; Peter et al., 1990) and depolymerization of intermediate filaments (Chou et al., 1990).

Just as activation of p34^{cdc2} induces entry into mitosis, inactivation of the enzyme during anaphase is required for mitotic exit (Murray et al., 1989; Ghiara et al., 1991). Inactivation involves proteolysis of B-type cyclins (Evans et al., 1983; Murray et al., 1989; Ghiara et al., 1991; Gallant and Nigg, 1992), and it has been suggested that cells may regulate ubiquitin attachment to cyclin or may transiently activate a cyclin protease (Glotzer et al., 1991). Protein phosphorylation/dephosphorylation also is involved, since mutations in genes encoding protein phosphatase type 1 (PP-1) yield a phenotype characteristic of metaphase arrest in a variety of organisms (Doonan and Morris, 1989; Axton et al., 1990; Kinoshita et al., 1990), and microinjected anti-PP-1 antibodies prolong M phase in starfish oocytes (Picard et al., 1989) and mammalian fibroblasts (Fernandez et al., 1992). The p34^{cdc2} binding protein, p13^{suc1}, may be important for cdc2 kinase inactivation as well: deletion of p13suc1 in fission yeast gives rise to cells that are unable to inactivate p34^{cdc2} or exit mitosis (Moreno et al., 1989).

To identify molecules that mediate inactivation of p34^{cdc2} at the metaphase/anaphase transition in mammalian cells, I have developed a cell-free system in which cyclin destruction and inactivation of cdc2 protein kinase activity associated with a chromosomal fraction are induced by cytosol from early interphase tissue culture cells. I show that inactivation requires ATP, Mg^{2+}, protein phosphatase activity and the joint action of at least two distinct cytosolic proteins that are differentially regulated during mitosis.

MATERIALS AND METHODS

Materials

Tissue culture medium and serum were purchased from Inovar Inc. (Gaithersburg, MD). Rabbit anti-p34^{cdc2} IgG was obtained from GIBCO/BRL (Gaithersburg, MD) and rabbit anti-human IgG was obtained from Pierce (Rockford, IL). Polyclonal rabbit antiserum raised against purified recombinant human cyclin B was the generous gift of Dr. Helen Piwnica-Worms. [γ-^{32}P]adenosine-5′-triphosphate (ATP) (7000 Ci/mmol; 160 mCi/ml) was from ICN Biomedicals (Irvine, CA). Nocodazole was purchased from

Aldrich (Milwaukee, WI); okadaic acid and microcystin-LR were from LC Services Corp. (Woburn, MA). Adenyl-imidodiphosphate (AMP-PNP) and adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS) were supplied by Boehringer Mannheim (Indianapolis, IN). All other reagents were obtained from Sigma (St. Louis, MO).

Cell culture methods

Adherent cultures of Chinese hamster ovary (CHO) cells were propagated at 37˚C in Joklik-modified Minimum Essential Medium supplemented with 10% fetal calf serum, 0.1 mM non-essential amino acids, 2 mg/ml sodium bicarbonate, 100 i.u./ml penicillin G and 100 μg/ml streptomycin sulfate. For experiments, 1750 cm^2 plastic roller bottles were inoculated with 10^7 cells, gassed with 5% carbon dioxide, sealed and cultured for 3 days rotating at 0.1 revs/min. Cultures were blocked in S phase of the cell cycle by addition of 2 mM thymidine for 11.5 hours. These were then rinsed and incubated in thymidine-free medium for 3 hours. At that time, 100 ng/ml nocodazole was added to arrest cells in mitosis (Zieve et al., 1980). One hour later, the roller bottles were rotated for 3 minutes at 300 revs/min to detach weakly adherent interphase cells. Fresh medium containing nocodazole was added, and mitotic cells (>95% mitotic index) were collected at 2.5 hour intervals using the same mechanical shake-off procedure (Tobey et al., 1967). Interphase cells remaining attached to roller bottles after the first mitotic harvest were scraped into Dulbecco's phosphate-buffered saline containing 1 mM $MgCl_2$ at 4˚C to provide a G_2-phase-enriched population (<10% mitotic). Early G_1 phase cells were obtained by washing mitotic cells twice with nocodazole-free medium at 4˚C, resuspending in 37˚C medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.2 (NaOH), and incubating for 30 minutes in suspension culture. During this period, the cells complete cytokinesis, reform nuclei and undergo chromosome decondensation.

Adherent HeLa cell cultures were maintained in supplemented Joklik's medium containing 0.9 mM $CaCl_2$. Roller bottles (1750 cm^2) were inoculated with 1.8×10^7 cells and cultured for 3-4 days before addition of 50 ng/ml nocodazole. After one hour, weakly adherent cells were detached by mechanical shake-off and discarded. Fresh medium containing nocodazole was added and mitotic cells (95% mitotic index) were collected by shake-off 16 hours later. A population enriched for early G_1 phase cells was harvested 110 minutes after nocodazole washout. The population also included some late mitotic cells, since recovery from nocodazole-induced metaphase arrest lasting 16 hours was less synchronous than for CHO cells arrested for 2.5 hours.

Preparation of subcellular fractions

All procedures were performed at 4˚C unless otherwise indicated. Cells pelleted by centrifuging 5 minutes at 250 ***g*** were washed twice with 25 volumes of homogenization buffer (50 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), pH 7.0 (KOH), 50 mM KCl, 10 mM ethylene glycol-bis(β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20 μM cytochalasin B), resuspended in 1 volume of homogenization buffer and lysed using a Dounce-type tissue homogenizer with tight-fitting pestle. Post-ribosomal supernatants ("cytosol") were prepared by centrifuging homogenates 1 minute at 12,000 ***g*** (Eppendorf microfuge), followed by 10 minutes centrifugation at 180,000 ***g*** (45,000 revs/min in a 50Ti rotor; Beckman Instruments Inc., Fullerton, CA). A low-speed pellet containing chromosomes (p1) was isolated from mitotic CHO cells by centrifuging homogenates 5 minutes at 1,000 ***g***. The pellet was washed twice with 10 volumes of homogenization buffer before use in cell-free assays. Both cytosol and p1 fractions were stored in liquid nitrogen for months with no loss of activity.

Prior to experiments, early G_1 phase HeLa cytosol was incubated with 1 mM MgATP, 5 mM phosphoenolpyruvate (PEP) and 10 units/ml pyruvate kinase (PK) for 15 minutes at 33˚C to inactivate low levels of cdc2 kinase activity resulting from residual mitotic cells (see above). The cytosol was then cooled to 4˚C and sonicated for 5 seconds. Similar treatment was administered to cytosol from G_2 and M phase HeLa cells when determining the cell cycle dependence of cdc2 kinase inactivation in cell-free assays (Table 1).

Fractions insoluble in 0-35% and 35-55% $(NH_4)_2SO_4$ were obtained by addition of a saturated solution of $(NH_4)_2SO_4$ (pH 7.2) to stirred cytosol at 4˚C. After an additional 30 minutes of incubation, samples were centrifuged for 5 minutes at 6,000 ***g*** and the resulting pellets resuspended in dialysis buffer (25 mM PIPES-KOH, pH 7.0, 25 mM KCl, 5 mM EGTA, 2.5 mM $MgCl_2$, 1 mM DTT). These were transferred to 12,000 to 14,000 M_r cutoff Spectrapor 2 dialysis tubing (Spectrum Medical Industries Inc., Los Angeles, CA) and dialyzed for at least 3.5 hours vs 2 × 200 volumes of dialysis buffer.

cdc2 H1 kinase assays

Aliquots (20 μl) of cdc2 kinase inactivation mixtures (see below) were diluted 75-fold with kinase assay buffer (25 mM HEPES-KOH, pH 7.4, 25 mM β-glycerophosphate, 10 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0.1 μM cyclic AMP-dependent protein kinase (PK) inhibitor, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin) and were incubated 4-5 minutes at 33˚C in the presence of 0.5 mg/ml histone H1 (Sigma type IIIS), 0.4 mM MgATP, and 0.1 mCi/ml [γ-^{32}P]ATP. Incorporation of ^{32}P into histone H1 was determined for 25 μl aliquots of kinase assays as described by Witt and Roskoski (1975), using 2.3 cm P81 cellulose phosphate discs (Whatman LabSales Inc., Hillsboro, OR). Under these conditions, incorporation was linear for at least 6 minutes and almost exclusively measured transfer of ^{32}P to histone H1; at least 10-fold lower incorporation was observed when H1 was omitted from assays.

cdc2 kinase inactivation

The p1 fraction from mitotic CHO cells was resuspended in an equal volume of homogenization buffer (minus cytochalasin B) using 2-3 brief (5 s) pulses of a Branson Sonifier equipped with stepped micro tip (Branson Ultrasonics Corp., Danbury, CT). A 25 μl sample of p1 suspension was combined with up to 105 μl of cytosol (or other soluble fraction), 1 mM MgATP, 10 mM PEP, 10 units/ml PK, 2 mM $MgCl_2$ and homogenization buffer (minus cytochalasin B) in a final volume of 140 μl. After determining initial cdc2 kinase activity as described, mixtures were incubated at 33˚C and $p34^{cdc2}$ inactivation followed by periodically assaying 20 μl aliquots to quantitate remaining cdc2 kinase activity. Rates of inactivation were calculated from duplicate kinase assays conducted 7-15 minutes apart, or from the slope of a semilogarithmic plot of cdc2 kinase activity determined at 4-5 successive times during the course of inactivation (see Results).

Immunoadsorption

The mitotic p1 fraction was resuspended in 15 volumes of homogenization buffer and brought to 250 mM KCl by addition of a 4 M stock solution. After 20 minutes at 4˚C, the suspension was centrifuged 5 minutes at 180,000 ***g***, yielding a postribosomal supernatant containing 90% of p1-associated histone H1 kinase activity. Aliquots of supernatant were mixed with an IgG fraction isolated from rabbits immunized with the peptide CDNQIKKM, corresponding to the deduced carboxyl terminus of human $p34^{cdc2}$ (Draetta and Beach, 1988), or were mixed with control rabbit anti-human IgG antibodies. After 90 minutes, fixed *Staphylococcus aureus* cells were added (Pansorbin standardized suspension used

at 6-fold excess binding capacity; Calbiochem Corp., San Diego, CA) and mixtures were incubated for an additional 60 minutes. Following centrifugation for 5 minutes at 2,300 ***g*** to pellet the *S. aureus*, H1 kinase activity was determined for supernatants diluted 30-fold with kinase assay buffer.

Western blots

Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose and labeled essentially as described by Burnette (1981), except that the concentration of bovine serum albumin in blocking solution was reduced to 2% (w/v). In addition, both the blocking and wash solutions contained 0.5% (w/v) Triton X-100. ^{125}I-labeled Protein A (30 mCi/mg Protein A; 100 μCi/ml) from Amersham (Arlington Heights, IL) was used at 10 ng/ml.

RESULTS

cdc2 kinase inactivation in cell-free extracts

Mitosis-specific Ca^{2+} and cyclic AMP-independent histone H1 kinase activity has been described in slime mold (Bradbury et al., 1974a,b), fission yeast (Moreno et al., 1989), starfish (Picard et al., 1987), sea urchins (Meijer et al., 1989), frogs (Dabauvalle et al., 1988) and mammalian cells (Lake and Salzman, 1972; Woodford and Pardee, 1986). The activity derives from a single major protein kinase identified immunologically as p34^{cdc2} (Arion et al., 1988; Labbe et al., 1988; Brizuela et al., 1989), and H1 kinase assays have been used widely to quantitate mitotic cdc2 protein kinase activity in vitro and in vivo (Dunphy and Newport, 1989; Moreno et al., 1989; Morla et al., 1989; Solomon et al., 1990, 1992). With the assay system employed here, CHO cells exhibited Ca^{2+} and cyclic AMP-independent histone H1 kinase activity that was greatly elevated in nocodazole-arrested mitotic cells (Fig. 1). The activity was 19-fold lower in lysates prepared 40 minutes after removing nocodazole; when the cells had undergone chromosome decondensation, nuclear envelope re-formation and cytokinesis. In contrast, cells cultured in the continued presence of nocodazole retained metaphase-like condensed chromosomes and exhibited only a 30% decrease in H1 kinase activity over 100 minutes (Fig. 1).

When mitotic CHO cell lysates were analyzed using differential centrifugation, approximately 50% of the H1 kinase activity was cytosolic (present in a postribosomal supernatant), while 50% was associated with a 1,000 ***g*** particulate fraction (p1) despite repeated washing with buffer containing 50 mM KCl (Fig. 2A; 0 minutes). Phase-contrast microscopy indicated that the p1 fraction consists primarily of chromosomes and membrane fragments; however, the H1 kinase appears to be bound to chromosomes, rather than to membranes, since 90% of this activity was solubilized by 250 mM KCl, but was not released by treatment with 0.5% Triton X-100 (data not shown). These observations are consistent with previous reports that have described the association of M phase-promoting factor (MPF) (Adlakha et al., 1982a,b) and mitosis-specific histone H1 kinase activity (Lake and Salzman, 1972; Woodford and Pardee, 1986) with chromatin. In mitotic HeLa cells, approximately 50% of the MPF is soluble, while 50% is bound to chromosomes but is extractable with 200 mM NaCl (Adlakha et al., 1982a,b).

To prove that p34^{cdc2} is the source of H1 kinase activity in the mitotic p1 fraction, p1-associated H1 kinase was solubilized in 250 mM KCl and incubated with antibodies recognizing the carboxyl terminus of human p34^{cdc2} (Draetta and Beach, 1988) or with control antibodies recognizing human IgG. While anti-p34^{cdc2} depleted 89% of the H1 kinase activity, anti-human IgG removed less than 10% (Fig. 3).

I investigated inactivation of mitosis-specific cdc2 kinase activity in vitro by incubating concentrated p1 or cytosol fractions from mitotic CHO cells at 33˚C in the presence of MgATP and an ATP-regenerating system. At intervals, aliquots of the incubations were diluted extensively, and immediately assayed to quantitate cdc2 kinase activity. While p1-associated cdc2 kinase activity remained unchanged for at least 45 minutes, cytosolic activity decreased by 93% within 30 minutes (Fig. 2A). Apparently, cdc2 kinase in the particulate fraction was resistant to inactivation, or the inactivating system was soluble. To distinguish between these possibilities, p1 and cytosol were combined and incubated at 33˚C as before. Initially, cdc2 kinase activity for the combined fractions equalled the sum of individual values, and fully reconstituted a level of activity found in the unfractionated lysate. However, within 45 minutes the activity decreased by 90% to the basal level seen for cytosol alone (Fig. 2A). Thus, cdc2 kinase in both the cytosol and p1 fractions could be inactivated, and the factor(s) responsible for inactivation are cytosolic.

cdc2 kinase inactivation in cell-free extracts was dependent on the cell cycle stage used to isolate soluble inactivator(s). Cytosol from early G_1 phase CHO cells did not posess endogenous cdc2 kinase activity, and inactivated p34^{cdc2} associated with the mitotic p1 fraction (Fig. 2B). In contrast, cytosol from G_2 phase cells also lacked cdc2 kinase activity, but did not inactivate the p1-associated kinase (Fig. 2B). That the cdc2 kinase is inactivated by early G_1 phase cytosol should not be surprising, since inac-

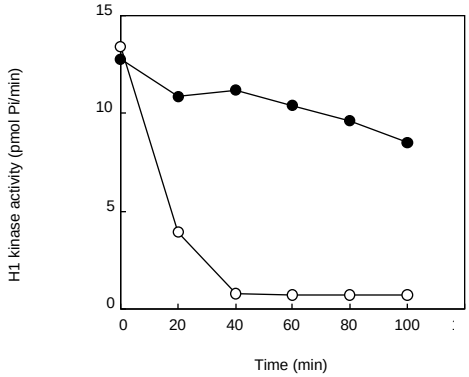

Fig. 1. Time-course of H1 kinase inactivation in cells released from mitotic arrest. Nocodazole-blocked CHO cells were washed twice at 4˚C and resuspended to 2 × 10^6 cells/ml in medium (○) or medium containing 100 ng/ml nocodazole (●) at 37˚C. For each time-point, 1.0 ml of stirred cell suspension was pelleted, resuspended in 1.0 ml kinase assay buffer and lysed using brief sonication at 4˚C. Histone H1 kinase activity was determined for 3-fold diluted lysates as described in Materials and Methods.

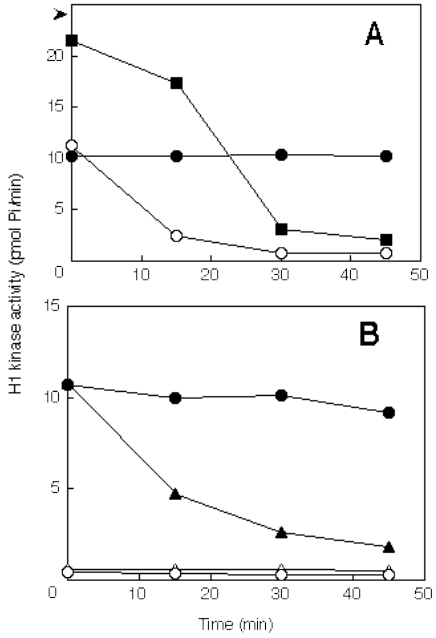

Fig. 2. (A) Subcellular distribution of H1 kinase and kinase inactivator(s) in mitotic CHO cells. A washed 1,000 ***g*** pellet (p1 fraction) was resuspended in homogenization buffer (●) or mitotic cytosol (■), and was incubated at 33˚C in the presence of an ATP-regenerating system (see Materials and Methods). In parallel, mitotic cytosol was similarly incubated without the p1 fraction (○). At intervals, aliquots of the incubations were diluted for quantitation of histone H1 kinase activity. Arrowhead indicates initial H1 kinase activity in the unfractionated homogenate. (B) Cell cycle dependence of H1 kinase inactivation in CHO cell extracts. H1 kinase activity was monitored as in (A) for cytosol isolated from G_2 phase (○) or G_1 phase (△) cells, and for the mitotic p1 fraction combined with G_2 phase (●) or G_1 phase (▲) cytosol.

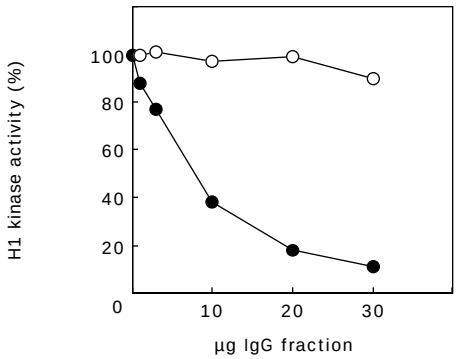

Fig. 3. Immunoadsorption of p1-associated H1 kinase. H1 kinase activity was quantitatively released from the p1 fraction of mitotic CHO cells in the presence of 250 mM KCl and was incubated with anti-$p34^{cdc2}$ antibodies (●) or control anti-human IgG antibodies (○). Plots show H1 kinase remaining after adsorption of the antibodies to fixed *S. aureus* cells (see Materials and Methods).

tivator(s) of MPF remain functional throughout early stages of interphase in HeLa cells (Adlakha et al., 1983) and frog embryos (Gerhart et al., 1984). A similar dependence of cdc2 kinase inactivation on the cell cycle in the cell-free system described here strongly argues against the possibility that it results from non-specific proteolysis.

A useful approach for studying how inactivation of the cdc2 kinase is regulated at the metaphase/anaphase transition is to compare early G_1 phase cytosol containing functional cdc2 kinase inactivator(s) with metaphase cytosol, in which the inactivators are inactive or absent. Unlike CHO cells, which return to interphase in the presence of nocodazole after 4-5 hours, HeLa cells can be arrested in a metaphase-like state for at least 24 hours (Zieve et al., 1980). I therefore tested whether or not p1-associated cdc2 kinase from mitotic CHO cells was inactivated by cytosol prepared from early G_1 phase and nocodazole-arrested mitotic HeLa cells. As shown in Table 1, mitotic HeLa cytosol inactivated the cdc2 kinase at only 9% of the rate obtained with cytosol from early G_1 phase cells. Inactivation of p1-associated $p34^{cdc2}$ also was negligible with cytosol from G_2 phase cells. Thus, cytosol from mitotic and early G_1 phase HeLa cells contain inactive and active forms of a cell cycle-regulated $p34^{cdc2}$ inactivating system.

Characterization of cdc2 kinase inactivation in vitro

In the cell-free system, p1-associated cdc2 kinase was inactivated at a rate proportional to the concentration of soluble inactivator(s). For all dilutions of G_1 phase HeLa cytosol tested, each individual timecourse of inactivation followed pseudo first-order kinetics (Jencks, 1969), so that log(H1 kinase activity) vs time was linear (Fig. 4). Rates of inactivation, determined from the slope of these plots, increased with the concentration of cytosol, verifying that the assay conditions quantitatively measure differences in activity of the $p34^{cdc2}$ inactivating system (data not shown).

In vivo, cdc2 kinase inactivation is tightly coupled to the proteolytic destruction of B-type cyclins at anaphase onset (Evans et al., 1983; Ghiara et al., 1991; Gallant and Nigg, 1992). The cell-free system reproduces this property of the inactivation process, as demonstrated by immunoblot analysis of samples taken during the course of inactivation in vitro. While the absolute level of $p34^{cdc2}$ remains constant, cdc2 protein kinase activity decreases concomitant with the disappearance of cyclin B (Fig. 5).

Luca and Ruderman (1989) have reported that the

Table 1. cdc2 kinase inactivation induced by HeLa cytosol

Phase of cell cycle	Endogenous H1 kinase activity (% of maximum)	Rate of cdc2 kinase inactivation (% of maximum)
G_2	19 ± 4	4 ± 4
M	100 ± 7	9 ± 1
G_1	3 ± 1	100 ± 20

The p1 fraction from mitotic CHO cells was incubated for 25 minutes at 33˚C with cytosol from G_2, mitotic, or G_1 phase HeLa cells in the presence of an ATP regenerating system. Aliquots were periodically withdrawn from the incubations and assayed to determine cdc2 H1 kinase activity.

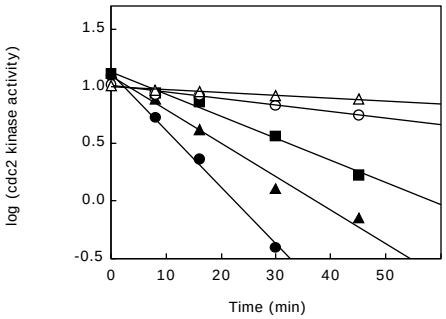

Fig. 4. Kinetics of cdc2 kinase inactivation in vitro. Identical aliquots of p1-associated cdc2 kinase were inactivated using undiluted cytosol from early G_1 phase HeLa cells (●), or cytosol diluted to 70% (▲), 50% (■), 30% (○) or 15% (△) strength with homogenization buffer. For each dilution, the logarithm of cdc2 kinase activity is plotted as a function of time.

destruction of clam cyclins A and B in vitro requires ATP and Mg^{2+}. To determine whether inactivation of cdc2 protein kinase activity requires a source of energy in the mammalian cell-free system described here, ATP and other low molecular mass components were removed from early G_1 phase HeLa cytosol by chromatography on Sephadex G-25. The depleted cytosol was then incubated at 33˚C with the p1 fraction from mitotic CHO cells. As shown in Table 2, inactivation was observed only when Mg^{2+} and ATP were present in the incubation: the cdc2 kinase remained active in the absence of additions, or in the presence of 5 mM EDTA. Inactivation also was not supported by the non-hydrolyzable ATP analog, AMP-PNP, by ATPγS, or by GTP.

Since protein phosphatase activity (Doonan and Morris, 1989; Picard et al., 1989; Axton et al., 1990; Kinoshita et al., 1990; Fernandez et al., 1992) and cdc2 kinase inactivation (Murray et al., 1989) are required for anaphase onset, it was of interest to know whether protein dephosphorylation was necessary for cdc2 kinase inactivation in the cell-free system. Sodium fluoride was used to inhibit all serine/threonine protein phosphatases, okadaic acid (Bialojan and Takai, 1988) and microcystin-LR (Honkanen et al., 1990; MacKintosh et al., 1990) were used to specifically inhibit type 1 and type 2A serine/threonine phosphatases, and sodium vanadate was employed as an inhibitor of tyrosine phosphatases (Swarup et al., 1982; Nelson and Branton, 1984) during inactivation of p1-associated cdc2 kinase mediated by early G_1 phase cytosol. As shown in Table 2, kinase inactivation was strongly inhibited by 25 mM sodium fluoride, 1 μM okadaic acid and 0.5 μM microcystin-LR, indicating that the dephosphorylation of serine or threonine residues by protein phosphatase 1 or 2A is an essential step in the inactivation process. Tyrosine dephosphorylation is not required, since kinase inactivation was not inhibited by 100 μM sodium vanadate.

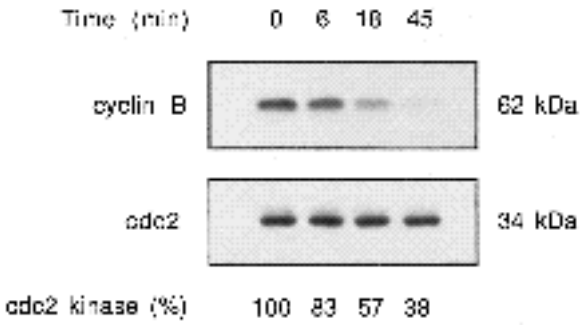

Fig. 5. Cyclin B is selectively destroyed during cdc2 kinase inactivation in vitro. Western blots were used to monitor levels of cyclin B and $p34^{cdc2}$ during inactivation of p1-associated cdc2 kinase induced by 60% strength G_1 phase HeLa cytosol. Diluted cytosol was employed to obtain slower kinetics of kinase inactivation, thereby facilitating immunoblot analysis of intermediate stages. cdc2 protein kinase activity is given for each time-point as the percentage of initial activity. Kinase activity remaining at 45 minutes correlates with a low level of cyclin B visible in longer autoradiographic exposures of the cyclin immunoblot.

cdc2 kinase inactivation requires multiple protein factors

As the first step towards identifying individual components of the cdc2 kinase inactivating system, early G_1 phase cytosol was separated into 0-35% and 35-55% $(NH_4)_2SO_4$-insoluble fractions. While neither fraction induced significant inactivation of the p1-associated cdc2 kinase when assayed separately, a 1:1 mixture of the two exhibited a rate of inactivation that was 3.5-fold greater than the sum of individual rates (Fig. 6A). This synergistic effect was not observed when the concentration of either individual fraction was doubled (data not shown). The combined fractions reconstituted 56% of the inactivation activity present in unfractionated cytosol (Fig. 6A); and addition of 55% $(NH_4)_2SO_4$-soluble material to the mixture did not stimulate inactivation further (data not shown). Therefore, it appears that the 0-35% and 35-55% fractions contain all cytosolic components of the cdc2 kinase inactivating system. Neither fraction is present in excess in these assays, since the rate of inactivation decreases when either is diluted (Fig. 6B). The combined $(NH_4)_2SO_4$ fractions also

Table 2. cdc2 kinase inactivation requires MgATP and serine/threonine phosphatase activity

Addition	Rate of cdc2 kinase inactivation (% of control)
5 mM ATP / 5 mM $MgCl_2$ (control)	100
No addition	13
5 mM ATP / 5 mM EDTA	2
5 mM AMP-PNP / 5 mM $MgCl_2$	11
5 mM ATPγS / 5 mM $MgCl_2$	7
5 mM GTP / 5 mM $MgCl_2$	17
1 μM okadaic acid / 5 mM MgATP	7
0.5 μM microcystin-LR / 5 mM MgATP	3
25 mM NaF / 5 mM MgATP	5
10 μM Na_3VO_4 / 5 mM MgATP	132
100 μM Na_3VO_4 / 5 mM MgATP	156

Following chromatography on Sephadex G-25 to remove endogenous nucleotides, early G_1 phase HeLa cytosol was incubated with the p1 fraction from mitotic CHO cells for 20 minutes at 33˚C in the presence of additions as shown.

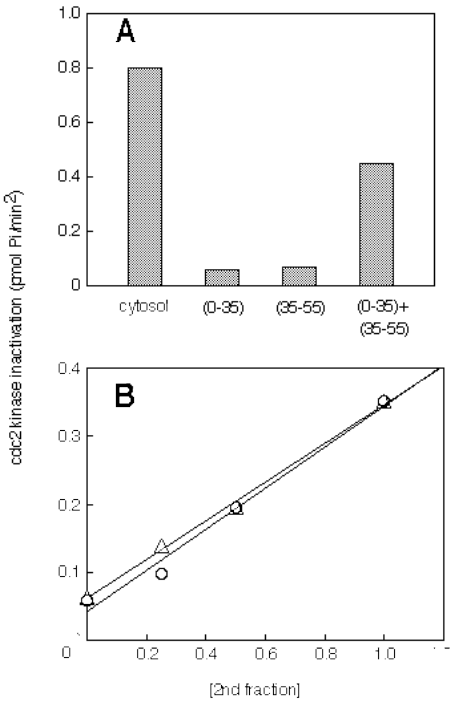

Fig. 6. $(NH_4)_2SO_4$ fractionation of cdc2 kinase inactivating factors. (A) 0-35% and 35-55% $(NH_4)_2SO_4$-insoluble fractions were isolated from 1 volume of G_1 phase HeLa cytosol and were resuspended in 0.5 volume of dialysis buffer. Inactivation of p1-associated cdc2 kinase subsequently was determined for cytosol, and for dialyzed 0-35% and 35-55% fractions mixed 1:1 with dialysis buffer or with each other. (B) The concentration of 0-35% (○) or 35-55% (△) fraction was held constant while the complimentary fraction was diluted.

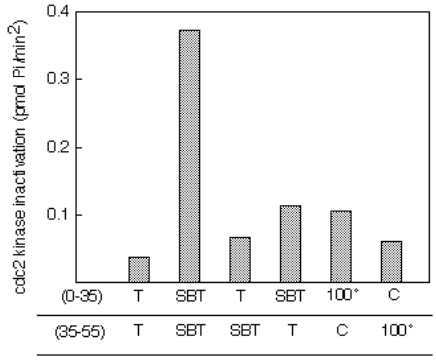

Fig. 7. cdc2 kinase inactivators are heat-labile proteins. 0-35% and 35-55% $(NH_4)_2SO_4$ fractions (isolated from G_1 phase HeLa cytosol) were supplemented with 1.2 mg/ml soybean trypsin inhibitor before (SBT) of after (T) incubation with 0.6 mg/ml trypsin for 25 minutes at 33˚C. Additional aliquots of the fractions were not pretreated (C), or were heated to 100˚C for 5 minutes (100˚). Following pretreatment, fractions were assayed for inactivation of p1-associated cdc2 kinase.

reconstitute the same mechanism of cdc2 kinase inactivation that G_1 phase cytosol provides: inactivation requires Mg^{2+} and ATP, is blocked by okadaic acid and microcystin-LR, and coincides with the degradation of cyclin B (data not shown).

cdc2 kinase inactivator(s) in the 0-35% and 35-55% $(NH_4)_2SO_4$ fractions are heat-labile proteins. Kinase inactivation was dramatically reduced when either fraction was heated to 100˚C, or was pretreated with trypsin (Fig. 7). In control experiments, trypsin had no effect when soybean trypsin inhibitor was present during pretreatment (Fig. 7).

Since cdc2 kinase inactivation is blocked by serine/threonine phosphatase inhibitors (Table 2), experiments were conducted to identify component(s) of the cell-free system that have to be dephosphorylated for inactivation to take place. Early G_1 phase cytosol was combined with the mitotic p1 fraction and was pre-incubated for 15 minutes at 33˚C in the presence of ATP or ATPγS, which promotes the stable thiophosphorylation of proteins by protein kinases (Eckstein, 1975). The p1 was then recovered by centrifugation and the cytosol resolved into 0-35% and 35-55% $(NH_4)_2SO_4$ fractions. As shown in Fig. 8A, 0-35% and 35-55% fractions derived from the pre-incubation with ATPγS inactivated fresh (not pre-incubated) cdc2 kinase at essentially the same rate as did control $(NH_4)_2SO_4$ fractions from the pre-incubation with ATP. In contrast, pre-incubating the p1 fraction with ATPγS inhibited subsequent inactivation of p1-associated cdc2 kinase by fresh G_1 phase cytosol (Fig. 8B). These results indicate that the inactivation of p1-associated cdc2 kinase requires dephosphorylation of one or more proteins in the p1. A protein kinase capable of thiophosphorylating these sites also is present in the p1, since cdc2 kinase inactivation was inhibited when the p1 fraction was pre-incubated solely with ATPγS and buffer (no cytosol) (Fig. 8B).

No increase in cdc2 kinase activity was observed when a 5 fold excess of ATPγS was added to incubations in which p1-associated cdc2 kinase had been inactivated by G_1 phase cytosol in the presence of ATP (data not shown). Thus, ATPγS does not promote higher levels of kinase activity in these experiments by converting an inactive precursor of $p34^{cdc2}$ to an active form, or by reactivating $p34^{cdc2}$ after it has been inactivated.

Differential regulation of factors mediating cdc2 kinase inactivation

To study how the inactivation of $p34^{cdc2}$ is regulated during mitosis, 0-35% and 35-55% $(NH_4)_2SO_4$ fractions were isolated from early G_1 phase HeLa cytosol, which contains a functional kinase inactivating system, and from nocodazole-arrested mitotic HeLa cytosol, which does not exhibit inactivation. Combinations of the fractions were then tested for inactivation of cdc2 kinase associated with the mitotic p1. As expected, inactivation was not observed for any 0-35% or 35-55% fraction assayed separately, nor for a mixture of the two fractions derived from mitotic cells (Fig. 9A). Inactivation did take place with combined 0-35% and 35-55% fractions from early G_1 phase cells, and with G_1 phase 0-35% plus mitotic 35-55% (Fig. 9A), indicating that the 35-55% component is active in nocodazole-arrested cells. The 0-35% component is active only in the early G_1 phase cells released from mitotic arrest, since mitotic 0-35% plus G_1 phase 35-55% did not inactivate the cdc2 kinase (Fig. 9A).

If cdc2 kinase inactivator(s) in the 35-55% $(NH_4)_2SO_4$

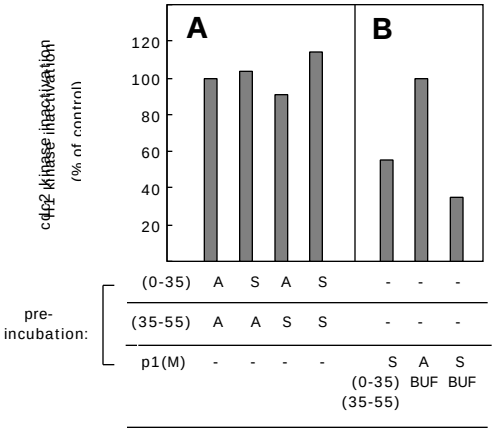

Fig. 8. (A) Pre-incubation of cdc2 kinase inactivators with ATPγS. A mixture of mitotic p1 and G_1 phase HeLa cytosol was incubated for 15 minutes at 33˚C in the presence of 5 mM MgATP (A) or 5 mM MgATPγS (S). After removing the p1 by centrifugation (10 minutes at 180,000 ***g***), supernatants were separated into 0-35% and 35-55% $(NH_4)_2SO_4$ fractions, which were used to inactivate fresh mitotic p1 in the presence of MgATP. (B) Pre-incubation of p1-associated cdc2 kinase with ATPγS. The mitotic p1 fraction was incubated with 0-35% and 35-55% $(NH_4)_2SO_4$ fractions [(0-35) + (35-55)], or dialysis buffer (BUF), in the presence of MgATP (A) or MgATPγS (S) as above. The p1 fractions were then recovered by centrifugation (5 minutes at 1,000 ***g***), washed with 10 volumes of dialysis buffer, and used as substrates for cdc2 kinase inactivation mediated by fresh G_1 phase $(NH_4)_2SO_4$ fractions.

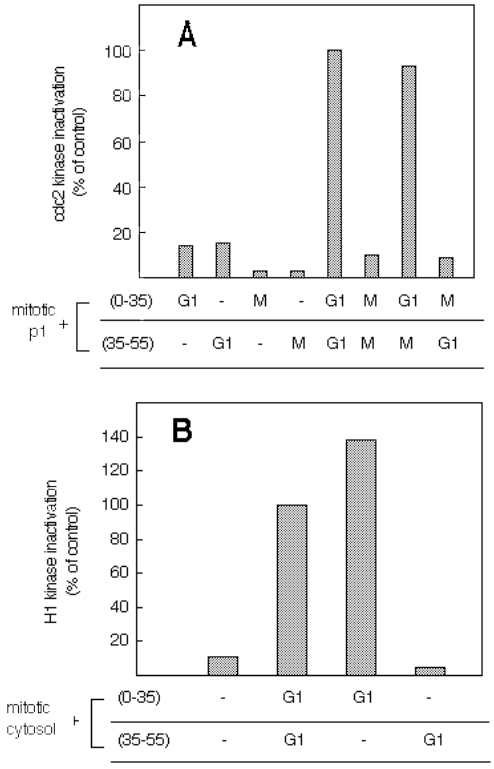

Fig. 9. Cell cycle regulation of cdc2 kinase inactivators. (A) 0-35% and 35-55% $(NH_4)_2SO_4$ fractions from nocodazole-arrested mitotic (M) or early G_1 phase (G1) HeLa cells were tested for inactivation of p1-associated cdc2 kinase. (B) G_1 phase 0-35% and 35-55% $(NH_4)_2SO_4$ fractions were added to mitotic HeLa cytosol containing soluble mitosis-specific H1 kinase activity.

fraction are constitutively active in nocodazole-arrested mitotic cells and in early G_1 phase cells, addition of the G_1 phase 0-35% $(NH_4)_2SO_4$ fraction to mitotic cytosol should trigger inactivation of cytosolic cdc2 H1 kinase activity. As shown in Fig. 9B, the G_1 phase 0-35% fraction was as effective as combined G_1 phase 0-35% and 35-55% fractions for inducing cdc2 H1 kinase inactivation in mitotic cytosol. Inactivation was not triggered by the G_1 phase 35-55% fraction; confirming the finding that component(s) of the inactivating system that partition in the 0-35% fraction are not functional in nocodazole-arrested HeLa cells (Fig. 9B).

Together, the data presented in Fig. 9 demonstrate that inactivation of particulate and soluble cdc2 kinase activity requires the action of at least two cytosolic proteins. One protein, present in a 35-55% $(NH_4)_2SO_4$ fraction, is constitutively active in nocodazole-arrested mitotic HeLa cells and in early G_1 phase cells. The second protein partitions into a 0-35% $(NH_4)_2SO_4$ fraction and is active only in the G_1 phase population released from metaphase arrest.

DISCUSSION

I have developed a cell-free system to identify factors that inactivate the cdc2 protein kinase during mitotic anaphase in mammalian cells. The design of this system is based on the finding that 50% of the cdc2 kinase activity in metaphase-arrested CHO cells is associated with a 1,000 ***g*** particulate fraction (p1), while factors that inactivate the kinase are cytosolic. Consequently, the p1-associated cdc2 kinase is stable in vitro and can be employed as a substrate to determine the functional status of soluble inactivators isolated at different stages of the cell cycle. Functional inactivators, exhibiting no cdc2 kinase activity, are isolated from early G_1 phase cells that already have inactivated endogenous $p34^{cdc2}$.

A major finding of this investigation is that at least two distinct cytosolic proteins are needed to inactivate the cdc2 kinase in vitro. Since inactivation also requires MgATP and the action of protein phosphatase 1 or 2A (PP-1 or PP-2A), one soluble inactivator may be a protein phosphatase and the second may be an ATP-dependent enzyme, such as a protein kinase, ATP-dependent protease, or mediator of ubiquitin attachment to cyclin (Glotzer et al., 1991). Alternatively, it is conceivable that the phosphatase or ATP-dependent protein is associated with the p1 fraction; in which case at least three factors are involved in the inactivation process. However, soluble cdc2 kinase is inactivated in the absence of p1 (Fig. 2A), so that inactivator(s) associated with the p1 are soluble as well.

It seems likely that PP-1 is the protein phosphatase involved in cdc2 kinase inactivation, since genes encoding PP-1 are required for anaphase onset in many organisms (Doonan and Morris, 1989; Axton et al., 1990; Kinoshita et al., 1990). In addition, microinjected anti-PP-1 antibod-

ies stabilize M phase-promoting factor (MPF) in meiotic starfish oocytes (Picard et al., 1989), and prevent exit from mitosis in mammalian fibroblasts (Fernandez et al., 1992). In skeletal muscle, PP-1 is activated in a MgATP-dependent manner resulting from multi-site phosphorylation of an associated regulatory subunit identified as phosphatase inhibitor-2 (Yang et al., 1981; Villa-Moruzzi et al., 1984; DePaoli-Roach, 1985; Vandenheede and Merlevede, 1985). If a similar mechanism regulates PP-1 in HeLa cells, MgATP may be required for $p34^{cdc2}$ inactivation because it is needed to activate PP-1.

In HeLa cells, both cytosolic proteins required for cdc2 kinase inactivation are functional in late mitotic and early G_1 phase cells, where inactivation of endogenous $p34^{cdc2}$ is ongoing or has been completed recently. One of these proteins also is functional in nocodazole-arrested metaphase cells that have not yet initiated kinase inactivation. This constitutively active factor may be the protein phosphatase involved in inactivation, since the activities of PP-1 and PP-2A are identical in mitotic and early G_1 phase extracts, and do not change after incubating these extracts at 33˚C in the presence of MgATP (data not shown). In fission yeast, the activities of PP-1 and PP-2A remain constant throughout the cell cycle (Kinoshita et al., 1990), and neither the abundance nor activity of PP-1 changes over the course of the cell cycle in rat fibroblasts (Brautigan et al., 1991). However, it is also possible that the inactivation of $p34^{cdc2}$ is regulated by changes in the action of a minor phosphatase species that accounts for only a small fraction of the activity detected in these assays. Indeed, mutations in one of four genes encoding PP-1 are sufficient to induce metaphase arrest in *Drosophila* (Axton et al., 1990).

The results of incubating the p1 fraction with ATPγS (Fig. 8) indicate that one or more proteins in the p1 have to be dephosphorylated in order for the p1-associated cdc2 kinase to be inactivated. A protein kinase capable of thiophosphorylating these sites also is present in the p1, since p1-associated cdc2 kinase is stabilized against inactivation when the p1 is pre-incubated solely with ATPγS and buffer (no cytosol). It may be that cyclin, which is phosphorylated by $p34^{cdc2}$ during entry into M phase (Pondaven et al., 1990), has to be dephosphorylated before it can be destroyed and $p34^{cdc2}$ inactivated at anaphase onset. Alternatively, inactivation may require dephosphorylation of $p34^{cdc2}$ at Thr161. Phosphorylation of this site is an essential step in cdc2 kinase activation (Solomon et al., 1992), and dephosphorylation results in a loss of kinase activity in vitro (Lee et al., 1991). The present study does not rule out the possibility that cdc2 kinase inactivation involves phosphorylation of $p34^{cdc2}$ at threonine 14 and/or tyrosine 15; however, in mammalian cells inactivation precedes tyrosine phosphorylation of $p34^{cdc2}$ by several hours (Morla et al., 1989).

It has been reported that cdc2 kinase activity is required for cyclin degradation and $p34^{cdc2}$ inactivation in cell-free extracts of *Xenopus* eggs (Felix et al., 1990). In that study, addition of purified $p34^{cdc2}$-cyclin complex to interphase extracts resulted in the destruction of cyclin after a lag phase lasting 15-25 minutes, implying that the kinase initiated a chain of events leading to the appearance of functional kinase inactivators. In the present investigation, the use of extracts from early G_1 phase cells in which cdc2 kinase inactivators already are functional undoubtedly bypasses some of these events: inactivation of p1-associated cdc2 kinase begins immediately after adding G_1 phase cytosol and warming to 33˚C. This simplified cell-free approach should facilitate the biochemical dissection of mechanisms controlling the metaphase/anaphase transition in mammalian cells.

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