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Evidence for a Pre-Restriction Point Cdk3 Activity

Susan M. Keezer and David M. Gilbert*

Department of Biochemistry and Molecular Biology, S.U.N.Y. Upstate Medical University, 750 East Adams Street, Syracuse, New York 13210

Abstract We have examined the activity of cyclin-dependent kinase 3 (cdk3) during G1-phase of the cell cycle in Chinese Hamster Ovary (CHO) fibroblasts. Histone H1 kinase activity associated with anti-cdk3 immunoprecipitates peaked during a brief window of time, 2–3 h prior to the restriction point. In vitro cdk3 activity was sensitive to roscovitine, a drug previously shown to inhibit cdks 1, 2, and 5, but not cdk4 or 6. Early G1-phase activation of cdk3 was downregulated by treatment of cells with MG132, an inhibitor of the proteasome, and by the protein synthesis inhibitor cycloheximide. These results provide evidence for a pre-restriction point cdk3 activity that requires both the synthesis of a regulatory subunit and degradation of an inhibitor. J. Cell. Biochem. 85: 545–552, 2002.

Key words: cell-cycle; cdk3; restriction point; origin decision point; G1-phase

Mammalian cell cycle progression is dependent, in part, on the tightly regulated activity of several cyclin dependent kinases (cdks). In G1-phase, intra-and extracellular signals provide an environment in which these enzymes become active to allow the cell's entry into S-phase. Opposing signals inhibit cdk activity, thus causing cell cycle arrest (and eventually quiescence) if the conditions are not ideal for proliferation. Cyclin D/cdk4/6 activity occurs in mid-late G1-phase, upstream of cdk2/cyclin E activity [Draetta, 1994; Pines, 1999]. Both of these activities are required for hyperphosphorylation of the retinoblastoma gene product (pRb). Rb phosphorylation leads to transcriptional activation of S-phase promoting genes and is indicative of the cell's commitment to enter S-phase [Hinds et al., 1992; Lundberg and Weinberg, 1998].

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*Correspondence to: David M. Gilbert, Department of Biochemistry and Molecular Biology, S.U.N.Y. Upstate Medical University, 750 East Adams Street, Syracuse, New York 13210. E-mail: gilbertd@mail.upstate.edu

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The replication origin decision point (ODP) is an early G1-phase event through which Chinese hamster ovary (CHO) cells must pass to become capable of utilizing the physiological origin of replication at the dihydrofolate reductase (DHFR) locus [Wu and Gilbert, 1996]. The ODP takes place after the formation of pre-replication complexes (Pre-RCs) and prior to the restriction point [Wu and Gilbert, 1997; Okuno et al., 2001], and is independent of the usual cellular signaling pathways required for cell growth [Wu and Gilbert, 1997; Keezer and Gilbert, 2002]. However, passage through the ODP is inhibited by the protein kinase inhibitors roscovitine and olomoucine, both selective inhibitors of cdks 1, 2, and 5 [Meijer et al., 1997]. This was surprising since cdk1 is activated at the end of S-phase, and is required for passage through mitosis [Clute and Pines, 1999], cdk2 is activated at the R-point [Hengstschlager et al., 1999; Ekholm and Reed, 2000; Ezhevsky et al., 2001], and cdk5 has been found in active form only in neuronal cells [Moorthamer et al., 1999]. Cyclin-dependent kinase 3 (cdk3), however, has not been tested for inhibition by roscovitine and olomoucine, but has a high degree of homology to cdk2 [Meyerson et al., 1992]. Cdk3 may have a unique function in G1-phase, as the arrest brought about by a kinase-dead dominant negative form of the protein [Hofmann and Livingston, 1996b; Braun et al., 1998c] could be rescued by wild type cdk3, but not wild type

cdk2 [van den Heuvel and Harlow, 1993]. The function and cyclin partner(s) of this enzyme, however, remain elusive. Although it can be activated by either cyclin E or A in vitro [Harper et al., 1995; Meikrantz and Schlegel, 1996], cdk3 does not appear to be active at times when these cyclins are expressed. The cip/kip family of cdk inhibitors (CKIs) may be involved in the regulation of cdk3 activity, as p21 and p27 can inhibit the enzyme in vitro, and p27 has been shown to interact with inactive cdk3 in vivo [Harper et al., 1995; Braun et al., 1998b]. Cdk3 may function in the induction of cell cycle regulated gene transcription, as it can activate the transcription factor E2F1, in an Rb-independent manner [Hofmann and Livingston, 1996a; Braun et al., 1998a].

We were interested in determining whether cdk3 might exhibit elevated activity prior to the restriction point, at or near the ODP. Here, we found that Cdk3 is sensitive to roscovitine and has a peak of histone H1 kinase activity 3–4 h after mitosis in CHO cells, which corresponds to the timing of the ODP in these cells. This activity was inhibited when cells were treated in culture with the proteasome inhibitor MG132, indicating that degradation (possibly of a CKI) is required for peak activity. MG132 also inhibits passage through the ODP [Keezer and Gilbert, 2002]. However, we were able to uncouple this activity from the ODP with cycloheximide, which inhibited activation of cdk3, but does not inhibit the ODP [Keezer and Gilbert, 2002]. This result suggests that the de novo synthesis of a protein(s), possibly a regulatory subunit (cyclin), is required for early G1-phase cdk3 activity. Together, our results provide evidence for the existence of a pre-restriction point cdk3 activity, but this activity is not necessary for passage through the ODP.

MATERIALS AND METHODS

Cell Culture and Synchronization

CHOC 400 cells are a derivative of CHO cells containing approximately 1,000 tandemly integrated copies of a 243-kb segment of the DNA surrounding the DHFR gene [Hamlin et al., 1994] and were cultured as described [Wu et al., 1997]. Cells were synchronized in metaphase by incubation with 0.05 μg/ml nocodozole for 4 h followed by isolation of metaphase cells by mitotic shake-off as described [Gilbert et al., 1995]. Cells were then frozen in complete medium plus 10% DMSO at a concentration of 5×10^6/ml. Frozen cells were thawed and re-plated for experiments in DMEM + 5% FBS at a concentration of 3.3×10^5 cells/ml. For G1/S-phase synchrony, cells in mitosis were plated to fresh medium to which 5 μg/ml aphidicolin was added at 1–2 h after plating, and cells were collected at 16–18 h after plating.

Cell Lysis and Immunoprecipitation

CHOC 400 cells were washed twice with cold (4°C) PBS and lysed on culture dishes with cold (4°C) RIPA lysis buffer (10 mM Na_2HPO_4, pH 7, 300 mM NaCl, 0.1% SDS, 1.0% Nonidet P40, 1.0% deoxycholic acid, 2 mM EDTA) supplemented with 10 μl/ml mammalian protease inhibitor cocktail (Sigma), 1 mM PMSF, 500 μMNa_3VO_4, and 50 mM NaFl. A volume of 250 μl lysis buffer was added to a 100-mm dish with approximately 5×10^6 cells. The cells were then scraped off the dish and the resulting lysate transferred to a chilled 1.5-ml tube. Lysates were sonicated on ice (fifteen 1-s pulses) and cleared by centrifuging for 10 min at 4°C in a microcentrifuge at full speed. The pellet was discarded and protein concentration was determined in the supernatant (Pierce Coomassie Plus protein assay kit). For each immunoprecipitation, 1 mg/500 μl of total protein was incubated on ice for 2 h with 1 μg antibody. Antibodies used were rabbit polyclonal anti-cdk3 (Neomarkers), anti-cdk2 (Santa Cruz), and anti-GFP (Santa Cruz). Next, 20 μl of a 50% slurry of protein A-sepharose beads (Sigma) was added to each reaction and the mixture was incubated at 4°C rotating for 1 h. The beads were then washed three times with lysis buffer. For analysis by Western blot, 30 μl sample buffer (80 mM Tris-HCl, pH 6.8, 2.0% SDS, 15% glycerol, 0.1% bromophenol blue, 100 mM DTT) was added to the beads. Beads were boiled for 5 min before the supernatant was subjected to SDS–PAGE.

Kinase Activity Assay

Cells were lysed and immunoprecipitations were carried out as described above. Following washes with lysis buffer, the beads were washed twice with equilibration buffer (50 mM HEPES, pH 7.4, 1 mM DTT). In some experiments, inhibitors were added at this point and incubated with the immunoprecipitate on ice for 10 min. Thirty microliters kinase reaction

buffer (50 mM HEPES, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$, 50 μM ATP, 0.5 μg histone H1, 0.5 μCi $^{32}P\gamma$-ATP) was added to each immunoprecipitate. Reactions were incubated at 37°C for 25 min with gentle mixing every 2 min. Reactions were stopped by adding 30 μl 2 × SDS sample buffer. Samples were boiled for 5 min, spun briefly in a microcentrifuge to pellet the beads, and the supernatant was loaded onto a 12% gel for SDS–PAGE. The resulting gel was dried at 80°C for 2 h and exposed to a Molecular Dynamics phosphorimager screen. The images were analyzed with Imagequant.

Western Blotting

SDS–polyacrylamide gels were transferred via BioRad semi-dry transfer apparatus to New England Nuclear Polyscreen PVDF transfer membrane according to the manufacturers' instructions. Polyclonal anti cdk3 (Neomarkers), polyclonal anti-cdk2 and cdk5, and monoclonal anti-p21 and p27 (Santa Cruz) were used at a dilution of 1:200. Polyclonal anti-cdk1 (generous gift of M. Pagano) was used at a dilution of 1:1,000. Monoclonal anti-PSTAIRE (Sigma) was used at a dilution of 1:2,000 after anti-cdk3 immunoprecipitation. Monoclonal anti-actin (Boehriger-Mannheim) was suspended at a concentration of 0.5 mg/ml in PBS + 0.1% sodium azide, and used at a dilution of 1:4,000. Secondary antibodies (anti-mouse and anti-rabbit horseradish peroxidase (HRP) conjugates (Sigma)) were used at a dilution of 1:4,000. Enhanced chemiluminescence (ECL) was carried out using Pierce Supersignal reagents according to the manufacturer's instructions.

Inhibitors

Roscovitine and olomoucine (Calbiochem) were suspended in DMSO at a concentration of 20 mM. MG132 (Biomol Research Laboratories) was dissolved at 50 mM in DMSO. Cycloheximide (Sigma) was dissolved at 10 mg/ml in ddH_2O. Aphidicolin (Calbiochem) was re-suspended in DMSO at 10 mg/ml.

RESULTS

Anti-cdk3 Immunoprecipitates a Roscovitine-Sensitive cdk Distinct From cdk1, 2, or 5

Rabbit polyclonal anti-cdk3 antibody recognized a CHO cell protein of the predicted apparent molecular weight of cdk3 (~36 kDa) that was totally solubilized by cellular lysis with RIPA buffer (Fig. 1A). Anti-cdk3 immunoprecipitates from this lysate exhibited histone H1 kinase activity that was approximately threefold higher than background levels seen with a mock immunoprecipitation with an anti-GFP antibody (Fig. 1B). This activity was sensitive to roscovitine in a dose-dependent fashion (Fig. 1B). Direct detection of cdk3 in immunoprecipitates using rabbit polyclonal anti-cdk3 antibody was not possible, as the signal from IgG bands in the immunoprecipitates was strong enough to mask the cdk3 signal. Since a monoclonal anti-cdk3 was not available, we used monoclonal anti-PSTAIRE to verify that the anti-cdk3 immunoprecipitates contained a 36-kDa cdk protein. The PSTAIRE sequence is conserved among cdks and distinguishes them from other protein kinases [Morgan, 1997; Tournier et al., 1997; Detivaud et al., 1999]. Anti-PSTAIRE antibody revealed four prominent bands in immunoblots of whole cell extracts (Fig. 1C), but none of these bands were of 36 kDa. It is likely that these bands correspond to molecules more abundant than cdk3, such as cdks 1 and 2. Cdk3 is known to be a very low abundance protein, and is probably masked by the signals of these other cdks. However, the only band recognized in the anti-cdk3 immunoprecipitates was ~36 kDa (Fig. 1C), indicating that a cdk of the same molecular weight as cdk3 is highly enriched in these anti-cdk3 immunoprecipitates. By contrast, no detectable cdk1, 2, or 5 could be found in these same immunoprecipitates (Fig. 1D). Together, these data strongly suggest that the roscovitine-sensitive histone H1 kinase activity detected in anti-cdk3 immunoprecipitates is cdk3 activity.

Cdk3-Asssociated Activity Peaks in Early G1-Phase

To evaluate cdk3 activity during G1-phase, cells were synchronized in mitosis by selective detachment, then released into G1-phase for various lengths of time. Cells were lysed, and the histone H1 kinase activity from either cdk3 or cdk2 immunoprecipitates was determined. Figure 2A shows that a peak of cdk3 activity was detected during early G1-phase that was greater than that observed at the G1/S border, whereas cdk2-associated activity was not detectable in early G1-phase, but was high at the G1/S-phase border, as expected. No activity was detected with anti-cdk5 immunoprecipitates (data not shown). Results of three independent

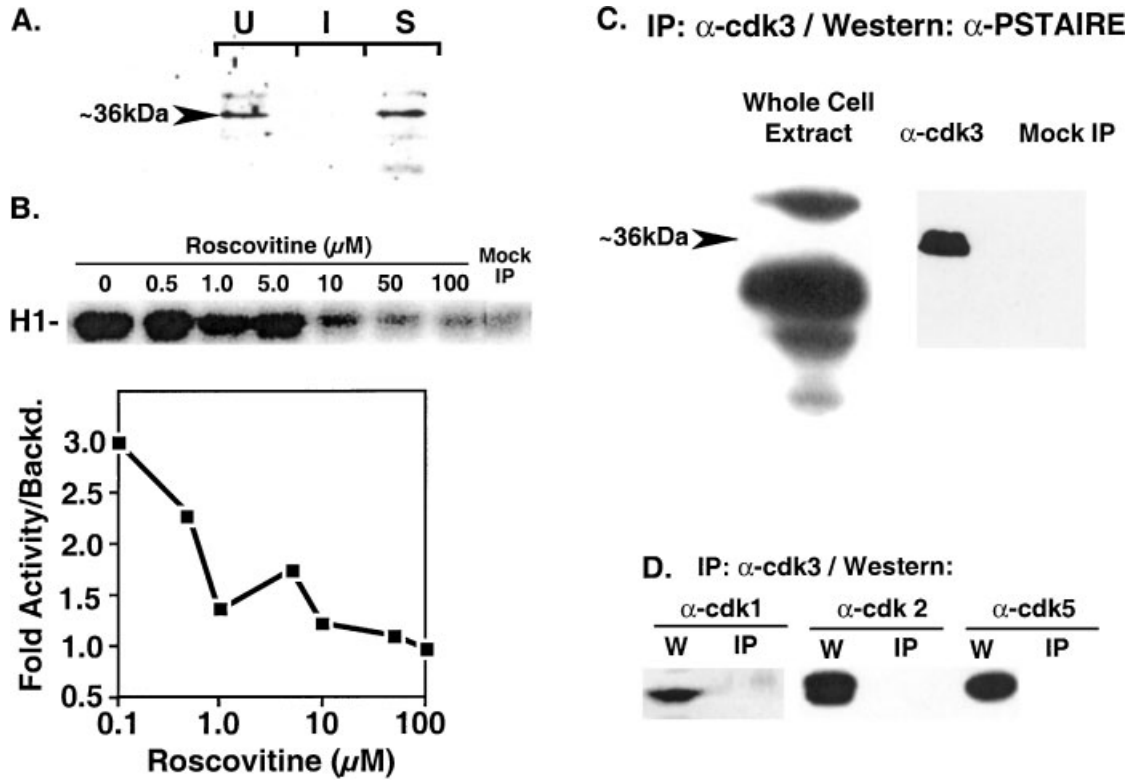

Fig. 1. Anti-cdk3 immunoprecipitates a cdk distinct from cdk1, 2, or 5. **A**: CHOC 400 cells were lysed in RIPA buffer (see Materials and Methods) and then part of the lysate was cleared by centrifugation. An equal number (2×10^5 cells) of cells corresponding to unfractionated (U), soluble (S), and insoluble (I) fractions were separated by SDS–PAGE, and immunoblots were probed with anti-cdk3 antibody. **B**: Cell lysates were immunoprecipitated with either anti-cdk3 or anti-GFP (Mock IP). Immunoprecipitates were incubated in the presence of the indicated concentration of roscovitine, and histone H1 kinase activity was assayed as described in Materials and Methods. The reactions were separated by SDS–PAGE, and the resulting gel was dried and exposed to a phosphorimager screen. Relative cpm were calculated using the Imagequant program, and ''Fold activity/background'' was obtained for each sample by normalizing to the cpm in the mock (anti-GFP) sample. **C**: Either whole cell extracts or anti-cdk3 immunoprecipitates were separated by SDS–PAGE and immunoblots were probed with anti-PSTAIRE antibody. Whole cell extract was included on the same gel, but a much shorter film exposure is shown relative to the anti-cdk3 and mock IP lanes. **D**: Either whole cell lysates (W) or anti-cdk3 immunoprecipitates (IP) were separated by SDS–PAGE, and immunoblots were probed with polyclonal anti-cdk1, 2, or 5.

experiments revealed that the peak of cdk3-associated H1 kinase activity was seen between 3 and 5 h after mitosis (Fig. 2B). This early G1-phase cdk3 activity was inhibited when roscovitine was included in the H1 kinase assay (Fig. 2C).

Proteasome Activity is Required for Early G1-Phase cdk3-Associated Activity

One means of regulation of cellular cdk activity is through natural cdk inhibitors (CKIs), including the cip/kip (p21, p27, p57) and ink4 (p15, p16) families [Vidal and Koff, 2000]. Since cdk3 activity has previously been shown to be inhibited by members of the cip/kip family [Harper et al., 1995; Braun et al., 1998b], whose levels are regulated by proteasome-dependent proteolysis [Pagano et al., 1995; Shirane et al., 1999], we tested whether an inhibitor of the proteasome would effect cdk3 activity. Cells were plated in mitosis, treated in culture with MG132 at 1 h after plating (virtually 100% of cells are in G1-phase by this time [Okuno et al., 2001]), and collected at 3.5–4.5 h after plating. Cdk3 was immunoprecipitated and its activity assayed as in Figures 1 and 2. MG132 inhibited cdk3 activity by greater than 50% (Fig. 3A), suggesting that a protein inhibitor of cdk3 is stabilized by MG132. It has been established that p27 can associate with and inhibit cdk3 activity [Braun et al., 1998b; Vidal and Koff, 2000], and is downregulated through ubiquitination-mediated proteolysis [Montagnoli et al., 1999; Nguyen et al., 1999] in late G1 and during S-phase [Slingerland and Pagano, 2000]. To determine whether p27 is proteasome regulated

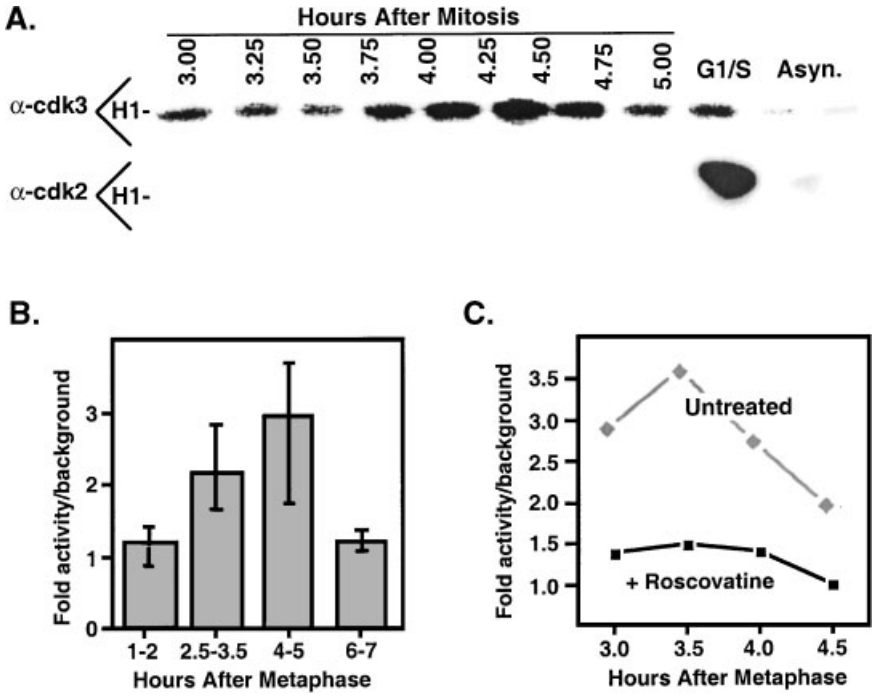

Fig. 2. Cdk3-associated activity peaks at 3–5 h after metaphase. **A**: CHOC400 cells were plated in mitosis and collected at the indicated times. For G1/S-phase cells, 5 μg/ml aphidicolin was added to cell medium at 2 h after mitosis. Cells were collected after 16 h. A sample of asynchronous cells (Asyn.) was also collected. Anti-cdk3 and anti-cdk2 immunoprecipitates from each cell population were analyzed for histone H1 kinase activity as in Figure 1. **B**: The fold H1 kinase activity over background for anti-cdk3 immunoprecipitates was normalized to mock (anti-GFP) immunoprecipitates as in Figure 1. Shown is the average of three independent experiments performed as in (A) with the standard deviation. **C**: Anti-cdk3 H1 kinase activity measured as in (A) and (B) except that half of each sample was treated with roscovitine during the in vitro kinase assay.

in early G1-phase, cells were treated at 1 h after mitosis with MG132 for a period of 2 h. As a control, cells were also treated with MG132 8 h after mitosis and collected 2 h later (50% of cells enter S-phase at 10 h after mitosis [Wu and Gilbert, 1997]). Steady-state levels of p27/kip were stabilized by MG132 at both time points, indicating that p27 levels are regulated by proteasome-mediated proteolysis during the period of time at which cdk3 activity rises to peak levels during early G1-phase.

Early G1-Phase cdk3-Associated Activity Requires De Novo Protein Synthesis

Cdk activation requires the binding of a regulatory partner (cyclin) to the catalytic subunit (cdk). Often, the regulatory subunit of a cyclin/cdk pair is synthesized in a cell cycle-dependent manner [Pines, 1999]. To determine whether the early G1-phase cdk3 activity is dependent on de novo protein synthesis, we treated cells with cycloheximide at 1 h after mitosis. This concentration is 10-fold higher than that required for inhibition of greater than 90% of protein synthesis in these CHO cells and is effective within 10 min of drug addition [Okuno et al., 2001]. Cells were collected at 3.5–4.5 h after mitosis and cdk3 activity was evaluated in immunoprecipitates as before. Cdk3 activity was reduced to near background levels in the cycloheximide-treated samples (Fig. 4). These data indicate that the de novo synthesis of a protein(s), possibly a cyclin partner, is required for cdk3 activation.

DISCUSSION

Here we show that G1-phase cdk3 activity peaks at 3–5 h after mitosis, prior to the restriction point in CHOC 400 cells [Wu and Gilbert, 1997]. This rise in cdk3 activity requires both proteasome-mediated proteolysis and protein synthesis, and is sensitive in vitro to roscovitine. A previous report detected a rise in cdk3 activity later in G1-phase and into S-phase [Braun et al., 1998b]. However, we found cdk3 levels to be reduced when cells were synchronized at the G1/S border with aphidicolin. We

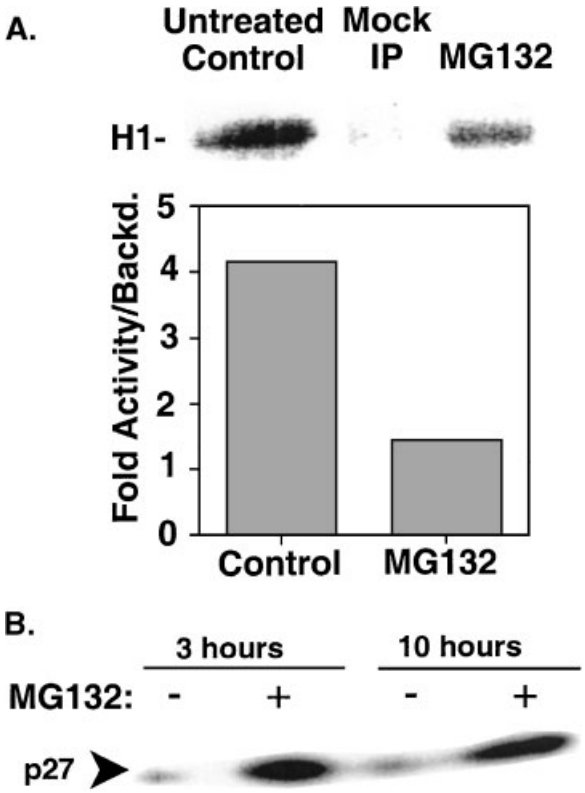

Fig. 3. Proteasome activity is required for early G1-phase cdk3-associated activity. **A**: CHOC400 cells were plated in mitosis and treated in culture with 10 μM MG132 or vehicle control at 1 h after plating. Cells were lysed at 4 h after plating, and immunoprecipitations, kinase assays, and analysis were performed as described in Materials and Methods. **B**: CHOC400 cells were plated in mitosis and treated with 10 μM MG132 or vehicle control at 1 or 8 h after plating. Cells were collected 2 h after treatment (3 or 10 h after mitosis), and whole cell lysates (2×10^5 cells) were separated by SDS–PAGE. Western blotting was carried out using monoclonal anti-p27/kip1. Similar results were obtained in three independent experiments.

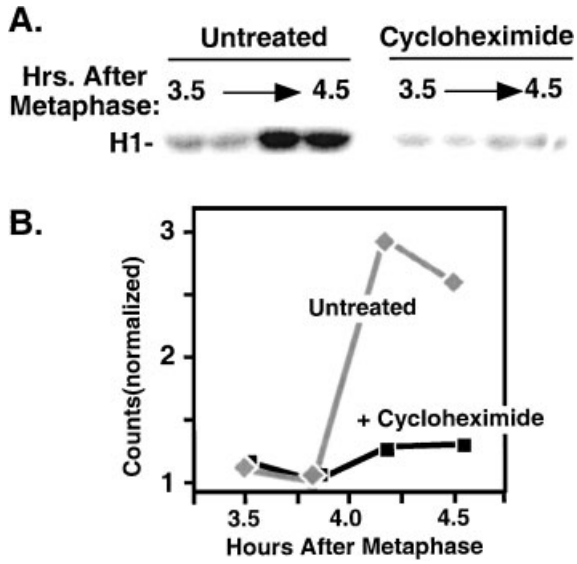

Fig. 4. Protein synthesis is required for cdk3-associated activity. **A**: CHOC400 cells were plated in mitosis and treated with 50 μg/ml cycloheximide 1 h after plating or left untreated. Cells were collected at 20-min intervals at 3.5–4.5 h after mitosis. Lysis, immunoprecipitation, kinase assays and analysis were carried out as described in Materials and Methods. **B**: Results from (A) were normalized by dividing counts from each sample by the lowest number for either cycloheximide treated or untreated samples. Similar results were obtained in three independent experiments.

suggest that this discrepancy may be due to different synchronization methods. The Braun et al. [1998b] report synchronized cells by centrifugal elutriation and by release from G0-phase by re-addition of serum to serum-starved cells. It is possible that the activity observed by this group was in early S-phase rather than late G1-phase. In fact, we detected a second increase in cdk3 activity when cells were allowed to proceed into S-phase cells (data not shown). In this report, we have focused on early G1-phase using mitotic selection.

Several lines of evidence indicate that the H1 kinase activity measured in this report is cdk3 activity. First, our anti-cdk3 antibody recognized a single band on Western blots that corresponds to the size of cdk3 and was completely solubilized in our immunoprecipitation conditions. Second, anti-PSTAIRE antibodies recognized a single band of protein in anti-cdk3 immunoprecipitates that also corresponded to the molecular weight of Cdk3. Third, the H1 kinase activity within these immunoprecipitates was sensitive to roscovitine, which is a selective inhibitor of cdk1, 2, and 5 [Meijer et al., 1997]. Cdk3, which had not previously been tested for sensitivity to roscovitine, is highly homologous to cdk2 [Meyerson et al., 1992] and anti-cdk3 immunoprecipitates did not contain detectable cdk1, 2, or 5. Finally, the activity measured in this report was sensitive to inhibitors of proteolysis and of protein synthesis, all consistent with properties of a cell-cycle regulated cyclin-dependent kinase activity.

The requirement for protein synthesis to induce cdk3 activity implies the existence of a cyclin partner. Although cyclin A or E can activate cdk3 in vitro [Harper et al., 1995; Meikrantz and Schlegel, 1996], it is unclear if either of these cyclins is required for cdk3 activation in the cell. A 70-kDa protein with cyclin homology was found to bind to cdk3 and to have a

G1-phase expression pattern as examined at the RNA level. The protein, $p70^{ik3-1}$, however, was unable to activate cdk3, leaving cdk3 yet without a known cyclin partner [Matsuoka et al., 2000]. Inhibition of cdk3 by proteasome inhibitors also suggests that cdk3 activity is regulated by a CKI. CKIs, particularly p27/kip1, are regulated by proteasome-dependent degradation. p27 has been shown to interact with inactive cdk3 [Braun et al., 1998b], and we demonstrate here that it can be stabilized in early G1-phase by inhibition of the proteasome. Of course, we cannot rule out the possibility that either or both of these inhibitors prevent the activation of cdk3 indirectly, through the inhibition of an upstream pathway.

Although cdk3 is required for entry into S-phase [van den Heuvel and Harlow, 1993], the function of this low-abundance protein is still unknown. It appears to be involved in E2F regulated transcription, and can bind E2F through its DP1 partner [Hofmann and Livingston, 1996a]. It has been shown that the G1-phase arrest brought about by dominant negative cdk3 can be overcome by wild type cdk3, but not by wild type cdk2 [van den Heuvel and Harlow, 1993], indicating that there is a unique function for cdk3 in G1-phase progression. Since its activity appears early in G1-phase, it is possible that cdk3 activity stimulates the transcription of genes required for events leading up to the restriction point, prior to the activation of cdk2, 4, and 6. Recently it was shown that many strains of laboratory mice (with a common progenitor) harbor a homozygous mutation that truncates cdk3 upstream of the conserved kinase domain [Ye et al., 2001]. Since the mice have no apparent defects, this finding indicates that cdk3 activity can be compensated for by another (as yet unidentified) activity. There is some precedent for this possibility, as cyclin E can compensate completely for cyclin D1 when transcribed from the cyclin D1 promoter (cyclin D1 knockout/cyclin E knockin) in mice [Geng et al., 1999]. Alternatively, the required function of cdk3 may be in a capacity other than a protein kinase activity. For example, D type cyclin/cdks participate in the activation of cdk2 by sequestering CKIs even after their role as a protein kinase is completed. Cdk3 would not be the first example of an enzyme whose essential function does not lie in its catalytic activity. The catalytic activity of the essential protein DNA polymerase ϵ is also dispensible for cell viability [Sugino, 1995; Kesti et al., 1999].

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