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$p55^{CDC25}$ is a nuclear protein required for the initiation of mitosis in human cells

(cell cycle/CDC2/CDC25/mitotic inducer)

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ABSTRACT **The *cdc25*$^+$ gene of fission yeast encodes a phosphotyrosine phosphatase that dephosphorylates tyrosine-15 of $p34^{cdc2}$ and thereby activates $p34^{cdc2}$/cyclin to bring about entry into M phase. We have recently cloned a human homolog, *CDC25*, which rescues the M-phase initiation defect of yeast *cdc25* temperature-sensitive mutants. Antibodies raised against the *CDC25* gene product specifically recognize human proteins of ≈55 and ≈52 kDa. Microinjection of affinity-purified anti-CDC25 antibodies into HeLa cells inhibits entry into mitosis. These observations suggest that the *CDC25* gene products are essential for the initiation of mitosis in human cells, similar to their homologs in fission yeast and *Drosophila*. *CDC25* gene products, like $p34^{CDC2}$, are localized primarily in the nucleus during interphase, suggesting that activation of $p34^{CDC2}$/cyclin by $p52/p55^{CDC25}$ occurs within the nucleus.**

The molecular events that trigger the onset of mitosis have for many years remained elusive. Recently, progress has been achieved primarily from two lines of research: the biochemical characterization of a protein complex called M-phase promoting factor that induces M phase in animal oocytes and genetic and molecular studies of cell cycle mutants in yeast (1). These studies have revealed that the onset of mitosis is brought about by a universally conserved serine/threonine protein kinase that consists of two subunits: a 34-kDa catalytic subunit encoded by the *cdc2*$^+$ gene and a 45- to 60-kDa regulatory subunit, which is member of the cyclin family of proteins (2).

The $p34^{CDC2}$/cyclin kinase is assembled in an inhibited state during interphase, and its activation is widely believed to be the critical step in the initiation of mitosis (1, 2). By using the genetically tractable fission yeast *Schizosaccharomyces pombe*, it has been possible to identify a number of proteins that are dose-dependent regulators of mitosis. The intracellular concentration of these proteins modulates the size at which cells initiate mitosis, thereby coordinating growth with cell cycle progression. The two most important dose-dependent regulators appear to be $p80^{cdc25}$ and $p107^{wee1}$ (3, 4). The *wee1*$^+$ product, $p107^{wee1}$, is a serine/tyrosine kinase that inhibits the initiation of mitosis by directly or indirectly promoting the phosphorylation of $p34^{CDC2}$ on tyrosine-15 and thereby rendering the kinase inactive (4–8). Acting in opposition to $p107^{wee1}$ is the $p80^{cdc25}$ mitotic inducer. Increased production of $p80^{cdc25}$ causes mitosis to initiate at an abnormally small size (3). In the absence of $p80^{cdc25}$, fission yeast cells arrest in late G_2 with an elongated phenotype and a tyrosine phosphorylated form of $p34^{cdc2}$ harboring low kinase activity (7, 9, 10). Recent evidence that purified cdc25 protein cannot only directly stimulate the dephosphorylation and activation of $p34^{CDC2}$/cyclin in enriched fractions (11) as well as purified complexes (12), but can also dephosphorylate *para*-nitrophenylphosphate and other protein substrates *in vitro*, has confirmed that it is a protein phosphatase (28). In wild-type cells, the level of cdc25 mRNA (13) and gene product $p80^{cdc25}$ (13, 14) increases during interphase, peaking at mitosis. Together these results show that activation of the $p34^{cdc2}$ kinase and the timing of mitosis in fission yeast is regulated by the cyclical accumulation of the $p80^{cdc25}$ mitotic inducer.

To determine whether the hierarchical gene network controlling mitotic entry in *S. pombe* is conserved among higher eukaryotes, we have identified homologs of the *cdc25*$^+$ gene by two strategies. A *cdc25*$^+$ homolog (*MIH1*) was cloned from the highly divergent budding yeast *Saccharomyces cerevisiae* by rescue of a fission yeast $cdc25^{ts}$ mutation (15). Sequence similarities between the two yeast *cdc25*$^+$ homologs and a *Drosophila melanogaster cdc25*$^+$ homolog (string), which was independently identified by genetic analysis (16), were then used to design oligonucleotide probes to successfully clone a human *cdc25*$^+$ homolog by polymerase chain reaction (17). The human *CDC25* gene was found to rescue a $cdc25^{ts}$ mutation in fission yeast. In this paper we have characterized the *CDC25* gene products and demonstrate an obligatory function for entry into mitosis in human cells. Moreover, we show that the CDC25 proteins are almost exclusively nuclear, implying that the $p34^{CDC2}$/cyclin kinase is activated within the nucleus.

MATERIALS AND METHODS

Cell Culture, Metabolic Labeling, and Synchronization. HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal bovine serum and subcultured onto either 90-mm Nunc Petri dishes or coverslips 2 or 3 days before use. For synchronization at S phase, cells were transferred into medium containing 2 mM thymidine for 12 hr at 37°C. For metabolic labeling, HeLa cells were grown to ≈70% confluency, washed, and incubated in methionine-free Eagle's minimal essential medium containing 10% dialyzed fetal bovine serum, 25 mM Hepes (pH 7.2), and [^{35}S]methionine (1000 Ci/mmol; 1 Ci = 37 GBq) to a final concentration of 0.5 mCi per 10^6 cells for 4 hr.

Antibody Production and Affinity Purification. The full-length human *CDC25* gene product was produced in *Escherichia coli* as a 55-kDa insoluble protein present in inclusion bodies. $p55^{CDC25}$ was purified by gel electrophoresis and injected into rabbits. IgGs were purified by passage of sera over a protein G column, eluted at pH 2.7, and immediately neutralized with 1 M Tris·HCl (pH 8.0). Purified IgGs were then passed over a column containing electrophoretically

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Abbreviation: PBS, phosphate-buffered saline.

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pure $p55^{CDC25}$ coupled to cyanogen bromide-activated Sepharose. The column was washed with 50 volumes of Tris-buffered saline (2.7 mM KCl/137 mM NaCl/25 mM Tris·HCl, pH 8.0) containing 0.3% Tween 20, and antibodies were eluted either with 4 M $MgCl_2$ or 0.1 M glycine (pH 2.7). The flow-through, containing antibodies that failed to bind to $p55^{CDC25}$, was passed over the column again to ensure complete depletion of CDC25-specific antibodies. Affinity-purified antibodies were generated from the sera of two different rabbits immunized with $p55^{CDC25}$, designated 8325 and 8326. The experiments in this paper were performed with 8326 unless otherwise stated.

***In Vitro* Transcription and Translation.** The plasmid pBSK-1 (17) containing the full-length CDC25 cDNA was linearized with *Bam*HI and then transcribed using polymerase T7 and a transprobe T kit (Pharmacia); the CDC25 mRNA (1 μg) was translated by incubation with [^{35}S]methionine (0.8 mCi/ml) in a rabbit reticulocyte lysate translation system (Promega) for 60 min at 30°C. Aliquots were analyzed by SDS/PAGE or subjected to immunoprecipitation.

Immunoblotting and Immunoprecipitation. SDS/PAGE gels were transferred electrophoretically to nitrocellulose filters using a semidry blotting apparatus and blocked by incubation in phosphate-buffered saline (PBS; 137 mM NaCl/20 mM Na_2PO_4/1.8 mM KH_2PO_4, pH 7.2) containing 10% dried milk. After incubation with primary antibody, the filters were washed with fresh PBS containing 0.5% Tween 20 prior to detection. Detection was performed using an alkaline phosphatase-conjugated anti-rabbit IgG (Promega).

For immunoprecipitations, metabolically labeled HeLa cells were washed with ice-cold PBS and lysed at 4°C in 1 ml of RIPA buffer (137 mM NaCl/10% glycerol/0.1% SDS/0.5% sodium deoxycholate/1% Triton X-100/2 mM EDTA/20 mM Tris·HCl, pH 7.4). Lysates were clarified by centrifugation for 30 min at 100,000 × *g*. After incubation for 1 hr with the appropriate IgG fraction preabsorbed to protein A-Sepharose beads, the beads were washed three times with RIPA buffer and twice with lysis buffer [0.1% Nonidet P-40/50 mM NaF/10 mM sodium pyrophosphate/250 mM NaCl/10% glycerol/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/aprotinin (5 μg/ml)/pepstatin A (5 μg/ml)/leupeptin (5 μg/ml)/20 mM Tris·HCl, pH 7.2]. Immunoprecipitated proteins were extracted from the beads by solubilization in 2× SDS sample buffer (6% SDS/10% glycerol/4% 2-mercaptoethanol/125 mM Tris·HCl, pH 6.8) at 100°C for 10 min and analyzed by SDS/PAGE.

Immunofluorescence Microscopy. Cells were washed twice with PBS, fixed by incubating coverslips for 4 min at room temperature in 4% paraformaldehyde in PBS containing 0.1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, permeabilized by incubation for 6 min in PBS containing 0.2% gelatin and 0.2% Triton X-100, and then incubated for sequential 20-min periods with primary antibody and a secondary rhodamine-conjugated anti-rabbit antibody (each diluted in PBS containing 0.2% gelatin at concentrations of 3–25 μg/ml). After each stage, the cells were washed three times with PBS containing 0.2% gelatin and finally mounted in the same medium containing 0.1% *p*-phenylenediamine.

Microinjection. Cells grown on coverslips for microinjection were sparsely plated such that injected cells were separated by a distance of at least three cell diameters. Prior to injection, cells were incubated in fresh Dulbecco's modified Eagle's medium containing 25 mM Hepes at pH 7.2 and various additions as detailed in the experiments. Antibodies for injection were dialyzed and concentrated against two changes of 50 ml of injection buffer (90 mM KCl/2 mM Pipes, pH 7), then clarified by centrifugation for 10 min at 4°C at 100,000 × *g* in a Beckman TL-100 ultracentrifuge, and stored at 4°C until used. Fluorescein isothiocyanate-conjugated 150-kDa dextran or fluorescein isothiocyanate-conjugated bovine serum albumin was added to the injectant solutions at a concentration of 2 mg/ml, and the IgG concentration was adjusted to 5 mg/ml. Injectants were loaded into femtotip needles (Eppendorf), and microinjection was performed at room temperature by using an Eppendorf microinjector. Cells were injected over a period of 20 min. Cells were then returned to a 37°C incubator for an additional 6 hr. After this time, the cells were fixed with 4% paraformaldehyde in PBS and then mounted on slides in PBS containing 50% glycerol and 0.1% *p*-phenylenediamine. Injected cells (between 21 and 118 per experiment) were visualized by fluorescence microscopy as detailed above. Cells that were undergoing mitosis were recognized by their rounded morphology and the presence of condensed chromosomes. Divided cells were readily recognized as adjacent pairs with approximately mirror-image rounded morphology.

Materials. Dulbecco's modified Eagle's medium, streptomycin, penicillin, and thymidine were from GIBCO. Tran^{35}S-label (1000 Ci/mmol) was purchased from ICN. Methionine-free Eagle's minimal essential medium was from Flow General (McLean, VA). Aprotonin and leupeptin were from Boehringer Mannheim. L-[^{35}S]Methionine (1000 Ci/mmol) was from New England Nuclear. Fetal bovine serum was purchased from HyClone. *p*-Phenylenediamine was obtained from J. T. Baker. Rhodamine-conjugated anti-rabbit IgG was from Organon. Reagents for *in vitro* transcription (transprobe kit) and protein A-Sepharose beads were obtained from Pharmacia LKB. Alkaline phosphate-conjugated anti-rabbit IgG and reagents for *in vitro* translation were from Promega. Isopropyl β-D-thiogalactoside, pepstatin A, phenylmethylsulfonyl fluoride, and the restriction enzymes *Bam*HI and *Nde* I were purchased from Bethesda Research Laboratories. All other chemicals were of the highest grade available.

RESULTS

Identification of CDC25 Protein in Human Cells. We raised antisera to bacterially produced human CDC25. Expression of the full-length *CDC25* gene in *Escherichia coli* gave rise to an insoluble 55-kDa protein that was used as an inoculant in two rabbits. IgGs highly specific for $p55^{CDC25}$ were purified by protein G chromatography followed by adsorption to an immobilized preparation of bacterially produced $p55^{CDC25}$. Antibodies that were eluted from the $p55^{CDC25}$-Sepharose column demonstrated strong cross-reactivity with bacterially produced $p55^{CDC25}$ by immunoblot analysis. Conversely, antibodies from the column flow-through showed little or no detectable cross-reactivity against $p55^{CDC25}$ and were used as a control in many of the experiments performed in this study. These preparations are referred to as "anti-CDC25" and "flow-through" antibodies, respectively. It should be noted that flow-through antibodies are an excellent experimental control because they are isolated by manipulations identical to that experienced by the anti-CDC25 antibodies, except that the flow-through preparation was not subjected to elution from the $p55^{CDC25}$ column.

The affinity-purified antibodies were first tested using CDC25 protein produced *in vitro*. The *CDC25* gene was transcribed and translated in an *in vitro* eukaryotic translation system. As compared to controls, the translation of CDC25 mRNA was rather inefficient. Nevertheless, major proteins of ≈55 and 57 kDa were synthesized, together with a weakly detectable 52-kDa species (Fig. 1*A*). A number of other proteins between 31 and 35 kDa were also observed. These are possibly truncated products of the *CDC25* gene. The 55- and 57-kDa protein species were efficiently immunoprecipitated by anti-CDC25 IgG (Fig. 1*B*). The flow-through antibodies failed to react with any of the *in vitro* synthesized proteins. The low abundance of the 52-kDa species prevented evaluation of its cross-reactivity. These results demonstrate

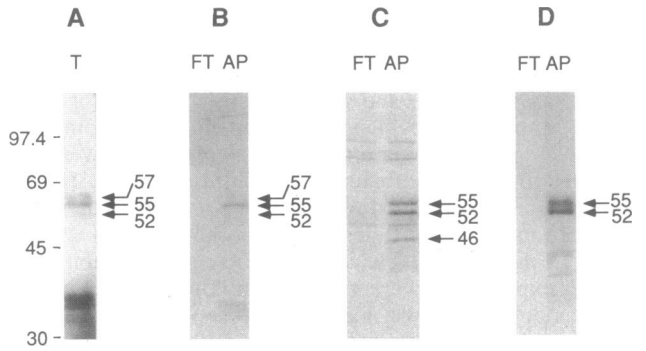

FIG. 1. Identification of *CDC25* gene products in human cells. (*A*) *In vitro* transcription and translation. pBSK-1 containing the full-length *CDC25* gene was transcribed and translated *in vitro* as described in *Materials and Methods*, and an aliquot was analyzed by SDS/PAGE (lane T). Arrows indicate the positions of the 57-, 55-, and 52-kDa proteins. (*B*) Immunoprecipitation of *in vitro*-translated *CDC25*. An aliquot (2 μl) of the *in vitro* translation reaction (in *A*) was immunoprecipitated with either affinity-purified (AP) or flow-through (FT) IgGs. (*C*) Immunoprecipitation from HeLa cells. Cultures of exponentially growing HeLa cells on 90-mm dishes were incubated for 4 hr in medium containing [^{35}S]methionine (0.5 mCi/ml). Cultures were washed, lysed, and then precipitated by either affinity-purified (AP) or flow-through (FT) IgGs. Arrows indicate the positions of the 55-, 52-, and 46-kDa proteins. (*D*) Immunoblotting of total HeLa cell extracts. HeLa cells grown on 90-mm dishes were washed and lysed by boiling in SDS sample buffer. One hundred micrograms of total cell extract was separated by SDS/PAGE, blotted on nitrocellulose, and probed with either affinity-purified (AP) or flow-through (FT) IgGs (1 μg/ml). Visualization was performed using an alkaline phosphatase detection system. Arrows indicate the position of the 55- and 52-kDa proteins.

that the affinity-purified antibodies specifically recognize protein products derived from the *CDC25* gene.

To further characterize the human *CDC25* gene products, lysates of exponentially growing HeLa cells labeled with [^{35}S]methionine were immunoprecipitated with either anti-CDC25 or flow-through antibodies under nondenaturing conditions. Two major proteins of 55 and 52 kDa were specifically precipitated with the anti-CDC25 antibodies. These comigrated with the similarly sized products of the *in vitro* translation reaction (Fig. 1*C*). Both species were also observed in immunoprecipitates of lysates denatured in SDS prior to immunoprecipitation (data not shown). We refer to these proteins as p55^{CDC25} and p52^{CDC25}. In addition, a minor protein of 46 kDa was weakly detected in nondenaturing immunoprecipitates; the minor protein did not comigrate with any species in the *in vitro* translation reaction (Fig. 1*C*) or appear under denaturing conditions and thus may represent either a protein complexed with p52/p55^{CDC25} *in vivo* or a protein having a cross-reactive epitope that is lost upon denaturation.

Two major proteins of 55 and 52 kDa were also recognized in immunoblots of total HeLa cell extracts by the affinity-purified antibodies but not by the flow-through IgG (Fig. 1*D*). In some experiments both bands could be resolved into doublets. Together these results indicate that p52^{CDC25} and p55^{CDC25} represent the major *in vivo* products of the *CDC25* gene.

Subcellular Localization of p52/p55^{CDC25}. The subcellular localization of the *CDC25* gene products was examined by immunofluorescence staining of exponentially growing HeLa cells. A prominent immunofluorescence signal with anti-CDC25 antibodies was observed over a range of concentrations (3–25 μg/ml). At an equivalent concentration, no signal was observed with either the flow-through antibodies (Fig. 2*B*), preimmune sera, or the secondary antibody alone (data not shown). In all interphase cells, anti-CDC25 antibodies

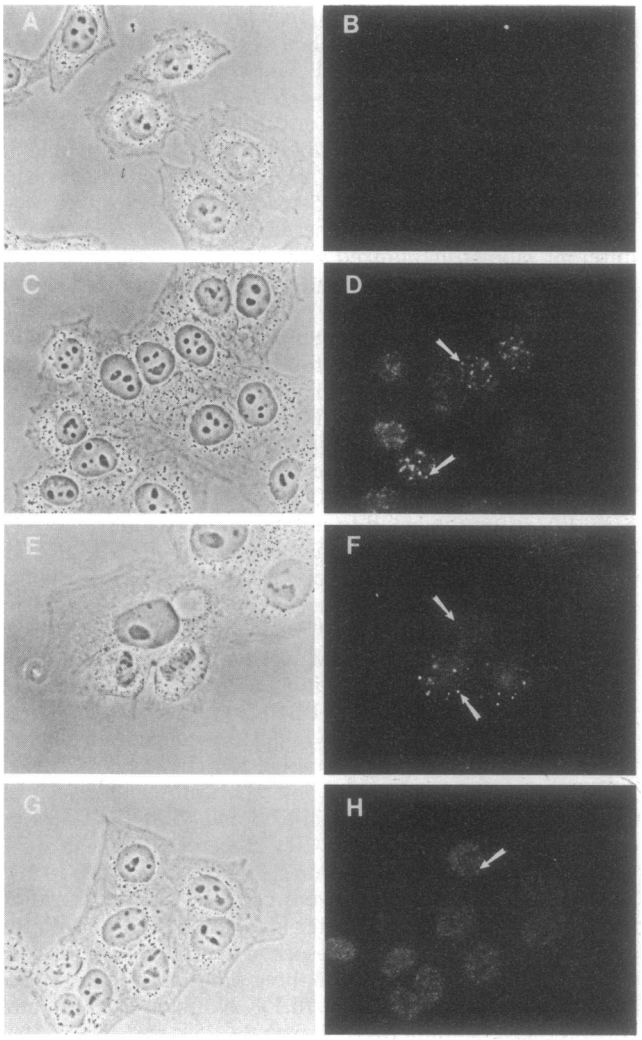

FIG. 2. Localization of p55^{CDC25} in human cells. Exponentially growing HeLa cells grown on coverslips were washed and fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and probed with either flow-through (*A* and *B*) or affinity-purified IgG 8326 (*C–F*) both at 6 μg/ml or affinity-purified IgG 8325 (*G* and *H*) at 15 μg/ml as described in *Materials and Methods*. Detection was performed using rhodamine-conjugated anti-rabbit IgG. Cells were visualized either by phase-contrast (*A*, *C*, *E*, and *G*) or fluorescence (*B*, *D*, *F*, and *H*) microscopy and are represented in pairs (*A* + *B*; *C* + *D*; *E* + *F*; *G* + *H*). Arrows indicate the position of intranuclear fluorescence.

gave rise to a prominent speckled or punctate staining throughout the nuclear interior in regions not occupied by the nucleoli (Fig. 2 *D* and *F*). The staining pattern varied somewhat from cell to cell; in a small proportion of the cells (≈20%), the nuclear spots were fewer in number but of greater intensity (Fig. 2*D*) and may represent a coalescence of the smaller spots. In contrast, cytoplasmic staining was weak and not substantially different from that observed with control antibodies (Fig. 2*D*). In cells undergoing mitosis, strong staining occurred throughout most areas of the cell but was excluded from the mitotic chromosomes (data not shown). No specific staining of centrosomes or spindles could be determined under these conditions, although this may have been obscured by the high level of cytoplasmic staining. However, cells in telophase consistently displayed a small number of brightly stained spots (Fig. 2*F*). HeLa cells stained with anti-CDC25 antibodies prepared in a second rabbit displayed almost identical immunofluorescence—namely, a speckled signal almost exclusively within the

nucleus of the cell (Fig. 2*H*). We conclude from these results that p52/p55^{CDC25} resides primarily within the nucleus of HeLa interphase cells. As elaborated in the *Discussion*, this result has important implications for the determination of the subcellular sites of p34^{CDC2}/cyclin activation.

CDC25 Is Required for Mitosis in Human Cells. Deletion of the fission yeast *cdc25*$^+$ gene results in a cell cycle arrest in the G_2 phase (3). Since a similar gene deletion analysis is not possible in human cells, we have adopted an alternative approach to determine the effect of loss of *CDC25* function. Our strategy was to inject cells of a G_2-enriched population with anti-CDC25 antibodies, with the aim of specifically neutralizing the *CDC25* products and evaluating the effect on the progression into M phase. Exponentially growing HeLa cells were transferred to medium containing 2 mM thymidine for 12 hr. Under these conditions, the rate of DNA replication was dramatically reduced and cells accumulate in S phase (18). The majority of the viable cells underwent mitosis between 4 and 8 hr after the removal of thymidine from the extracellular medium (Fig. 3*A*).

Four hours after release from a thymidine block, cells sparsely plated on a coverslip were microinjected with a concentrated preparation of affinity-purified anti-CDC25 IgG. As a control, an equal amount of flow-through antibodies depleted of p55^{CDC25} immunoreactivity was microinjected in an identical manner into a second set of cells. Coinjection with either dextran or bovine serum albumin conjugated to fluorescein isothiocyanate enable injected cells to be visualized by fluorescence microscopy after an additional 6-hr incubation (see *Materials and Methods*). In four independent experiments, 46.8% ± 4.1% of the cells injected with flow-through antibodies had already entered or progressed through mitosis (Fig. 3*B*). In contrast, only 8.2% ± 3.0% of the cells microinjected with anti-CDC25 antibodies had undergone mitosis (Fig. 3*B*). As a control, we showed that injection of cells with purified IgG directed against gp120, a major nuclear pore protein, had no effect on mitosis (data not shown). In other experiments, we confirmed that the majority of cells injected with anti-CDC25 antibodies did not undergo mitosis when analyzed at any time less than 6 hr postinjection. These data indicate that neutralization of p55^{CDC25} function inhibits mitosis in human cells.

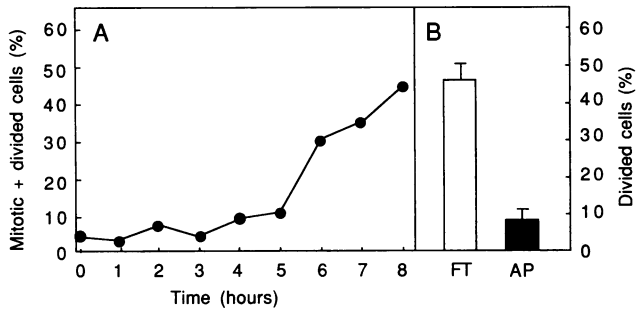

FIG. 3. Inhibition of mitosis by microinjection of anti-p55^{CDC25} IgG. (*A*) Time course of entry into mitosis. HeLa cells grown on coverslips were incubated in medium containing 2 mM thymidine for 12 hr. After this time, the cells were washed and placed in fresh medium. At various times cells were scored for the appearance of condensed chromatin and rounded morphology (mitotic) or the appearance of divided cells. Values represent the mean obtained from a representative experiment. (*B*) Microinjection of antibodies. HeLa cells grown on coverslips were released from a thymidine block and placed in fresh medium for 4 hr. At this time cells were injected with either affinity-purified (AP) or flow-through (FT) antibodies both at 5 mg/ml into the cytoplasm of ≈150 cells (total time of injection was 20 min). The cells were incubated for an additional 6 hr, fixed, and visualized by fluorescence microscopy and scored for passage through mitosis. Values represent the mean ± SD of four independent experiments.

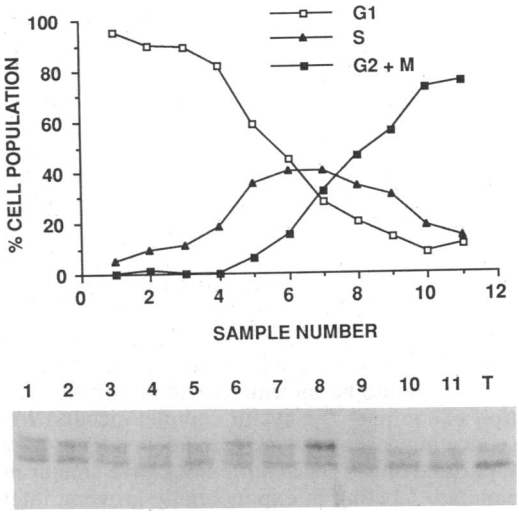

FIG. 4. Immunoblot analysis of p52/p55^{CDC25} levels during the cell cycle. HeLa cells grown in suspension were separated on the basis of size by centrifugal elutriation (17), and fractions were divided for flow cytometry analysis of DNA content and for immunoblot analysis using anti-CDC25 IgG.

p52/p55^{CDC25} Levels Remain Constant During the HeLa Cell Cycle. We have previously reported that in HeLa cells the level of CDC25 mRNA is low in G_1 and increases as cells approach M phase (17). If p52/55^{CDC25} functions as a dose-dependent M-phase inducer in HeLa cells, this observation suggests that the timing of mitosis could be influenced by the periodic accumulation of p52/55^{CDC25} during interphase. To determine whether the level of p52/55^{CDC25} parallels the oscillation in CDC25 mRNA, we used immunoblot analysis to monitor the appearance of p52/55^{CDC25} during the HeLa cell cycle. Cells grown in suspension culture were separated on the basis of size by centrifugal elutriation (17). Cell cycle profiles of the fractions were determined by flow cytometry analysis of propidium iodide-stained cells. Equal amounts of protein were immunoblotted with anti-CDC25 antibodies. This analysis clearly showed that the level of p52/55^{CDC25} remained relatively constant during the cell cycle, fluctuating less than 2-fold (Fig. 4). This pattern of expression during the cell cycle, in which mRNA levels fluctuate but protein levels remain steady, has previously been observed for CDC2 in HeLa cells (19). As mentioned in the *Discussion*, models have been proposed to suggest how this pattern of expression could play a role in cell cycle regulation, with the presumption that there may be a functional distinction between old and newly synthesized protein (19, 20).

DISCUSSION

A key goal of these experiments was to provide formal evidence regarding the requirement for CDC25 function in human cells. One of the most successful techniques available to evaluate the *in vivo* function of proteins in cultured mammalian cells has been microinjection of reactive antibodies. For example, microinjection of anti-p34^{cdc2} antibodies was shown to inhibit entry into mitosis in rat fibroblasts (21). Using the same approach, we have shown that microinjection of purified anti-CDC25 antibodies into cultured HeLa cells strongly inhibited the onset of mitosis. These results suggest that p52/p55^{CDC25} plays an important role in the initiation of mitosis in human cells. We cannot, however, completely exclude the possibility that microinjected anti-CDC25 antibodies recognized an unrelated protein *in vivo* and that coincidentally its function is also required for mitosis. This is an inherent limitation of the technique.

Nevertheless, the fact that the purified anti-CDC25 antibodies were highly specific for the products of the *CDC25* gene by the assay of immunoprecipitation of native proteins, which must most closely simulate the circumstances of microinjected antibodies, greatly supports the conclusion that anti-CDC25 antibodies inhibited mitosis by specifically neutralizing CDC25 protein function. Thus we conclude that CDC25 normally plays an important role in the induction of mitosis in human cells, as do its counterparts in fission yeast and *D. melanogaster* (3, 16, 22).

Previous studies have shown that cdc25 product is the protein phosphatase that dephosphorylates $p34^{cdc2}$ on tyrosine-15 and activates the $p34^{cdc2}$/cyclin M-phase kinase (12, 28). Therefore, the subcellular localization of the *CDC25* gene product should be informative about the sites at which activation of the $p34^{CDC2}$/cyclin complex occurs. Immunofluorescence microscopy using two independently generated affinity-purified anti-CDC25 antibodies gave similar intranuclear punctate labeling in exponentially growing interphase HeLa cells. These results strongly suggest that the *CDC25* gene products function within the nucleus to regulate activation of M-phase promoting factor, although it remains possible that a small, undetectable cytoplasmic fraction of CDC25 protein plays an important role in the M-phase promoting factor activation process. Intriguingly, a speckled nuclear staining pattern similar to that observed for $p52/p55^{CDC25}$ has been observed in HeLa cells with antibodies directed against $p34^{CDC2}$ and, in particular, human homologs of $p13^{suc1}$, a protein that interacts with $p34^{CDC2}$ (21, 23). Although we have no direct evidence indicating whether these three proteins colocalize, the similarity in staining patterns suggests that it is a strong possibility that warrants further investigation.

Our data demonstrate that CDC25 function is required for M-phase onset in human cells. However, they do not address the issue of whether the level of CDC25 activity is normally rate limiting for determining the timing of M-phase initiation. In fission yeast the cellular concentration of $cdc25^+$ mRNA and protein product increases as cells approach M phase, and changes in the level of $cdc25^+$ expression modulate mitotic timing (3, 13). We have previously observed that the level of CDC25 mRNA increases as HeLa cells proceed into G_2 phase (17), and thus we might have expected to observe a gradual accumulation of $p52/p55^{CDC25}$ during the cell cycle. No such variation was observed. Our data indicate that expression of CDC25 follows a pattern similar to that seen for CDC2, in which $p34^{CDC2}$ is produced periodically but turned over slowly, thus generating only a mild periodicity in the steady-state level of $p34^{CDC2}$ during the cell cycle (19). If there was a mechanism of distinguishing new and old CDC25 protein, then periodic production of $p52/p55^{CDC25}$ could play an important role in the control of mitotic timing. It is also possible that a small periodic change in the level of a critical rate-limiting element could be sufficient to regulate the timing of M phase. Nevertheless, it is clear that cell cycle pattern of $p80^{cdc25}$ accumulation in fission yeast is at least quantitatively different from that of $p52/p55^{CDC25}$ in HeLa cells. Although CDC25 is clearly required for mitosis, it remains to be determined whether it regulates the timing of mitosis in human cells. Other candidate regulators include cyclins (24–26) and human homologs of fission yeast mitotic inducer $nim1^+$ and inhibitor $wee1^+$ (4, 27).

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