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The cdc25B phosphatase is essential for the G_2/M phase transition in human cells

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SUMMARY

Cdc25 phosphatases play key roles in cell cycle progression by activating cyclin-dependent kinases. In human cells, cdc25 proteins are encoded by a multigene family, consisting of cdc25A, cdc25B and cdc25C. While cdc25A plays a crucial role at the G_1/S phase transition, cdc25C is involved in the dephosphorylation and activation of the mitotic kinase, cdc2/cyclinB. In addition, cdc25C itself is regulated by cdc2/cyclinB which then creates a positive feedback loop that controls entry into mitosis. In this study we show that the activity of cdc25B appears during late S phase and peaks during G_2 phase. Both in vitro and in vivo cdc25B is activated through phosphorylation during S-phase. Using a cell duplication, microinjection assay we show that ablation of cdc25B function by specific antibodies blocks cell cycle progression in Hs68 cells by inhibition of entry into mitosis. Cdc25B function neither plays a role in later stages of mitosis nor for the inititation of DNA replication. These results indicate that cdc25B is a mitotic regulator that might act as a 'starter phosphatase' to initiate the positive feedback loop at the entry into M phase.

Key words: cdc25, Cell cycle, Phosphorylation, cdk, Phosphatase

INTRODUCTION

Progression through the eukaryotic cell cycle involves the sequential activation of cyclin-dependent kinases (cdk) (Nigg, 1995; Morgan, 1995). The activity of these kinases is regulated by both binding to regulatory proteins and by phosphorylation. Activation of a cdk is dependent upon its association with a cyclin regulatory subunit (Pines, 1993). A complex formed by association of cdc2 (cdk1) and cyclinB plays a major role at entry into mitosis in most if not all organisms. In yeast, the cdc2 gene product regulates both entry into the cell cycle (START) and progression into mitosis. In higher eukaryotic cells other cdks control the G_1/S transition and progression through S phase in association with the cyclins D, E and A (Pines, 1995). Cdk inhibitors also interact with members of the cdk family. The expression of these proteins increases in response to extracellular growth inhibitory signals or DNA damage and negatively regulates cdk kinase activity (Sherr and Roberts, 1995). Another major mechanism of cdc2 regulation occurs by reversible phosphorylation (Lew and Kornbluth, 1996). In addition to cyclin binding, complete activation of cdc2 requires phosphorylation of Thr-161. In contrast, phosphorylation of Thr-14 and Tyr-15 suppresses kinase activity thus preventing premature activation of the cdc2/cyclinB complex. Phosphorylation of Tyr-15 is carried out by the wee1 protein kinase (McGowan and Russell, 1993) while another kinase, myt1, can phosphorylate cdc2 on both sites (Mueller et al., 1995; Liu et al., 1997).

In fission yeast, the cdc25 phosphatase catalyzes the dephosphorylation on Tyr-15 of cdc2. Cdc25 represents a key determinant of mitotic timing, where genetic defects that reduce Tyr-15 phosphorylation lead to premature mitosis (Gould and Nurse, 1989). In mammalian cells, cdc25 is a multigene family comprised of cdc25A, cdc25B and cdc25C (Sadhu et al., 1990; Galaktionov and Beach, 1991; Nagata et al., 1991). The three phosphatases share approximately 50% similarity at the amino acid sequence level. Cdc25A and cdc25C function at the G_1/S transition and G_2/M transitions, respectively (Hoffmann et al., 1993, 1994; Jinno et al., 1994). Cdc25C becomes phosphorylated by cdc2/cyclinB at mitosis and this directly stimulates its ability to dephosphorylate cdc2 thus creating a positive feedback loop (Hoffmann et al., 1993; Izumi and Maller, 1993; Strausfeld et al., 1994). However, the question of how this autocatalytic mechanism is initiated remains to be elucidated. In addition, the activity of cdc25C is regulated by other kinases. Plx1, a kinase purified from *Xenopus* egg extracts phosphorylates and activates *Xenopus* cdc25C in vitro (Kumagai and Dunphy, 1996). Recently it has been shown that cdc25C is phosphorylated by a novel kinase, Chk1, at Ser-216 which represents a binding site for 14-3-3 proteins in response to DNA damage (Funari et al., 1997; Sanchez et al., 1997; Peng et al., 1997). This implicates Chk1 as possibly regulating the interaction between 14-3-3 and

cdc25C during a DNA damage checkpoint response. A feedback loop similar to that described for cdc25C and cdc2/cyclinB might exist at the G_1/S transition involving cdc25A and cdk2/CyclinE as partners (Hoffmann et al., 1994). The cdk2/cyclinE complex is activated by cdc25A. Activated cdk2/cyclinE in turn phosphorylates and activates cdc25A. Cdc25A and B are potential human oncogenes since they are able to transform primary mouse fibroblasts in cooperation with the Ras oncogene (Galaktionov et al., 1995). Three splicing variants of the human cdc25B gene have been identified (Baldin et al., 1997a). Recent work indicates that cdc25B is an unstable protein that might be important for the G_2/M phase transition (Nishijima et al., 1997; Baldin et al., 1997b).

In this study we have investigated the function and regulation of human cdc25B during the cell cycle. We show that the cdc25B phosphatase is activated during late S phase/early G_2 phase prior to the activation of cdc25C. Both in vivo and in vitro the activity of cdc25B increases by phosphorylation during S-phase. Cdc25B shows a high substrate specificity for cdc2/cyclinA during S-phase while during G_2-phase the activity increases towards cdc2/cyclinB as substrate. Moreover, we show that microinjection of specific antibodies against cdc25B into Hs68 human fibroblasts block entry into mitosis, indicating a crucial role of this phosphatase at the G_2/M transition.

MATERIALS AND METHODS

Cell culture and extract preparation

Human foreskin fibroblasts (Hs68), obtained from the American Type Culture Collection (Rockville, MD), were cultured for no longer than seven passages in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/l glucose supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 mg/ml), in a humidified atmosphere containing 5% CO_2 at 37°C.

HeLa cells were obtained from the Cold Spring Harbor Laboratory Tissue Culture facility (Cold Spring Harbor NY) and cultured as previously described (Giordano et al., 1989). Conditions for immunoprecipitation have been previously described (Draetta and Beach, 1988). Briefly, cell extracts were prepared by addition of 3-5 volumes of lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.1% Triton X-100, 50 mM NaF, 1 mM DTT, 0.1 mM Na_3VO_4) to a cell pellet. The following protease inhibitors were added: 0.1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, L-1chlor-3-(4-tosylamido)-4 phenyl-2-butanon (TPCK); 10 μg/ml L-1 chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK); 1 μg/ml aprotinin. For cell fractionation, cell monolayers were washed twice with ice-cold PBS, and then cells were harvested with a rubber policeman. Total protein concentrations were determined using the Bio-Rad protein assay system and BSA as calibration standard.

For in vivo labelling, HeLa cells were incubated with 2 mCi/plate of [^{32}P]orthophosphate in phosphate free DMEM medium for 4 hours at 37°C.

Elutriation and cell synchronization

G_1 HeLa cells were obtained by centrifugal elutriation as described by Draetta and Beach (1988). For the experiment described in Fig. 3 fractions enriched in G_1 cells were re-added to the culture at a density of 3×10^5 cells/ml. Fractions were taken at the times indicated in Fig. 3A. Progression through the cell cycle in the elutriated cells was monitored by flow cytometry. Mitotic shake-off of nocodazole treated HeLa cells was perfomed as described (Morla et al., 1989). Briefly, cells were treated for 12 hours with nocodazole (50 ng/ml). After one wash with PBS, mitotic cells were prepared by gently pipetting up and down several times. Subsequently, plates were rinsed with ice-cold PBS containing 0.4 mM EDTA (>90% in G_2) scraped off the plates with PBS containing 0.4 mM EDTA and 50 ng/ml nocodazole. Cell cycle stage was monitored by flow cytometry and mitotic index determination. In the experiment for Fig. 5, 10 mM hydroxyurea or 1 mg/ml aphidicolin were added to asynchronous cells which were then incubated for 18-20 hours. This treatment allowed recovery of cells that were 85-90% arrested in early S phase with a 2N DNA content, as assessed by flow cytometry. For a double-thymidine block to arrest cells in S phase 2 mM thymidine (Sigma) was added to a HeLa cell population that was 50-70% confluent. Cells were incubated for 17 hours. They were then extensively washed and further incubated in drug-free medium for 6 hours. To optimize synchronization cells were re-incubated for 15 hours in 2 mM thymidine. Hs68 cells were arrested in G_0 by incubating them for 3 days in DMEM without serum. They were stimulated to re-enter the cell cycle by adding medium supplemented with 20% FCS. Cell cycle progression was monitored by measuring BrdU incorporation.

Expression and purification of recombinant cdc25 proteins

Human cdc25B2 (Galaktionov and Beach, 1991) was produced from the bacterial strain Xl1-blue using the pGEX-2T expression vector. After isopropyl-β-D-thiogalactoside (IPTG) induction for 4 hours, (1 mM final concentration) the 89 kDa GST-cdc25B2 fusion protein was recovered as described by Smith and Johnson (1988) on glutathione-Sepharose beads and eluted with 20 mM glutathione in 50 mM Tris-HCl, pH 8.0.

Phosphatase and kinase assays

For phosphatase assays, cdc25B or cdc25C immunoprecipitates or recombinant GST-fusion proteins were incubated with 300 μM OMFP (3-O-methyl fluorescein phosphate; Sigma) in 50 mM Tris-HCl, pH 8.2, 50 mM NaCl, 1 mM DTT, 20% glycerol for 15 minutes at room temperature. Hydrolysis of OMFP to OMF was monitored at 477 nm (Gottlin et al., 1996). Treatment of cdc25B2 with 0.2 U/ml lambda protein phosphatase (λ-PPase; New England BioLabs) was performed in 50 mM Tris-HCl, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35 supplemented with 2 mM $MgCl_2$ for 30 minutes at 30°C. The inhibitor cocktail used contained 100 mM NaF, 300 mM Na_2MoO_4, 100 mM β-glycerophosphate (Hoffmann et al., 1993).

In vitro protein kinase assays were performed as described by Hoffmann et al. (1993). Briefly, after immunoprecipitations pellets were incubated at 30°C in the presence of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 500 μM ATP and 50 μCi [γ-^{32}P]ATP for 10 minutes. GST-cdc25B2 was added at 0.3 mg/ml.

Activation of cyclin-dependent kinases by cdc25B

To prepare the substrates for the cdc25B phosphatase cdc2/cyclinB or cdk2/cyclinA complexes were prepared as described using bacterially expressed GST-cyclinB or GST-cyclinA as described by Pan et al. (1993). To produce inactive complexes both kinases were phosphorylated on Tyr-15 with bacterially expressed GST-wee1 as described by Parker et al. (1993). Cdk2/cyclinA or cdc2/cyclinB were incubated with immunoprecipitated cdc25B from HeLa cells arrested in G_1, S and G_2/M phases for 15 minutes at 30°C in 1 ml of phosphatase buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM DTT. The activity of the kinases was measured by phosphorylation of histone H1 as described (Hoffmann et al., 1993).

Immunochemistry

The anti-cdc25B antibodies were generated by injecting rabbits with a full-length human cdc25B-GST fusion protein. The first injection was carried out with 200 μg GST-cdc25B protein mixed with

complete Freund's adjuvant. Subsequently they were injected with the same amount of protein in incomplete Freund's adjuvant every two weeks until a significant immune signal was detected by immunoblotting of total cell extracts. The resulting polyclonal antiserum was affinity purified using the GST-cdc25B fusion protein covalently coupled to CNBr-activated Sepharose and subsequently on coupled GST alone to remove antibodies specific to the glutathione-S-transferase. In order to avoid crossreactivity of the antibodies with the other cdc25 phosphatases the affinity purified cdc25B antibodies were purified on both cdc25A and cdc25C Sepharose. Cdc25C and cdc25A antibodies were used as described (Hoffmann et al., 1993, 1994). For immunoblotting 150 μg of total protein from HeLa or Hs68 cell lysates were loaded per lane. Proteins were transferred from gels by semi-dry blotting as described by Harlow and Lane (1988). As secondary antibodies horseradish peroxidase (HRP)-labelled anti-rabbit or (HRP)-labelled anti-mouse antibodies were used.

Microinjection and immunofluorescence microscopy

For microinjection of asynchronous cells, Hs68 cells were grown on coverslips to 50% confluency and injected with antibodies using an Eppendorf micromanipulator and microinjector (AIS, Zeiss). Alternatively, cells were injected after serum starvation for 36 hours (G_1 injections). Coverslips were transferred to fresh medium shortly before injection of the appropriate IgG preparation (3.2 mg/ml for anti-cdc25B antibodies, 3.5 mg/ml and 2.9 mg/ml for control IgG and cdc25C, respectively, in PBS) into the cytoplasm or the nucleus. Injections were either clustered for DNA synthesis experiments or widely scattered (one cell per microscopic field) so that any effects on the ability of injected cells to divide could be clearly observed. After injection coverslips were placed into fresh medium containing 10% FCS (for asynchronous injections) or 20% FCS (for G_0 injections). For measurements of DNA synthesis bromodeoxyuridine (BrdU) was added to the medium to a final concentration of 100 μM. Cells were fixed at room temperature for 10 minutes with 3.7% formaldehyde in PBS and permeabilized for 30 seconds with 100% acetone at −20°C. Fixed and permeabilized cells were incubated for 1 hour at 37°C in a humidified atmosphere with affinity-purified anti-cdc25B antibodies. For analysis of DNA synthesis coverslips were incubated for 10 minutes at room temperature in 1.5 M HCl followed by extensive washing with PBS. Cells were then washed three times with 0.5% BSA in PBS and incubated for 30 minutes at room temperature with Texas Red-conjugated goat anti-rabbit antibodies (dilution 1:500; Amersham, UK). To detect BrdU incorporation cells were incubated with a monoclonal anti-BrdU antibody (dilution 1:100, Boehringer, Mannheim). After three washes with 0.5% BSA in PBS, cells were incubated for 30 minutes with fluorescein-conjugated anti-mouse antibodies (dilution 1:200, Dianova, Hamburg). Cells were finally washed for 5 minutes in PBS containing Hoechst 33258 (Sigma; final concentration 1 mg/ml) and mounted on glass slides with Moviol. Cells were observed with a microscope (Axiophot, Carl Zeiss) using ×63 immersion oil objectives.

RESULTS

Human cdc25B is activated by phosphorylation during late S phase

To perform functional studies on the role of cdc25B in the human cell cycle, we first characterized the antibodies to be used. A rabbit polyclonal antibody was raised against the full-length human cdc25B-GST fusion protein and affinity-purified from serum. Fig. 1 illustrates the specificity of this reagent. Cell extracts from exponentially growing HeLa cells (Fig. 1A, lane 2) or human foreskin fibroblasts (Hs68) (Fig. 1A, lane 3) were analyzed by SDS-PAGE and immunoblotting with the anti-cdc25B antibody. The antibody specifically recognizes a protein at 63 kDa. The ability of this reagent to immunoprecipitate cdc25B from ^{35}S-labelled HeLa cells is demonstrated in Fig. 1B (lane 1). The protein was not recognized by the preimmune serum (lane 2). We note that a protein of ~85 kDa reproducibly coprecipitates with cdc25B. It will be interesting to identify this protein and determine if it is involved in the regulation of cdc25B. The antibody was absorbed out both on GST-cdc25C and GST-cdc25A columns. No crossreactivity with either cdc25A or cdc25C could be detected (data not shown).

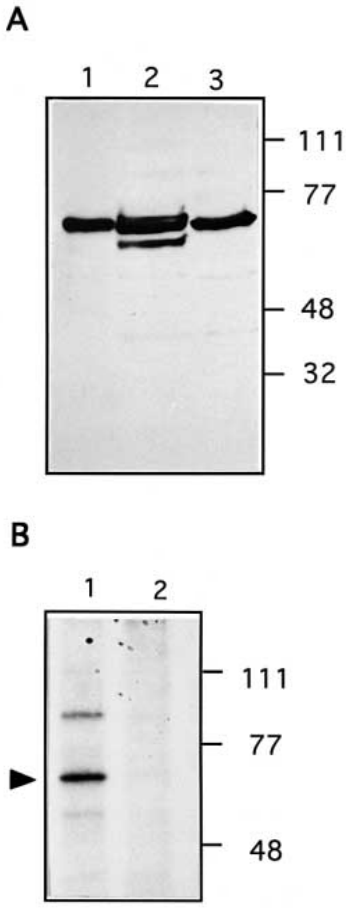

Fig. 1. Characterization of anti-cdc25B antibodies. (A) Extracts of HeLa cells (lane 2) or Hs68 human fibroblasts (lanes 3) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with cdc25B antiserum; [^{35}S]methionine-labeled in vitro-translated human cdc25B was used as a marker (lane 1) (B) HeLa cells were labelled in vivo with [^{35}S]methionine and cdc25B was immunoprecipitated with the cdc25B antiserum (lane 1, arrowhead). Control immunoprecipitations were performed with a preimmune serum (lane 2). Molecular mass of standard proteins (kDa) is indicated.

We then proceeded to perform a detailed analysis of cdc25B phosphatase activity. HeLa cells were either synchronized using drug arrest-release protocols or by size fractionation. In a first experiment, HeLa cells were synchronized by using a double thymidine block. After release from drug arrest, cells were collected at regular intervals. Cdc25B or cdc25C was immunoprecipitated with specific antibodies from each time point and the phosphatase activity was measured using the chromophoric substrate 3-0-methylfluorescein phosphate (OMFP) (Fig. 2A). Gottlin et al. (1996) have recently shown that OMFP is a better substrate for cdc25 phosphatases than the previously used substrate para-nitrophenylphosphate (pNPP). In parallel to the immunoprecipitation experiments, the DNA content of each sample was analyzed by flow cytometry (Fig. 2B). Fig. 2A shows a comparison of the phosphatase activities of cdc25B and cdc25C. Both activities were low in cells at early S phase. Cdc25B phosphatase

activity appeared in late S phase, four hours after release from the thymidine block and increases about five-fold during G_2. Cdc25B activity decreased again when the cells had entered mitosis. On the basis of these results we conclude that the human cdc25B phosphatase is maximally active during G_2. Cdc25C activity appeared four hours later than cdc25B when the cells entered prophase as assessed by chromosome staining (not shown). These results clearly indicate that human cdc25B is activated in late S phase clearly before cdc25C is activated.

To confirm the data in Fig. 2 we used another method of cell synchronization. HeLa cells were subjected to size fractionation by centrifugal elutriation. Cells in G_1 were collected and re-inoculated into fresh medium. The activity of immunoprecipitated cdc25B and cdc25C was assayed in each fraction on OMFP as substrate (Fig. 3A). Samples from each fraction were analyzed by flow cytometry (Fig. 3B). Consistent with the data shown in Fig. 2 we found that the cdc25B phosphatase activity appears during S phase and shows a peak in activity during G_2 (Fig. 3A).

In order to understand how the activity of cdc25B is regulated we first analyzed the protein expression levels during the cell cycle in HeLa cells. Equal amounts of proteins were immunoblotted with anti-cdc25B antibodies. The analysis clearly showed that the level of cdc25B remained relatively constant during the cell cycle, fluctuating less than 2-fold (Fig. 3C), suggesting that a regulatory mechanism other than protein expression is responsible for the activation of cdc25B at the late S-phase/early G_2 phase. The reason for the discrepancy between our results and those of Gabrielli et al. (1996), who showed a cell cycle specific expression during the G_2 phase in HeLa cells, is unknown. One possible explanation might be that the antibodies used recognize different alternatively spliced forms of the cdc25B phosphatase. However, the expression pattern where the protein levels remain constant has been previously demonstrated for the other members of the cdc25 phosphatase family, cdc25A and cdc25C (Millar et al., 1991; Hoffmann et al., 1994).

To determine if there was a change in phosphorylation level of cdc25B phosphatase during the cell cycle we incubated HeLa cells arrested in G_1, S and G_2/M phases with [^{32}P]orthophosphate. Using anti-cdc25B antibodies a band at 63-66 kDa was immunoprecipitated from S (lane 2) and G_2/M phase (lane 3) cells but was absent in cells from G_1 phase (lane 1; Fig. 4). No incorporation of phosphate was observed in

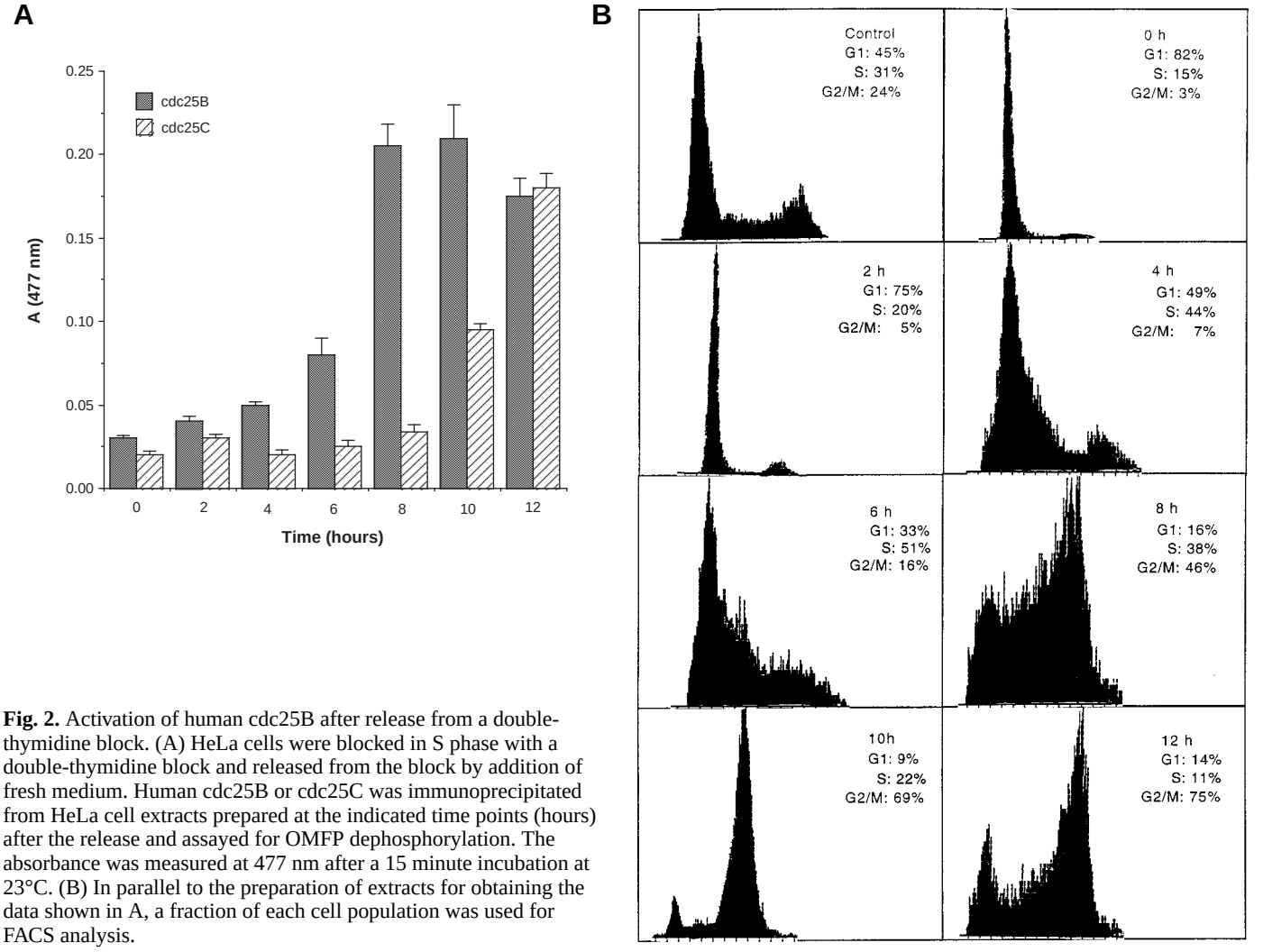

Fig. 2. Activation of human cdc25B after release from a double-thymidine block. (A) HeLa cells were blocked in S phase with a double-thymidine block and released from the block by addition of fresh medium. Human cdc25B or cdc25C was immunoprecipitated from HeLa cell extracts prepared at the indicated time points (hours) after the release and assayed for OMFP dephosphorylation. The absorbance was measured at 477 nm after a 15 minute incubation at 23°C. (B) In parallel to the preparation of extracts for obtaining the data shown in A, a fraction of each cell population was used for FACS analysis.

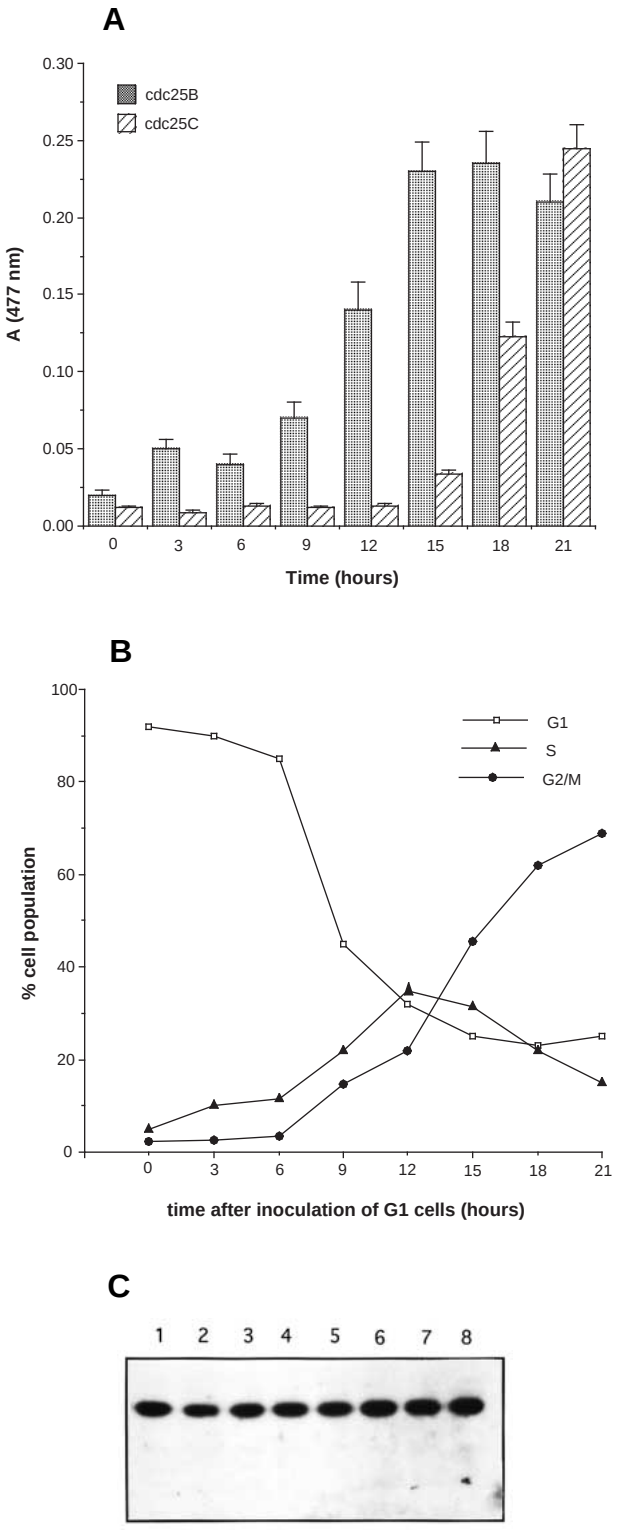

Fig. 3. Cell cycle-dependent activity of cdc25B in cells synchronized by centrifugal elutriation. HeLa cells grown in suspension were separated on the basis of size by centrifugal elutriation. Cells enriched in G_1 were re-inoculated in fresh medium and sampled at the indicated time intervals. The fractions were divided (A) to analyze the activities of cdc25B and cdc25C immunoprecipitations on OMFP as substrate, or (B) for flow cytometry analysis of DNA content, and (C) to determine the abundance of cdc25B in each sample by immunoblotting with anti-cdc25B antibodies.

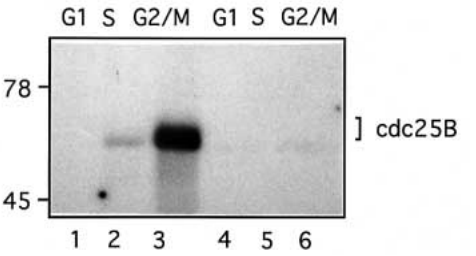

Fig. 4. Phosphorylation of cdc25B in vivo. Immunoprecipitations of HeLa cells labelled in vivo by [^{32}P]orthophosphate using the affinity-purified anti-cdc25B antibodies. Lanes 1 and 4, HeLa cells in G_1; lanes 2 and 5 hydroxyurea-arrested HeLa cells in S-phase; lanes 3 and 6, nocodazole arrested HeLa cells in G_2/M phase. Immunoprecipitations were performed with either cdc25B antibodies (lanes 1-3) or preimmune serum (lanes 4-6).

control immunoprecipitations during the cell cycle (Fig. 4, lanes 4-6). The phosphorylated band observed in G_2/M phase was slightly shifted in molecular mass and stronger than the phosphorylated band observed in immunoprecipitations from S-phase cells, indicating that additional or different kinase(s) are involved in the G_2/M phosphorylation of cdc25B. Taken together these data suggest that human cdc25B undergoes activation through phosphorylation during S-phase.

cdc25B phosphatase is activated by phosphorylation in vitro

To test whether the GST-cdc25B2 fusion protein could be modulated by phosphorylation in vitro, we incubated it with extracts from cells at different stages of the cell cycle in the presence of [γ-^{32}P]ATP. The products were repurified on GSH-Sepharose beads and analyzed on SDS-PAGE. The autoradiogram is shown in Fig. 5A. In cell extracts from G_2 and M phases radioactive phosphate was incorporated into the protein. We observed a lower level of phosphate incorporation into cell extracts from S phase, whereas little was incorporated into cdc25B incubated in G_1 phase cells. To study the possible effect of phosphorylation on the activity of cdc25B2, the protein was incubated either in extracts from G_1, S, G_2 or M phase HeLa cells, repurified on GSH-Sepharose and then assayed for OMFP hydrolysis. We found that phosphorylation of GST-cdc25B2 in S, G_2 and M phase extracts but not in G_1 phase extracts resulted in an increase in its phosphatase activity (Fig. 5B). Although we observed an increase of phosphorylation levels during G_2 phase these data indicate that the activating phosphorylation might occur in extracts from S-phase and that additional phosphorylations during G_2 and M phases do not lead to a further activation. The activation of cdc25B2 in extracts from S, G_2 and M phase was abolished by treatment of the GST-cdc25B2 fusion protein bound to GSH Sepharose with lambda protein phosphatase (Fig. 5B) which completely dephosphorylated the cdc25B2 band. Fig. 5C shows the cdc25B protein levels under the conditions used to synchronize the cells. In extracts from mitotic HeLa cells, a slight upshift in molecular mass of the cdc25B2 protein was observed. A similar pattern was observed by Gabrielli et al. (1997a). These results suggest that human cdc25B2 undergoes activation upon phosphorylation during S phase in vitro.

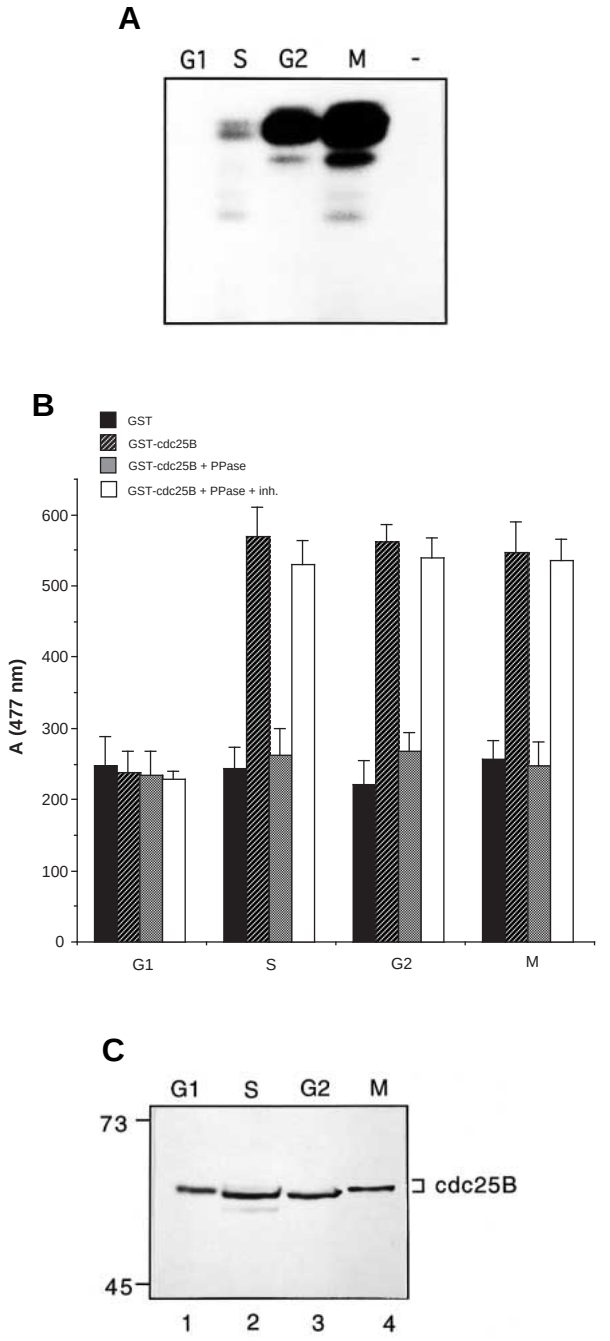

Fig. 5. Phosphorylation and activation of human cdc25B in vitro. (A) For the in vitro-phosphorylation experiments, GST-cdc25B2 protein (5 μg) was incubated in the presence of 0.4 mM ATP, 40 μCi [γ-^{32}P]ATP and 10 mM $MgCl_2$ in extracts prepared from synchronized HeLa cells, (-) control without addition of GST-cdc25B. (B) In vitro activation of cdc25B during the cell cycle. GST-cdc25B2 (hatched bars) or GST protein alone (black bars) was incubated in extracts from HeLa cells synchronized in G_1, S, G_2 and M phase (see Materials and Methods for details) in the presence of 200 μM ATP, bound to GSH Sepharose and assayed for OMFP dependent phosphatase activity. Fractions of phosphorylated GST-cdc25B2 protein was further incubated with λ phosphatase (New England BioLabs) in the presence (dotted bars) and absence (open bars) of inhibitors. The absorbance was measured at 477 nm after a 15 minute incubation at 23°C (C) levels of cdc25B protein in HeLa cell extracts from G_1 (lane 1), S (lane 2), G_2 (lane 3) and M-phase (lane 4).

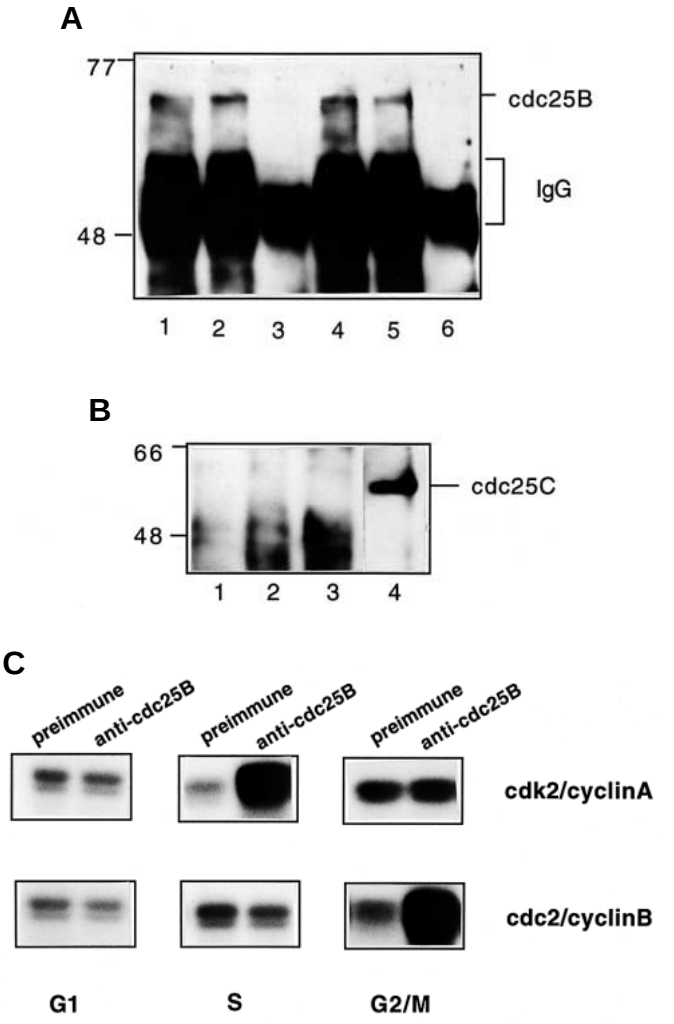

Fig. 6. Activation of cyclin-dependent kinases by cdc25B. (A) immunoprecipitations of cdc25B from HeLa cell extracts with anti-cdc25B antibodies (lanes 1, 2 and 4, 5) or control antibodies (lanes 3 and 6) followed by immunoblotting with cdc25B antibodies; lane 1: exponentially growing HeLa cells; lane 2: HeLa cells in G_1-phase; lane 3: control immunoprecipitations from HeLa cells in S-phase using preimmune serum; lane 4: HeLa cells in S-phase; lane 5: HeLa cells in G_2/M phase; lane 6: control immunoprecipitations from G_2/M phase HeLa cells using preimmune serum (B) cdc25C levels in HeLa cells extracts from G_1 (lane 1), S (lane 2) and G_2/M phase cells (lane 3) as measured by immunoprecipitation of cdc25B and subsequent western blotting, in vitro translated cdc25C protein (lane 4), (C) Cdc25B phosphatase was immunoprecipitated from extracts of HeLa cells in S-phase (aphidicolin block) or G_2/M-phase (aphidicolin block and release for 6 hours) and incubated with either soluble cdk2/cyclinA or cdc/cyclinB as substrates. As a control preimmune serum was used in the precipitation experiments. The ability of cdc25B to activate cyclin-dependent kinases was monitored by histone H1 phosphorylation.

cdc25B from S-phase and M-phase exhibits different substrate specificities towards cyclin-dependent kinases

To investigate the substrate specificity of cdc25B we immunoprecipitated cdc25B phosphatase from different phases of the cell cycle and assayed the activity on inactive

cdc2/cyclinB and cdk2/cyclinA (both were phosphorylated on Tyr15) complexes as substrates. Fig. 6A demonstrates that equal amounts of cdc25B were immunoprecipitated from HeLa cell extracts in G_1, S or G_2/M phase. The immunoprecipitates did not contain any detectable cdc25C protein (Fig. 6B). Activation of the cyclin-dependent kinases by cdc25B was monitored upon their ability to phosphorylate histone H1. We found that cdc25B when immunoprecipitated from S-phase has a higher substrate specificity for cdk2/cyclin A than for cdc2/cyclinB kinase. Cdc25B phosphatase immunoprecipitated from G_2/M phase HeLa cells, however, reveals a higher substrate specificity for the cdc2/cyclinB complex (Fig. 6C). Cdc25B immunoprecipitated from G_1-phase was not active towards either cdk2/cyclinA or cdc2/cyclinB kinases. These results indicate that the substrate specificity of cdc25B is cell cycle dependent.

Microinjection of cdc25B antibodies blocks entry into mitosis

On the basis of the activation studies it is conceivable that cdc25B might act as a mitotic regulator. To study the in vivo function, we used the protocol of scattered microinjection described by Lane and Nigg (1996). Equal concentrations (3.2 mg/ml) of affinity purified antibodies against cdc25B or control antibodies (rabbit IgG) were microinjected into the cytoplasm or the nucleus of asynchronously growing human foreskin fibroblasts (Hs68) with the aim of specifically neutralizing the cdc25 products and evaluating the effect on the progression of M phase. After the injection cells were incubated for 48 hours and were then fixed and stained with secondary antibodies to visualize the injected proteins by immunofluorescence. In five independent experiments of the control (IgG)-injected cells, >85% proceeded through mitosis whereas only 6% of the cdc25B injected cells did (Table 1). Neutralising the antibody by prior incubation with the recombinant GST-cdc25B fusion protein abolished the inhibition. As a positive control for a mitotic block antibodies against cdc25C were injected into exponentially growing Hs68 cells. Similar data were also obtained from HeLa cells (data not shown). These results clearly indicate that inhibition of cdc25B function blocks entry into mitosis.

In another series of microinjection experiments we asked whether cdc25B may play an additional role for the progression through mitosis. We used exponentially growing Hs68 cells that had already established a mitotic spindle as assessed by phase contrast microscopy. We found that 95% of the cells had completed cytokinesis if they were either microinjected with cdc25B or control antibodies (Table 2).

We have shown previously that cdc25B phosphatase activity increases during S phase. We therefore wished to determine whether microinjection of antibodies specific to cdc25B would also interfere with DNA synthesis. Hs68 human fibroblasts were arrested during G_0 by serum starvation for three days and then released into the cell cycle by serum addition. Cdc25B antibodies were microinjected into the cytoplasm or the nucleus of Hs68 cells at five hours (mid G_1 phase) after the addition of FCS. As a positive control we also microinjected cdc25A antibodies to block DNA synthesis while microinjection of cdc25C antibodies and rabbit IgG served as negative controls. Immediately after the injections BrdU was added to the cells and these were further cultured for 40 hours.

Table 1. Effect of anti-cdc25B antibodies on the division of Hs68 human fibroblasts

Antibody	Cells injected	Normally divided cells	%
Anti-cdc25B	276	16	6
Anti-cdc25B + cdc25B-GST	205	177	83
Control IgG	243	211	87
Anti-cdc25C	196	17	9

Asynchronously growing Hs68 cells were microinjected during interphase with either control IgG (rabbit IgG) or cdc25B antibodies pretreated with excess of recombinant cdc25B-GST protein. As a positive control microinjections were performed with anti-cdc25C antibodies. Cells were fixed after 48 hours and analyzed by immunofluorescence microscopy.

The total results from 5 independent experiments are shown. Cells have gone through at least one normal cell division.

Table 2. Effect of anti-cdc25B antibodies on the division of Hs68 cells injected during metaphase

Antibody	Cells injected	Normally divided cells	%
Control IgG	174	156	89.5
Anti-cdc25B	161	148	92

Metaphase Hs68 human fibroblasts were microinjected with anti-cdc25B antibodies or control IgG. 24 hours after injection, cells were fixed and analyzed by immunofluorescence microscopy. The percentage of injected cells that had divided were calculated. The results from four independent experiments are shown.

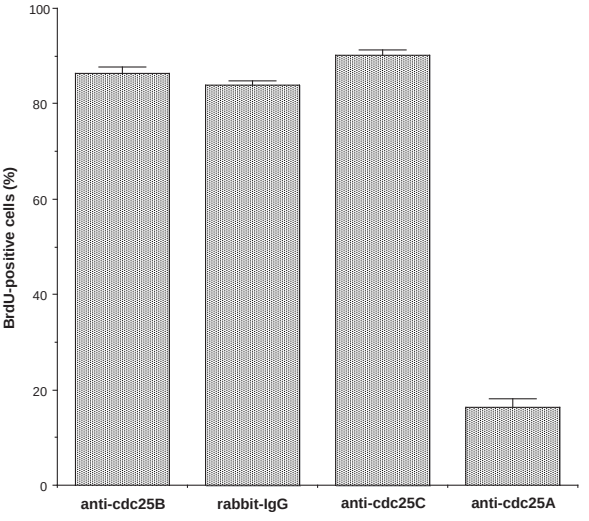

Fig. 7. Injection of anti-cdc25B antibodies has no effect on S phase entry. Asynchronously growing Hs68 cells were arrested in G_0 by serum withdrawal and released back into the cell cycle by readdition of serum. Cells were microinjected in either the nucleus or the cytoplasm at 5 hours after the readdition of serum. BrdU was added at the time of microinjection and the cells were fixed 24 hours after serum stimulation. BrdU-positive cells (%) were calculated as the ratio of injected BrdU-positive cells to BrdU-positive surrounding cells ×100. The results are the mean of four independent experiments.

After fixation, the ability of injected cells to incorporate BrdU into DNA was assessed using an anti-BrdU monoclonal antibody. For all injections (except cdc25A antibody injections), similar proportions of cells had incorporated BrdU, regardless whether they had been injected with cdc25B or control antibodies (Fig. 7). Similarly, there was no difference on the effect of S-phase entry whether cdc25B antibodies were microinjected into the nucleus or the cytoplasm. These data indicate that cdc25B antibodies do not block DNA synthesis.

DISCUSSION

cdc25B is specifically required for entry into mitosis

Unlike yeast, mammalian cells contain at least three distinct cdc25 homologs. Mammalian cdc25 proteins share a highly conserved C-terminal catalytic domain but a distinct N-terminal regulatory domain suggesting that they exert different functions during the cell cycle. It has been reported previously that cdc25A is required for entry into S phase (Hoffmann et al., 1994; Jinno et al., 1994) while cdc25C acts as a mitotic inducer (Millar et al., 1991).

In this study, we have explored the function of the human cdc25B phosphatase. Parallel measurements of cdc25B and cdc25C phosphatase activities show that cdc25B phosphatase activity first appears during late S phase and peaks during G_2. Cdc25B activation clearly occurs prior to the activation of the mitotic inducer cdc25C. These findings raise the question of how cdc25B activity is regulated. The protein levels of cdc25B appear to be constant during the cell cycle in HeLa cells, a pattern of expression that has previously been observed for both cdc25A and cdc25C (Millar et al., 1991; Jinno et al., 1994). The activity of these phosphatases is regulated by cell cycle dependent phosphorylation (Hoffmann et al., 1993, 1994). Therefore it seems conceivable that the activity of cdc25B may be regulated by post-translational modifications as well. We show that cdc25B undergoes phosphorylation during S phase. In fact, we found that the phosphatase activity of cdc25B in vitro is dependent upon phosphorylation. This phosphorylation increases cdc25B phosphatase activity threefold. Using highly specific antibodies against human cdc25B in microinjection experiments we show that cdc25B function is required for entry into mitosis. The fact that human cdc25A is activated during G_1 and our findings showing that cdc25B is active earlier in the cell cycle than cdc25C implies that the individual members of the cdc25 phosphatase family regulate distinct cell cycle checkpoints.

Human cdc25B as a putative trigger of the positive feedback loop at the G_2/M phase transition

Our knowledge of how entry into mitosis is regulated is becoming more clear, although it is still not complete. It is now widely accepted that this transition is governed by an autocatalytic mechanism which is responsible for the rapid activation of cdc2/cyclinB kinase through dephosphorylation by cdc25C (Kumagai and Dunphy, 1992; Izumi et al., 1992; Hoffmann et al., 1993). Phosphorylation and activation of cdc25C is dependent on active cdc2/cyclinB complex (Hoffmann et al., 1993; Izumi and Maller, 1993) creating an autocatalytic feedback loop. The mystery of how the loop is initiated is still unsolved. Small amounts of either active cdc25C or cdc2/cyclinB would be sufficient to trigger the initiation of the feedback loop.

A possible candidate for the activity that triggers the positive feedback mechanism would be Plx1, a kinase related to the Polo family of protein kinases (Plk). Plx1 has recently been described as a stimulator of cdc25 phosphatase activity in *Xenopus* (Kumagai and Dunphy, 1996). In this respect, depletion and reconstitution experiments will be required to show that Plks are essential for the activation of cdc25C in *Xenopus*. Plk1, the human homolog of *Xenopus* Plx1, is required for entry into M phase, the pattern of activation of this kinase seems to be indistinguishable from that of cdc2/cyclinB (Goldsteyn et al., 1995; Lane and Nigg, 1996). In this respect it has to be proven if Plk1 can activate human cdc25C by phosphorylation. Furthermore it will be necessary to study the exact timing and activation of cdc25C in comparison to that of Plk1.

Alternatively, the feedback loop may be initiated by dephosphorylation of cdc2 by phosphatases other than cdc25C. Our results show that cdc25B activity precedes that of cdc25C indicating that cdc25B would be a good candidate for the trigger that starts the positive feedback mechanism. According to this model cdc25B activates small amounts of cdc2/cyclinB which then leads to phosphorylation and activation of cdc25C thus starting off the autocatalytic feedback mechanism. When this work was in progress Nishijima et al. (1997) reported that cdc25B is an unstable protein and could therefore act as a trigger for the entrance of mitosis. Furthermore, immunodepletion of hamster cdc25B from extracts of BHK1 cells prevented the activation of the cdc2/cyclinB complex. Our results are in agreement with the latter findings. However, these results only indicate that cdc25B might be the starter phosphatase. To prove that cdc25B can trigger the initiation of the feedback mechanism it is essential to show that entry into mitosis is dependent on active cdc25B phosphatase. To demonstrate that cdc25B is a starter for the mitotic activation of cdc2/cyclin B we show that cdc25B exerts a crucial function at the onset of mitosis. Ablation of cdc25B function by microinjection of specific antibodies that neutralize cdc25B phosphatase activity prevents entry into mitosis. In addition the results by Gabrielli et al. (1996) support our findings since overexpression of a dominant-negative mutant of cdc25B resulted in a accumulation of G_2/M phase HeLa cells. Although the data presented in this work and by Nishijima et al. (1997) strongly suggest that cdc2/cyclin B is the direct target of cdc25B we cannot exclude the possibility that cdc25B activates a kinase upstream of cdc2/cyclinB or a subpopulation of cdc2 as suggested by Gabrielli et al. (1996).

Since cdc25B is already active in extracts from S phase a mechanism must exist that prevents premature activation of the cdc2/cyclinB complex. This problem could be solved by a different subcellular localization or compartmentalization of the two proteins. Cdc25B is predominantly localized to the nucleus during S-phase and translocates to the cytoplasm during G_2 (Gabrielli et al., 1996, 1997b). Since cyclin B is cytoplasmic throughout interphase these data would suggest that cdc25B activates cdc2/cyclinB in the cytoplasm thus initiating the feedback mechanism. In addition cdc25B when immunoprecipitated from G_2-phase has an increased affinity for cdc2/cyclinB. In vitro, hyperphosphorylation of cdc25B by mitotic extracts also leads to an increased activity towards cdc2/cyclinB but not cdk2/cyclinA (Gabrielli et al., 1997b).

Cdc25B from S-phase extracts has a higher substrate specificity towards cdk2/cyclinA than cdc2/cyclinB suggesting that both substrate specificity and subcellular localization might be critical parameters to prevent premature activation of the mitotic kinase cdc2/cyclin B.

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