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Activation of the phosphatase activity of human cdc25A by a cdk2–cyclin E dependent phosphorylation at the G_1/S transition

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Progression through the cell cycle is monitored at two major points: during the G_1/S and the G_2/M transitions. In most cells, the G_2/M transition is regulated by the timing of $p34^{cdc2}$ dephosphorylation which results in the activation of the kinase activity of the cdc2–cyclin B complex. The timing of $p34^{cdc2}$ dephosphorylation is determined by the balance between the activity of the kinase that phosphorylates $p34^{cdc2}$ (wee1 in human cells) and the opposing phosphatase (cdc25C). Both enzymes are regulated and it has been shown that cdc25C is phosphorylated and activated by the cdc2–cyclin B complex. This creates a positive feedback loop providing a switch used to control the onset of mitosis. Here, we show that another member of the human cdc25 family, cdc25A, undergoes phosphorylation during S phase, resulting in an increase of its phosphatase activity. The phosphorylation of cdc25A is dependent on the activity of the cdc2–cyclin E kinase. Microinjection of anti-cdc25A antibodies into G_1 cells blocks entry into S phase. These results indicate that the cdc25A phosphatase is required to enter S phase in human cells and suggest that this enzyme is part of an auto-amplification loop analogous to that described at the G_2/M transition. We discuss the nature of the *in vivo* substrate of the cdc25A phosphatase in S phase and the possible implications for the regulation of S phase entry.

Key words: cdc25/cdk/cell cycle/cyclin E/phosphorylation

Introduction

In eukaryotes, cell cycle progression is regulated at two major checkpoints: just before the G_1/S transition [a point called START in yeast and 'Restriction point' (R) in vertebrate cells] and at the G_2/M transition. These checkpoints involve the regulation of serine/threonine protein kinases belonging to the cyclin-dependent kinase (cdk) family. Genetic and biochemical studies have shown that in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* the cdc2 (CDC28) protein kinase is required at both the G_1/S and G_2/M transitions (for review see Forsburg and Nurse, 1991). In mammalian cells, the homologue of the yeast cdc2 gene product is involved in controlling the G_2/M transition in association with a cyclin B regulatory subunit (Draetta, 1991). Other cdks control the G_1/S transition and progression through S phase in association with various cyclins (D, E and A; Pines, 1993).

The timing of entry into mitosis is mostly determined by post-translational control of the kinase activity of the cdc2–cyclin B complex. In this complex, the cdc2 subunit contains phosphorylation sites on tyrosine, threonine and serine residues (Draetta and Beach, 1988), the phosphorylation of which varies during the cell cycle (Draetta *et al.*, 1988; Gautier *et al.*, 1989; Morla *et al.*, 1989). Phosphorylation on Thr14 and Tyr15, close to the ATP binding site (Gould and Nurse, 1989; Krek and Nigg, 1991a,b; Solomon *et al.*, 1992) is inhibitory and prevents immediate activation of newly formed cdc2–cyclin B complexes. Phosphorylation of Tyr15 is carried out by homologues of the *S.pombe* protein kinases wee1 and mik1 (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren *et al.*, 1991; Parker and Piwnica-Worms, 1992; Parker *et al.*, 1992; McGowan and Russell, 1993). Dephosphorylation of Tyr15 and Thr14 is achieved by the *S.pombe* cdc25 gene product or its homologues (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Millar *et al.*, 1991a; Strausfeld *et al.*, 1991; Lee *et al.*, 1992). Three such homologues have been identified in both human and murine cells, denoted cdc25A, B and C (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Nagata *et al.*, 1991; Kakizuka *et al.*, 1992; Gautier, 1993). The timing of activation of the cdc2–cyclin B complex, and therefore of entry into mitosis, depends on the relative activities of wee1 and cdc25C, which are both regulated by phosphorylation. cdc25C becomes phosphorylated during mitosis and this stimulates directly its ability to dephosphorylate cdc2 (Kumagai and Dunphy, 1992; Izumi *et al.*, 1992; Hoffmann *et al.*, 1993; Izumi *et al.*, 1993; Strausfeld *et al.*, 1994). The predominant kinase responsible for the phosphorylation of cdc25C in mitotic HeLa cells or human fibroblasts is cdc2–cyclin B itself and this creates an autocatalytic positive feedback loop (Hoffmann *et al.*, 1993; Strausfeld *et al.*, 1994). This loop can function as a switch to govern the entry into mitosis and it is probably at the heart of the regulation of M phase entry by unreplicated DNA or other checkpoint controls monitoring the completion of the S and G_2 phases (Clarke *et al.*, 1993).

It is still unclear exactly how the G_1/S transition is governed. In higher eukaryotes, the successive expression of different cdks associated with specific cyclins seems to drive progression through G_1, beyond R and during S phase (Ninomiya-Tsuji *et al.*, 1991; Tsai *et al.*, 1991; Matsushime *et al.*, 1992; Meyerson *et al.*, 1992). The cdk2 kinase, for instance, which is involved in the activation of DNA synthesis (Pagano *et al.*, 1993), associates with cyclin A or cyclin E and its activity is maximal during the S and G_2 phases (Rosenblatt *et al.*, 1992). Before it becomes active, the cdk2–cyclin A complex is phos-

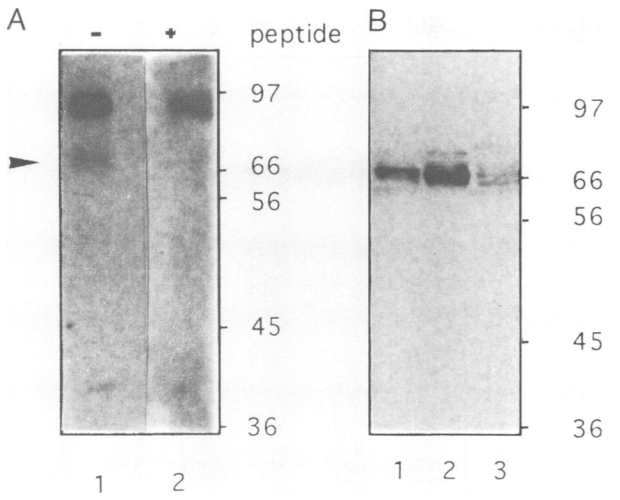

Fig. 1. Characterization of anti-cdc25A antibodies. (**A**) Immunoprecipitation of cdc25A from asynchronous HeLa cells with anti-cdc25A antiserum in the absence (−; lane 1) or presence (+; lane 2) of antigenic peptide, followed by immunoblotting with the same antiserum. The cdc25A protein is marked by an arrow. The band at 90 kDa corresponds to immunoglobulins. Molecular weight markers are given in kDa. (**B**) Immunoblot of cell extracts with the anti-cdc25A antibody. Lane 1, [^{35}S]methionine-labelled *in vitro*-translated cdc25A marker; lane 2, 100 μg of HeLa cell extract; lane 3, 100 μg of IMR-90 cell extract. Lanes 2 and 3 are immunoblots carried out with affinity-purified anti-cdc25A antibodies.

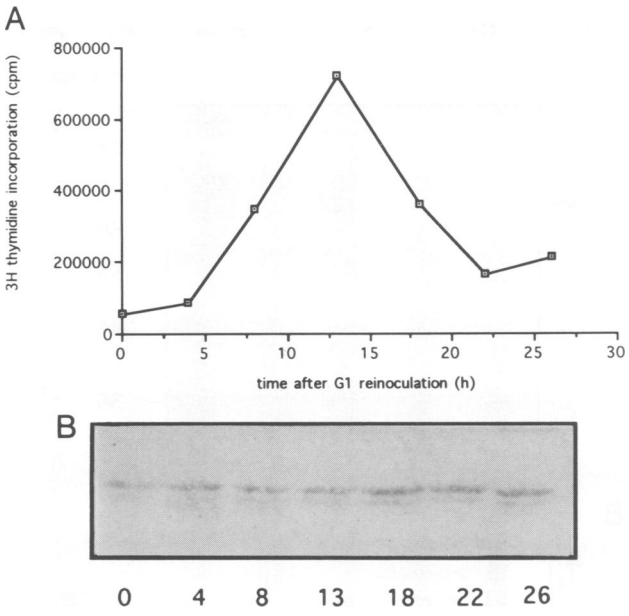

Fig. 2. Level of cdc25A protein during the cell cycle. HeLa cells grown in suspension were separated on the basis of size by centrifugal elutriation. G_1-enriched cells were re-inoculated in fresh medium and sampled at the indicated time intervals. (**A**) The graph shows the rate of DNA synthesis determined by [^{3}H]thymidine incorporation; (**B**) immunoblot analysis of cdc25A protein levels using the anti-cdc25A antibodies, 100 μg of total proteins from HeLa cell lysates were loaded per lane.

phorylated on sites analogous to those found in cdc2 (Gu *et al.*, 1992), suggesting that its mechanism of activation is similar. cdc25C dephosphorylates cdk2 on Thr14 and Tyr15 *in vitro* and increases its kinase activity (Gu *et al.*, 1992). However, cdc25C becomes activated much later in the cell cycle and it seems therefore unlikely that it is the actual enzyme that dephosphorylates and activates cdk2 during S phase.

We report that cdc25A undergoes phosphorylation and activation in S phase of the cell cycle and that this phosphorylation is dependent on the activity of the cdk2–cyclin E complex. Moreover, microinjection of specific antibodies against cdc25A into human fibroblasts blocks their progression into S phase, indicating that cdc25A is essential at this transition point.

Results

The level of cdc25A protein remains constant throughout the cell cycle

To study the regulation and function of cdc25A in the human cell cycle, we raised antibodies against a specific peptide modelled upon the cdc25A N-terminal sequence. The affinity-purified anti-cdc25A antibody immunoprecipitated a protein of M_r 67 000 from asynchronous HeLa cell extracts and stained the immunoprecipitated protein on immunoblots (Figure 1A, lane 1). This protein was not immunoprecipitated in the presence of an excess of the antigenic peptide (lane 2) and a protein of similar size was also detected by immunoblotting of total HeLa or human lung fibroblast (IMR-90) cell extracts. The protein was expressed in much greater amounts in HeLa cells than in the fibroblast cell line IMR-90 (Figure 1B, lanes 2 and 3). No cross-reactivity of the antibody with either cdc25B or cdc25C could be detected (data not shown).

The cdc25A mRNA is expressed mostly in late G_1 and early S phases (Okayama *et al.*, 1992). To determine whether the level of cdc25A protein paralleled the oscillation in cdc25A mRNA level, we monitored its abundance during the HeLa cell cycle. Cells grown in suspension culture were separated by centrifugal elutriation (Draetta and Beach, 1988). As shown in Figure 2 (top), the peak of HeLa cells in S phase appears at 13 h after reinoculation into fresh medium (monitored by [^{3}H]thymidine pulse labelling). Equal amounts of total cell proteins were separated on SDS gels and immunoblotted with the anti-cdc25A antibody. The level of cdc25A remained fairly constant during S phase, with a slight increase (<2-fold) when cells entered G_2 (Figure 2, bottom). This pattern of expression during the cell cycle, in which mRNA levels fluctuate but protein levels do not vary greatly, has previously been described for cdc25C in HeLa cell extracts (Millar *et al.*, 1991b).

Human cdc25A undergoes phosphorylation and activation during S phase

To examine whether there was a change in the phosphorylation level of cdc25A during the cell cycle, we incubated HeLa cells arrested in G_1, S and M phases with [^{32}P]orthophosphate (Figure 3A). A M_r 67 000 phosphorylated band was immunoprecipitated by anti-cdc25A antibodies from S phase (Figure 3A, lane 4) and M phase (lane 6) but not from G_1 phase HeLa cell lysates (lane 2). Addition of excess antigenic peptide to the reaction blocked the immunoprecipitation of the phosphorylated band (Figure 3A, lanes 1, 3 and 5). A strong band of M_r 105 000 co-precipitating with cdc25A in mitotic cells was also observed. On overexposed autoradiograms, a weak

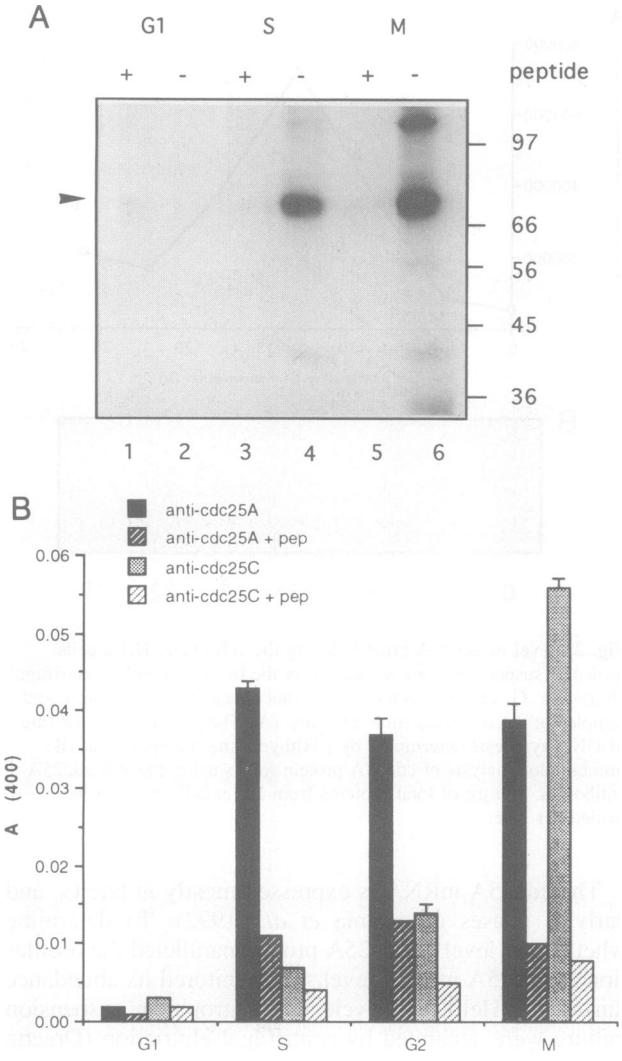

Fig. 3. Phosphorylation and activation of cdc25A *in vivo*. (**A**) Immunoprecipitations of HeLa cells labelled *in vivo* by [^{32}P]orthophosphate using the anti-cdc25A antiserum. Lanes 1 and 2, HeLa cells in G_1; lanes 3 and 4, hydroxyurea-arrested HeLa cells (in S phase); lanes 5 and 6, nocodazole-arrested cells (in mitosis). Immunoprecipitations were performed in the absence (−) or presence (+) of the antigenic peptide. (**B**) Comparison of cdc25A or cdc25C phosphatase activities during the cell cycle. Immunoprecipitates from G_1, S, G_2 or M phase arrested cells were prepared in the presence or absence of antigenic peptide and assayed for pNPP dephosphorylation at 30°C. The absorbance was measured at 400 nm after a 10 min incubation. Results of triplicate assays (± SEM) are shown.

signal was also detected in a protein migrating like phosphorylated cdc25A in immunoprecipitates from G_1 HeLa cell extracts. This may mean that cdc25A is weakly phosphorylated during G_1 or that there is a slight contamination with S phase cells.

To determine whether the phosphorylation of cdc25A was concommitant with an activation of its phosphatase activity, we performed immunoprecipitations with the cdc25A antiserum and assayed the phosphatase activity present in the pellet using *p*-nitrophenylphosphate (pNPP), a chromogenic molecule that is structurally related to phosphotyrosine, as a substrate. As shown in Figure 3B, a weak phosphatase activity was detected in G_1 HeLa cells. Cells in early S phase however, showed a 15- to 20-fold higher phosphatase activity. A similar level of

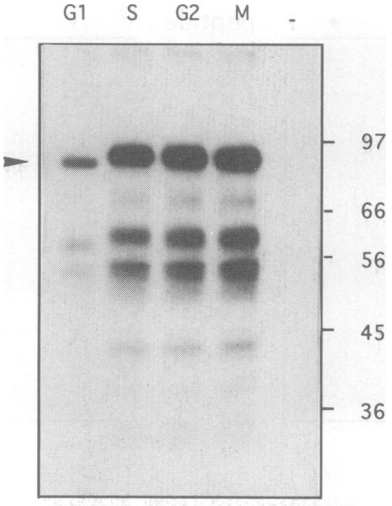

Fig. 4. *In vitro* phosphorylation of cdc25A. Recombinant GST−cdc25A (1 mg) was incubated in the absence (−) or in the presence (other lanes) of 0.5 mM ATP, 50 μCi [γ-^{32}P]ATP and 10 mM $MgCl_2$, in extracts prepared from synchronized HeLa cells (see Materials and methods for details).

activity was detected in G_2 and mitotic cells. The activity of cdc25A in S phase was abolished by prior treatment of the immunoprecipitated pellet with potato acid phosphatase, which completely dephosphorylated the cdc25A band (data not shown). Figure 3B also shows a comparison with the phosphatase activity of cdc25C during the cell cycle. The weak cdc25C activity detected in interphase increased 4-fold during mitosis as previously described (Hoffmann *et al.*, 1993).

Clearly, cdc25A undergoes phosphorylation and activation at the onset of DNA replication, much earlier than cdc25C.

Cdk2−cyclin E-dependent phosphorylation of cdc25A in S phase

In order to examine the nature of the kinase responsible for phosphorylation and activation of cdc25A during S phase, we expressed cdc25A as a fusion protein with glutathione (GST)-*S*-transferase and purified it to near homogeneity by passage over a glutathione (GSH)−Sepharose column. The isolated GST−cdc25A fusion protein had a molecular weight of 93 kDa and was recognized by the cdc25A antiserum. We first tested whether the cdc25A−GST fusion protein could be phosphorylated *in vitro*, by incubating it with extracts prepared from HeLa 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 analysed by SDS−PAGE. The autoradiogram is shown in Figure 4. In cells from S, G_2 and M phases, radioactive phosphate was incorporated into the protein, which also showed a small upshift in molecular weight from 93 to 95 kDa. This upshift did not occur after incubation in G_1 cell extracts in which GST−cdc25A was weakly phosphorylated. No phosphorylation of the GST part alone was observed in control experiments (data not shown). The phosphorylated bands migrating at 54 and 60 kDa, respectively, correspond to C-terminal truncations of the GST−cdc25A protein. These cdc25A fragments are typical degradation products of the

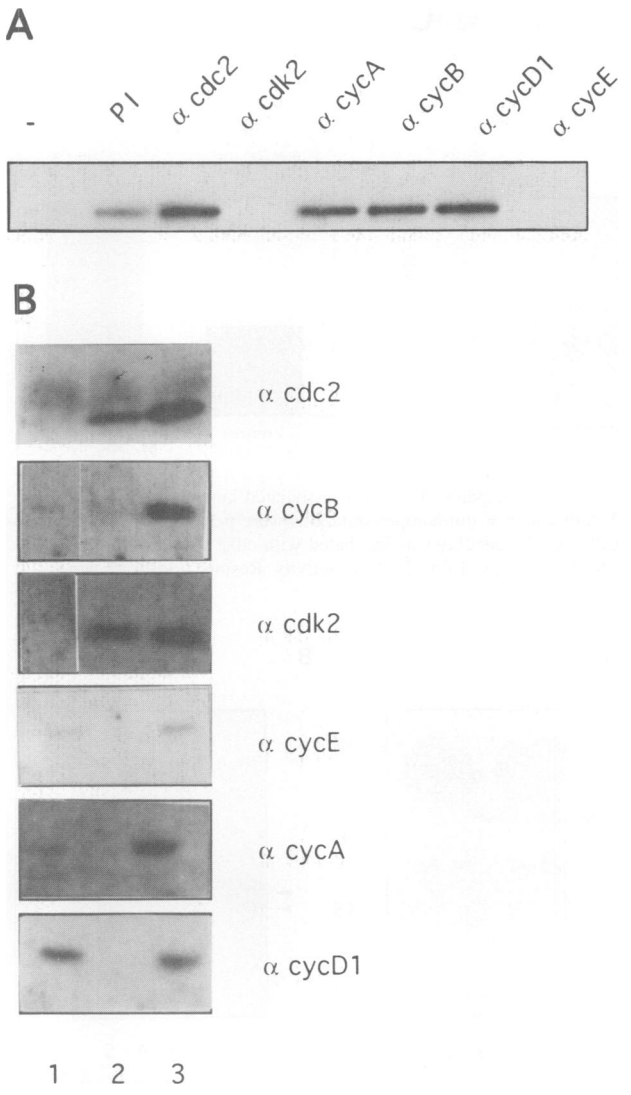

Fig. 5. Depletion of cdk2 or cyclin E from S phase HeLa cell extracts prevents cdc25A phosphorylation. (**A**) Phosphorylation of GST–cdc25A in the absence of extract (–) or in the presence of extracts depleted by control serum (PI), anti-cdc2 antibodies, anti-cdk2 antibodies, anti-cyclin A antibodies, anti-cyclin B antibodies, anti-cyclin D1 antibodies or anti-cyclin E antibodies. (**B**) The depleted S phase extracts were immunoblotted with anti-cdc2, anti-cyclin B, anti-cdk2, anti-cyclin E, anti-cyclin A or anti-cyclin D1 antibodies. Anti-cdc2 and anti-cyclin B panels: lane 1, anti-cdc2 depletion; lane 2, anti-cyclin B depletion; lane 3, control treatment. Anti-cdk2 and anti-cyclin E panels: lane 1, anti-cdk2 depletion; lane 2, anti-cyclin E depletion; lane 3 control treatment. Anti-cyclin A and anti-cyclin D1 panels: lane 1, anti-cdk2 depletion; lane 2, anti-cyclin A or anti-cyclin D1 depletion; lane 3, control treatment.

preparation of the fusion protein that are still able to bind to GSH–Sepharose. Phosphorylation of GST–cdc25A in S phase cell extracts also resulted in an increase in its phosphatase activity (data not shown). Having shown that recombinant cdc25A could be phosphorylated in S phase extracts in the same way as *in vivo*, we set out to determine whether one of the known cdk–cyclin complexes was responsible for this event. We depleted cdks or cyclins from extracts prepared from S phase HeLa cells with specific antibodies and tested the capacity of the depleted extracts to phosphorylate cdc25A (Figure 5A). Extracts treated with anti-cdk2 or anti-cyclin E antibodies lost their ability to phosphorylate cdc25A, but removal of cdc2, cyclin A, cyclin B or cyclin D1 had no effect. The immunodepletion efficiency in each case is shown on the Western blot of Figure 5B (see figure legend for details).

This result indicated that a cdk2–cyclin E kinase was essential during S phase for the phosphorylation of cdc25A. To test whether the cdk2–cyclin E complex could phosphorylate cdc25A directly, we immunoprecipitated the complex from S phase cells using cyclin E antibodies and assayed its kinase activity on both cdc25A (Figure 6A) and histone H1 (Figure 6B). As shown in Figure 6A, incubation of GST–cdc25A with cdk2–cyclin E resulted in a strong incorporation of radioactive phosphate into the protein. Phosphorylation of the GST part of the fusion protein by cdc2–cyclin E kinase was not observed (data not shown). To confirm further the role of cdk2–cyclin E in the activation of cdc25A, we incubated cdc25A with immunocomplexes of cdk2–cyclin E from S phase extracts, repurified cdc25A and then assayed its phosphatase activity. Figure 6C shows that cdc25A was activated 2- to 3-fold when phosphorylated by the cdk2–cyclin E complex. These data showed that cdk2–cyclin E could phosphorylate cdc25A and suggested that it was one of the kinases involved in its activation at the G_1/S transition *in vivo*.

To investigate further the interaction between cdc25A and cdk2, we tested the possibility that cdc25A directly associates with cdk2 in S phase. Extracts of HeLa cells in S phase were immunoprecipitated with anti-cdc25A or anti-cdk2 antibodies and probed with the same anti-cdc25A antibodies on the immunoblot. Figure 7A (lane 2) shows that the cdk2 immunoprecipitate contains a 67 kDa band that co-migrates with cdc25A. Addition of the antigenic peptide to the immunoprecipitation reaction with cdk2 antibodies abolished the appearance of the cdc25A band. These results indicate that cdc25A stably associates with cdk2 in S phase. We also tested if cdc25A physically interacts with cyclin E. Figure 7B lane 4 shows that a doublet of 45–50 kDa co-precipitates with cdc25A antibodies and is recognized by monoclonal anti-cyclin E antibodies. The experiments described above suggest that cdc25A associates with cdk2–cyclin E kinase *in vivo*.

cdc25A is required for entry into S phase

The activation of cdc25A in S phase suggested that this enzyme could perform an essential function during this phase of the cell cycle. We tested this hypothesis by microinjecting cdc25A antibodies into G_1 cells to test whether they could still enter S phase. We chose to use a human diploid fibroblast (IMR-90) cell line for these studies because they can easily be arrested in G_0 by serum deprivation and released into the cell cycle by serum re-addition. Affinity-purified cdc25A or cdc25C antibodies were microinjected into G_1 cells (11 h after serum re-addition) and bromodeoxyuridine (BrdU) was added just after microinjection to monitor DNA synthesis. Twenty-four hours after serum addition, the cells were fixed and stained to detect the injected anti-cdc25 antibodies and BrdU incorporation.

The results of a typical microinjection experiment are shown in Figure 8. Photographs of cells microinjected with anti-cdc25C (A, C and E) and anti-cdc25A (B, D and F) are shown. Microinjection of anti-cdc25A blocked

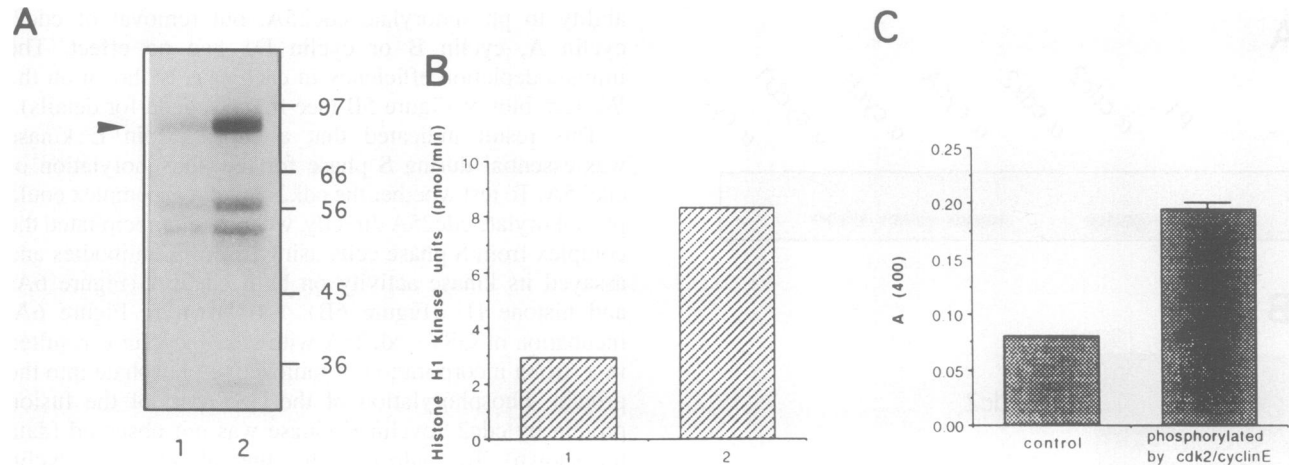

Fig. 6. cdk2−cyclin E kinase phosphorylates and activates cdc25A *in vitro*. (**A**) Immunoprecipates of cyclin E-associated kinase were mixed with recombinant cdc25A or (**B**) with histone H1 in the presence of radioactive ATP. Anti-cyclin E immunoprecipitations were performed from 0.5 mg (lanes 1) or 2.5 mg (lanes 2) of S phase HeLa cell extract, respectively. (**C**) Purified GST−cdc25A was incubated with cdk2−cyclin E immunocomplex or with control immunocomplexes, re-purified on GSH−Sepharose and assayed for pNPPase activity. Results from triplicate samples (± SEM) are shown.

DNA synthesis while cdc25C antibodies did not have an effect. We quantified the data by counting the number of cells having incorporated BrdU in non-injected cells, in cells injected with anti-cdc25A or anti-cdc25C in three different experiments (Figure 9). When the microinjection of cdc25A antibodies was carried out at 11 h after serum addition, a strong inhibition of DNA synthesis was observed while there was no effect of cdc25C antibodies detected. The efficiency of DNA synthesis inhibition decreased in cells microinjected at later time points (18 h) after restimulation of the cells (not shown). The specific inhibitory effect of the affinity-purified anti-cdc25A antibodies was also demonstrated by the fact that co-injection of the antigenic cdc25A peptide completely abolished the inhibition of entry into S phase (Figure 9). These results clearly indicate that inhibition of cdc25A function during G_1 blocks entry into S phase.

Subcellular localization of cdc25A

In asynchronous cells, the immunofluorescence signal was found predominantely in the nucleus (Figure 10). This staining was specific as pre-incubation of the antibody with the antigen abolished the staining. We did not find a significant change in subcellular distribution of cdc25A during the cell cycle (data not shown).

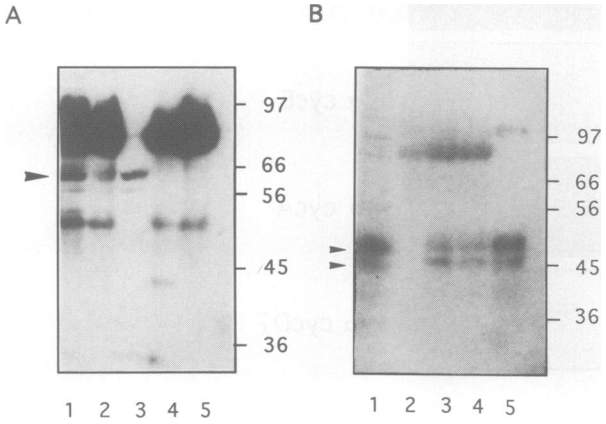

Fig. 7. Cdc25A interacts with cdk2−cyclin E during S phase. (**A**) Immunoprecipitates (IP) prepared with cdc25A or cdk2 antibodies were separated on gels and immunoblotted with cdc25A antibodies. Lane 1, anti-cdc25A IP; lane 2, anti-cdk2 IP; lane 3, anti-cdc25A IP boiled for 20 min at 95°C; lanes 4 and 5, anti-cdc25A or anti-cdk2 IP prepared in the presence of the corresponding antigenic peptide. (**B**) Immunoprecipitates prepared with cdc25A, cdk2, cyclin E or a control serum (preimmune) or an Hela extract were separated on gels and immunoblotted with cyclin E antibodies. Lane 1, HeLa cell extract; lane 2, control serum; lane 3, anti-cdk2; lane 4, anti-cdc25; lane 5, anti-cyclin E. The bands at 90 and 50 kDa are immunoglobulins. The positions of cdc25A and cyclin E proteins are marked by arrowheads.

Discussion

Regulation of cdc25A phosphatase activity in S phase

In this paper, we have examined the regulation of the human cdc25A phosphatase. This protein seems to be localized predominantly in the nucleus, as determined by immunofluorescence, in keeping with a nuclear function. We found that the phosphatase activity of cdc25A is increased ~20-fold during S phase, at the same time as it becomes phosphorylated. This phosphorylation is dependent on the activity of the cdk2−cyclin E kinase. cdc25A is essential for the G_1 to S phase transition, as demonstrated by microinjection of specific antibodies into human fibroblasts. This demonstrates that a cdc25 phosphatase, cdc25A, is active at an early stage in the cell cycle (G_1/S transition) and that it is involved in the control of DNA replication most probably through dephosphorylation of cdks required at the G_1/S border.

When this work was in progress Jinno *et al.* (1994) reported that microinjection of antibodies against human cdc25A into normal rat kidney (NRK) cells blocked cell cycle progression at the G_1/S transition. Our results are in agreement with this finding and further show that cdc25A is regulated at this transition. The protein level of cdc25A remains constant during the HeLa cell cycle although in normal rat kidney cells it seems to fluctuate, showing a peak in late G_1 (Jinno *et al.*, 1994). This difference might be explained by the fact that the latter

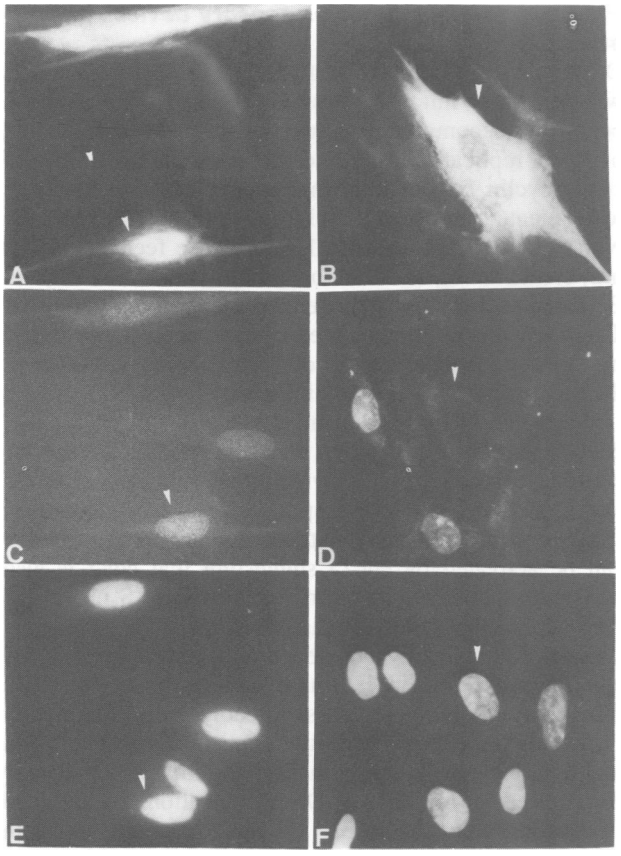

Fig. 8. Microinjection of anti-cdc25A and anti-cdc25C rabbit antibodies into human fibroblasts. Microinjections were performed 11 h after the addition of serum. BrdU was added after the injections and the cells were incubated for another 13 h. Cells were then fixed and stained as described in Materials and methods. (**A** and **B**) Detection of injected cells with Texas red-conjugated anti-rabbit antibodies (cdc25). (**C** and **D**) staining with mouse anti-BrdU antibodies and FITC-conjugated anti-mouse antibody. (**E** and **F**) Hoechst staining. In A, C and E the cells were injected with affinity-purified anti-cdc25C antibodies and in B, D and F with affinity-purified anti-cdc25A antibodies.

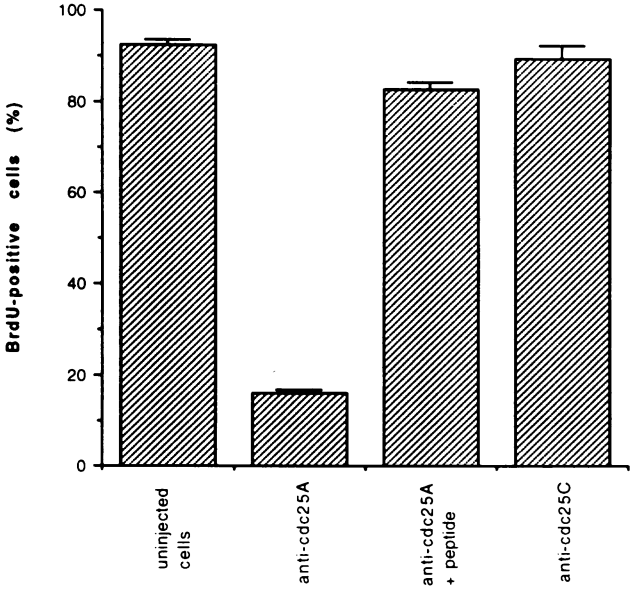

Fig. 9. Quantification of DNA synthesis inhibition after injection of anti-cdc25 antibodies into IMR-90 cells. For each experiment, 100–200 IMR-90 cells were microinjected with anti-cdc25 antibodies 11 h after serum addition. BrdU was added at the time of microinjection and the cells were fixed 24 h 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 (± SEM) of three independent experiments.

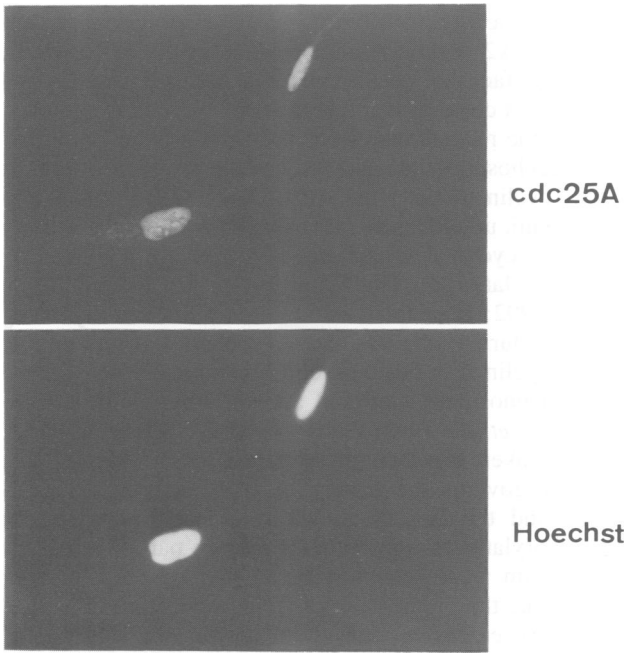

Fig. 10. Localization of cdc25A in human cells. Asynchronous HeLa cells grown on coverslips were washed and fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and probed with (top) affinity-purified anti-cdc25A antibodies and (bottom) Hoechst 33258 as described in Materials and methods. Controls in which the antibodies were pre-incubated with antigenic peptides were negative (not shown).

cell line, in contrast to HeLa cells, is non-transformed. Galaktionov and Beach (1991) reported that microinjection of anti-cdc25A antibodies into exponentially growing HeLa cells caused a mid-mitosis arrest. Our results do not exclude that cdc25A could also act during mitosis since we have not examined this point.

During S phase, phosphorylation of cdc25A is certainly dependent on the activity of cdk2−cyclin E kinase since the GST−cdc25A protein is not phosphorylated when added to extracts depleted from this kinase. Moreover, the purified cdk2−cyclin E complex can phosphorylate directly cdc25A. The cdc25A protein remains phosphorylated and highly active during G_2 and M phases. The cdk2−cyclin E kinase is mostly active in early S phase and rapidly inactivated during S and G_2 phases. Therefore, it is not a good candidate to phosphorylate cdc25A at late S phase and during G_2 phase. We found that the mitotic phosphorylation of cdc25A is partially dependent on the activity of cdc2−cyclin B. However, cdc25A is not a good substrate for cdc2−cyclin B *in vitro* (I.Hoffmann and E.Karsenti, unpublished results). Our results suggest that another kinase may contribute to the phosphorylation and possible activation of cdc25A during mitosis and perhaps also during S phase. This is supported by the difference in activity of cdc25A observed between G_1 and S phase: it is very high (20-fold) when assayed on

immunoprecipitates made from lysed cells, whereas *in vitro* phosphorylation by cdk2–cyclin E causes a modest activation (3-fold). Therefore, it is possible that during the G_1/S transition, another as yet unidentified kinase contributes to the activation of cdc25A in addition to cdk2–cyclin E. On the other hand, a weak phosphorylation of cdc25A was observed during G_1. This phosphorylation could occur at a distinct site and might be required for full cdc25A activation. We still do not know the nature of the phosphatase that dephosphorylates cdc25A and whether it becomes inactivated at the onset of S phase and remains inactive during G_2 and M. However, inactivation of the phosphatase that dephosphorylates cdc25A cannot be the only reason why cdc25A remains phosphorylated beyond S phase because G_2 and M phase extracts retain the capacity to phosphorylate the GST–cdc25A fusion protein.

Substrates of cdc25A phosphatase

One obvious question that comes to mind concerns the nature of the *in vivo* substrate of cdc25A. We know that cdc25C dephosphorylates and activates cdc2–cyclin B (Clarke *et al.*, 1993; Hoffmann *et al.*, 1993; Izumi and Maller, 1993; Strausfeld *et al.*, 1994), but we have not addressed the nature of the *in vivo* substrate of cdc25A experimentally in this paper. *In vitro*, cdc25A can dephosphorylate and activate various cdk–cyclin complexes (I.Hoffmann and E.Karsenti, unpublished). Given the timing of activation of cdc25A, its substrate could very well be cdk2–cyclin A and/or cdk2–cyclin E. However, given the fact that cdc25A remains active during G_2 and M phase, it could also act on the cdc2–cyclin B complex. In fact, the recombinant GST–cdc25A protein can efficiently dephosphorylate and activate the pro-kinase form of cdc2–cyclin B *in vitro*. (P.R.Clarke, I.Hoffmann and E.Karsenti, unpublished). It is also known that the activity of cdk2–cyclin A complexes is negatively regulated by phosphorylation on Thr14 and Tyr15, just like cdc2 (Gu *et al.*, 1992; Rosenblatt *et al.*, 1992), its activity being highest during the S and G_2 phases. Furthermore, cdk2–cyclin A or cdk2–cyclin E complexes were shown to be dephosphorylated and activated by cdc25 *in vitro* (Gabrielli *et al.*, 1992; Gu *et al.*, 1992; Sebastian *et al.*, 1993). Taken together, these data strongly suggest that cdc25A governs the timing of cdk2 activity during S phase and the fact that cdc25A is itself regulated by phosphorylation suggests that it may be part of a sensory mechanism which couples the activity of cdk2 to other events like the progression of DNA replication in early S phase. An extensive analysis of the *in vivo* substrate of cdc25A remains difficult due to the lack of powerful genetics in human cells. Recently, the existence of hitherto unknown cell cycle control proteins, cdk inhibitors (CKIs) have been identified (Gu *et al.*, 1993; Harper *et al.*, 1993; Serrano *et al.*, 1993; Xiong *et al.*, 1993; Dulic *et al.*, 1994). These proteins have been found to form complexes with various cdks and cyclins and prevent the activation of the kinases by a new mechanism. It seems that there are two mechanisms for regulating cdks at least at the G_1 to S transition: the pathway involving cdc25A regulation and that involving CKIs. We propose that they link the cell cycle engine to different aspects of cell growth.

Positive feedback loops as sensory devices and switches?

During the G_2/M transition, it is widely accepted that cells will not enter mitosis unless all S and G_2 events have been fully accomplished (Murray, 1992). In fact, the end of DNA replication seems to feedback on the timing of cdc2–cyclin B activation through the positive feedback loop created by cdc2–cyclin B–cdc25C (Enoch and Nurse, 1991; Clarke *et al.*, 1993). These feedback loops may create timing devices. For example, once cdc2–cyclin B starts to become activated by dephosphorylation of Thr14 and Tyr15, the complex cannot become inactivated by the same mechanism. Cyclin degradation seems to be the mechanism that triggers inactivation of MPF.

We have not formally demonstrated in this article that cdc25A is part of a positive feedback loop as we did for cdc25C. However, this enzyme is phosphorylated at the G_1/S transition and its phosphorylation is dependent on cdk2–cyclin E. Moreover, the cdk2–cyclin E complex can be activated by cdc25 suggesting that cdk2 is also transiently phosphorylated on Tyr15 in early S phase and then dephosphorylated (Sebastian *et al.*, 1993). This may suggest that a positive feedback loop involving cdc25A and the cdk2–cyclin E kinase also exists at the G_1/S transition. cdk2–cyclin A is probably involved directly in the regulation of the progression of DNA replication and cdk2–cyclin E may function slightly earlier. It may be required for initiation of DNA replication and involved in transcriptional control. If cdc25A regulates both enzymes, the loop to which it may belong would be a key player in the temporal control of S phase onset. It may respond to the level of transcription of cyclin E and/or to the progression of DNA replication. It could for example check that all initiation sites have fired to allow cdk2–cyclin A to become activated and trigger the elongation process. In this context, it is interesting that cyclin A does not seem to be required to phosphorylate and activate cdc25A. Since cyclin E seems to function before cyclin A, it could indeed initiate the activation of cdc25A which may then activate cdk2–cyclin A.

These results also give the impression that the cell cycle is composed of blocks of enzymes that function together at different phases of the cell cycle. cdc2–cyclin B functions in concert with cdc25C during the G_2/M transition, whereas cdk2–cyclin E would function in concert with cdc25A at the G_1/S transition. The mechanisms discussed herein might be part of a growing list, all based on the same principle: a cdk stimulates its own activation involving a positive feedback control.

Materials and methods

Cell culture and extract preparations

IMR-90 cells, obtained from the American Type Culture collection, were cultured for no longer than seven passages in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml), in a humidified atmosphere containing 5% CO_2 at 37°C. 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 20% FCS. Cell cycle progression was monitored by measuring BrdU incorporation.

HeLa cells were obtained from the Cold Spring Harbor Tissue Culture facility and cultured as previously described (Giordano *et al.*, 1989). Conditions for cell extract preparations have been previously described

by (Draetta and Beach, 1988; Giordano *et al.*, 1989). Briefly, cells were lysed by adding 3–5 vol of lysis buffer (50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF and 0.1 mM Na_3VO_4) to a cell pellet. The following protease inhibitors were added: 0.1 mM PMSF, 1 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor, 10 mg/ml L-1-chloro-3-(4-tosylamido)-7 amino-2-heptanone hydrochloride (TLCK), 10 mg/ml L-1-chloro-3-(4-tosyl-amido)-4 phenyl 2-butanone (TPCK) and 1 mg/ml aponitin.

For *in vivo* labeling, HeLa cells were incubated with 2 mCi/plate of [^{32}P]orthophosphate in phosphate free DMEM medium for 4 h.

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 Figure 2 fractions enriched in G_1 cells were re-added to the culture at a density of 3×10^5/ml. Progression through the cell cycle was monitored by flow cytometry and [^{3}H]thymidine incorporation. Mitotic shake-off of nocodazole-treated HeLa cells was performed as described by Morla *et al.* (1989). Briefly, cells were treated for 14–18 h with nocodazole (50 ng/ml). After one wash with PBS, mitotic cells were collected by gently pipetting up and down five 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 indicated experiments, 10 mM hydroxyurea or 1 mg/ml aphidicolin were added to asynchronous cells which were then incubated for 18–20 h. This treatment allowed recovery of cells that were 85–90% arrested in early S phase, as assessed by flow cytometry.

Expression and purification of recombinant human cdc25

Human cdc25A (Galaktionov and Beach, 1991) was produced from the bacterial strain DH5α using the pGEX-2T expression vector. After isopropyl β-D-thiogalactoside (IPTG) induction for 4 h, (1 mM final concentration), the 93 kDa GST–cdc25A protein was recovered as described by (Smith and Johnson, 1988) on glutathione–Sepharose beads and eluted by 10 mM glutathione in 50 mM Tris–HCl, pH 8.0, 50 mM NaCl and 1 mM DTT.

Immunochemistry

Monoclonal anti-cyclin E antibodies (HE III, Santa Cruz Biotechnology Inc.) were used as described by Lees *et al.* (1992). The anti-cdc25A antibodies were generated by injecting rabbits with a peptide corresponding to the N-terminal region of the cdc25A protein. The peptide with the amino acid sequence (H_2N-CELGPSPAPRRLLFA) was synthesized by the EMBL peptide synthesis facility and coupled to keyhole limpet hemocyanine as described by Draetta *et al.* (1988). Rabbits were injected with 200 μg of keyhole limpet hemocyanine–peptide conjugate mixed with complete Freund's adjuvant. Subsequently, they were injected with the same amount of peptide in incomplete Freund's adjuvant every 2 weeks until a significant immune signal was detected by immunoblotting total cell extracts. The resulting polyclonal antiserum was affinity purified using the N-terminal cdc25A peptide (10 mg) covalently coupled to CNBr-activated Sepharose 4B (1 g); 5 ml of antiserum were incubated with 1 ml of cdc25A peptide–Sepharose pre-equilibrated with 50 mM Tris–HCl, pH 7.4, 400 mM NaCl, 0.1% NP-40. After overnight incubation at 4°C, the Sepharose was poured into a column and washed with 20 ml of equilibration buffer. Ten millimolar glycine–HCl, pH 2.3, in 0.5 ml aliquots was applied to elute the antibodies. To neutralize the fractions, 0.05 ml of 1 M Tris–HCl, pH 9.5, was added. Fractions were individually checked for purity before being pooled. The preparation of the anti-cdc25C antibody has been described earlier (Hoffmann *et al.*, 1993). Affinity purification and the use of the anti-cdc25C antibody were performed in the same way as described above. For microinjection experiments, the antibodies were concentrated in PBS by centrifugation using Centricon 10 tubes (Amicon). For immunoblotting, 100 μg of total protein from HeLa or IMR-90 cell lysates were loaded per lane. Proteins were transferred from gels by semi-dry blotting as described in Harlow and Lane (1988). As secondary antibodies [^{125}I]protein A or [^{125}I]anti-mouse immunoglobulin (Amersham) were used. For immunodepletions, 25 ml of protein A–Sepharose (or protein G–Sepharose for cyclin E immunodepletions) were incubated with antiserum for 1 h at 4°C and washed three times with lysis buffer. Extracts were incubated twice with the prepared immunopellets for 1 h at 4°C on a rotator and recovered after centrifugation for 10 s in a microfuge.

Phosphatase and kinase assays

For phosphatase assays, cdc25A or cdc25C immunoprecipitates were incubated with 20 mM pNPP (Sigma), 0.1% β-mercaptoethanol and 1 mM EDTA for 10 min. The reaction was stopped by addition of 0.2 M NaOH and absorbance at 400 nm was determined. Protein kinase assays were performed as described by Hoffmann *et al.* (1993). Briefly, after immunoprecipitation, pellets were incubated at 30°C in the presence of 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 40 μM ATP and 5 μCi [γ-^{32}P]ATP for 10 min. Histone H1 (Boehringer, Mannheim) was added at 0.2 mg/ml.

Microinjection experiments and immunofluorescence

Affinity-purified antibodies (anti-cdc25A or anti-cdc25C, 2 mg/ml) or a control marker (purified rabbit antibodies, 2 mg/ml) were microinjected into the cytoplasm or the nucleus of cells using an Eppendorf micromanipulator and microinjector (AIS, Zeiss; Ansorge and Pepperkok, 1988; Pepperkok *et al.*, 1988). Using this system the percentage of successfully microinjected cells was >90% for cytoplasmic and 85% for nuclear microinjections (Pepperkok *et al.*, 1988). Immediately after injection, the growth medium was supplemented with BrdU (Sigma) to a final concentration of 100 μM. Cells were fixed 24 h after serum addition. They were washed once with PBS, fixed for 5 min at room temperature with 100% cold methanol and permeabilized with 0.25% Triton X-100 in PBS for 5 min. Fixed and permeabilized cells were incubated for 1 h at 37°C in a humidified atmosphere with affinity-purifed anti-cdc25A or anti-cdc25C antibodies. They were then washed three times with 0.5% BSA in PBS and incubated for 30 min with Texas red-conjugated goat anti-rabbit antibodies (dilution 1:200; Amersham, UK). Coverslips were then washed with PBS and incubated for 10 min at room temperature in 1.5 M HCl. After three washes with PBS, cells were incubated for 30 min with monoclonal anti-BrdU (dilution 1:100; Partec). After three washes with 0.5% BSA in PBS, cells were incubated for 30 min with fluorescein-conjugated anti-mouse antibodies (dilution 1:100; Sigma), washed and mounted with Moviol.

For immunofluorescence, coverslips were treated in the same way as described above except for the fixation steps. Cells were instead fixed for 10 min in 4% paraformaldehyde.

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