

Cdc25B and Cdc25C Differ Markedly in their Properties as Initiators of Mitosis

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Abstract. We have used time-lapse fluorescence microscopy to study the properties of the Cdc25B and Cdc25C phosphatases that have both been implicated as initiators of mitosis in human cells. To differentiate between the functions of the two proteins, we have microinjected expression constructs encoding Cdc25B or Cdc25C or their GFP-chimeras into synchronized tissue culture cells. This assay allows us to express the proteins at defined points in the cell cycle. We have followed the microinjected cells by time-lapse microscopy, in the presence or absence of DNA synthesis inhibitors, and assayed whether they enter mitosis prematurely or at the correct time. We find that overexpressing Cdc25B alone rapidly causes S phase and G2 phase cells to enter mitosis, whether or not DNA replication is complete, whereas overexpressing Cdc25C does not cause premature mitosis. Overexpressing Cdc25C together

with cyclin B1 does shorten the G2 phase and can override the unreplicated DNA checkpoint, but much less efficiently than overexpressing Cdc25B. These results suggest that Cdc25B and Cdc25C do not respond identically to the same cell cycle checkpoints. This difference may be related to the differential localization of the proteins; Cdc25C is nuclear throughout interphase, whereas Cdc25B is nuclear in the G1 phase and cytoplasmic in the S and G2 phases. We have found that the change in subcellular localization of Cdc25B is due to nuclear export and that this is dependent on cyclin B1. Our data suggest that although both Cdc25B and Cdc25C can promote mitosis, they are likely to have distinct roles in the controlling the initiation of mitosis.

Key words: cell cycle • mitosis • green fluorescent protein • nuclear export

THE Cdc25 phosphatase has a crucial and conserved role in the initiation of mitosis in eukaryotic cells; it activates the major mitotic protein kinase (composed of cyclin B and its partner catalytic subunit cyclin-dependent kinase 1 [CDK1])¹ (Nurse, 1990; Dunphy, 1994). Cyclin B/CDK1 causes profound changes in the cell architecture such as nuclear envelope breakdown and reorganization of the microtubule and actin filament networks in part by directly phosphorylating a number of important structural components of the cell (for review see Jackman and Pines, 1997; Nigg, 1993). In general, with the exception of budding yeast (Amon et al., 1992; Sorger and Murray, 1992), a pool of cyclin B-CDK1 complexes accumulates through late S and G2 phase that is kept inactive

by phosphorylation on residues in the ATP binding site of CDK1 (for review see Lew and Kornbluth, 1996). One of these residues, tyrosine 15, is phosphorylated in all cells by the Wee1/Mik1 kinase (Russell and Nurse, 1987a; Gould and Nurse, 1989; Lundgren et al., 1991; Parker et al., 1992; McGowan and Russell, 1993) and phosphorylation at this site appears to interfere with phosphotransfer to a bound substrate (Atherton Fessler et al., 1993). In animal cells, and in fission yeast under certain conditions (Den Haese et al., 1995), the adjacent residue, threonine 14, is phosphorylated by the membrane-bound Myt1 kinase (Kornbluth et al., 1994; Mueller et al., 1995; Booher et al., 1997; Liu et al., 1997) that interferes with ATP binding (Booher et al., 1997). The Cdc25 phosphatases are able to remove the phosphate from both Y15 and T14 and thereby activate cyclin B/CDK1 (Dunphy and Kumagai, 1991; Gautier et al., 1991; Kumagai and Dunphy, 1991; Millar et al., 1991b; Strausfeld et al., 1991; Sebastian et al., 1993).

Initial experiments using fission yeast showed that *cdc25* is a dosage-dependent regulator of mitosis (Russell and Nurse, 1986) whose level is partially regulated by proteolysis mediated by the *pub1* gene product (Nefsky and Beach, 1996). Cells with a defective *cdc25* gene product have a

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1. *Abbreviations used in this paper:* CDK1, cyclin-dependent kinase 1; DIC, differential interference contrast; GFP, green fluorescent protein; LMB, leptomycin B; MmGFP, modified green fluorescent protein; PCC, premature chromosome condensation; YFP, yellow fluorescent protein.

pronounced G2 delay before they enter mitosis, therefore, they grow to a longer size than wild-type cells (Russell and Nurse, 1986). Conversely, cells that overexpress *cdc25* or have a defect in the *wee1* kinase that antagonizes *cdc25*, enter mitosis prematurely and, therefore, divide at a smaller size than normal cells (Russell and Nurse, 1987b; Enoch and Nurse, 1990). Thus, the balance between the activities of *cdc25* and *wee1* determines when a cell enters mitosis. This control mechanism is responsive to the state of the DNA. Unreplicated or damaged DNA prevents cells from entering mitosis by altering the balance between the activities of *cdc25* and *wee1*, such that fission yeast cells with a mutant CDK1 (*cdc2*) gene that cannot be phosphorylated by *wee1/mik1* enter mitosis regardless of the state of the DNA (Gould and Nurse, 1989; Enoch and Nurse, 1990). Recently, the molecular mechanism linking damaged DNA to the activation of CDK1 has been partially elucidated. The *Cds1* and *Chk1* genes, which are activated by unreplicated DNA and DNA damage (Boddy et al., 1998; Blasina et al., 1999), phosphorylate a conserved site on *cdc25* to create a binding site for a 14-3-3 protein that prevents *cdc25* from activating cyclin B/CDK1 (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Lopez-Girona et al., 1999). In fission yeast, *cds1* also phosphorylates the *wee1* kinase and is required for the accumulation of the *mik1* kinase in response to unreplicated DNA (Boddy et al., 1998).

The fundamental importance of the balance between *cdc25* and *wee1* in preventing premature mitosis has also been demonstrated in frog egg extracts (Smythe and Newport, 1992) and in hamster (Heald et al., 1993) and human tissue culture cells (Hagting et al., 1998). The complication in human cells is that there are three different *Cdc25* genes: *Cdc25A*, *B*, and *C* (Galaktionov and Beach, 1991; Nagata et al., 1991). Of these, *Cdc25A* is present in G1 phase cells and appears to play a part in initiating DNA replication (Hoffmann et al., 1994; Jinno et al., 1994). However, both *Cdc25B* and *Cdc25C*, which are 45% identical at the amino acid level (85% identical in the catalytic domains), are present in G2 phase cells; each appears to be necessary for cells to enter mitosis, because microinjecting either anti-*Cdc25B* or anti-*Cdc25C* antibodies, or transfecting cells with an inactive mutant of *Cdc25B* or *Cdc25C* will block cells in the G2 phase (Millar et al., 1991b; Gabrielli et al., 1996; Lammer et al., 1998). Nevertheless, there are indications that *Cdc25B* and *Cdc25C* may not have redundant roles in the initiation of mitosis. First, anti-*Cdc25B* immunoprecipitates have phosphatase activity from S phase onwards that increases two- to four-fold at mitosis, whereas anti-*Cdc25C* immunoprecipitates are inactive until late G2/M phase (Gabrielli et al., 1996; Nishijima et al., 1997; Lammer et al., 1998). In vitro, *Cdc25B* is also more active than *Cdc25C* towards cyclin B/CDK1 unless *Cdc25C* is activated by prior phosphorylation on its amino terminus. Second, *Cdc25C* is a nuclear protein in human cells (Millar et al., 1991a; Girard et al., 1992), whereas *Cdc25B* is reported to be cytoplasmic, but to translocate to the nucleus at mitosis or when overexpressed (Gabrielli et al., 1996, 1997b). Third, in transient transfection experiments overexpressing *Cdc25C* had no effect on the cell cycle profile of cells as analyzed by flow cytometry, whereas overexpressing *Cdc25B* caused a slow

increase in the population of G2/M cells and these cells had abnormal mini-spindles (Gabrielli et al., 1996).

There are a number of problems associated with performing cell cycle experiments in tissue culture cells. The effects of introducing mutant proteins, or overexpressing wild-type proteins, by transient transfection have to be interpreted in a heterogeneous population of cells at fixed points after transfection. Therefore, judging how a particular procedure has affected the cell cycle of any one cell is mostly a matter of informed guesswork. Furthermore, it is difficult to introduce a protein by transfection into cells at a precise stage in the cycle, and, most importantly, progress through the cell cycle, is often affected by the transfection procedure itself (Winters et al., 1998). In an effort to overcome these problems, we have begun to analyze the cell cycle by time-lapse fluorescence microscopy (Hagting et al., 1998). This allows us to follow individual cells through an entire cell cycle. We have combined this with microinjection into synchronized cells to introduce cell cycle regulators at defined points in the cell cycle. Furthermore, we introduce regulators in the form of fusion proteins with a modified form of green fluorescent protein (MmGFP) (Zernicka-Goetz et al., 1996, 1997). This allows us to follow the subcellular localization of specific proteins in real time and gives us a dynamic view of their behavior when the cell enters mitosis. Using this experimental regime, we find that *Cdc25B* is a rate-limiting component of mitosis whose overexpression will overcome the unreplicated DNA checkpoint. In contrast, *Cdc25C* does not accelerate cells into mitosis unless it is coexpressed with cyclin B1, and even this combination does not override the S phase checkpoint. We confirm that there is a marked difference in subcellular localization between *Cdc25B* and *Cdc25C* and show that the cytoplasmic localization of *Cdc25B* is regulated by nuclear export that is dependent on cyclin B1.

Materials and Methods

Cell Culture

HeLa cells were grown in DME supplemented with 5% newborn calf serum, 5% FCS, and antibiotics. HeLa cells were synchronized using a thymidine-aphidicolin protocol (Pines and Hunter, 1989). Cells were cultured on the microscope stage in a CO₂-independent medium without phenol red (GIBCO BRL). The medium was overlaid with mineral oil (Sigma Chemical Co.) to prevent evaporation. Hydroxyurea was used at a final concentration of 2.5 mM and was added immediately after release from the aphidicolin block.

Plasmid Constructs and Protein Purification

The cyclin B1-MmGFP, *cdc25C*(S216G), and *Wee1* constructs have been previously described (Hagting et al., 1998). Cyclin B1^{R42A}-MmGFP was the gift of Paul Clute (Wellcome/CRC Institute). *Cdc25B3* and *Cdc25C* were tagged at the amino terminus with MmGFP (Zernicka-Goetz et al., 1996, 1997) by PCR using *Taq* polymerase and cloned into the pCMX vector (Umesono et al., 1991). The stop codon of GFP was mutated to a Hind III site to link it with the first amino acid of *Cdc25B* or *Cdc25C* creating a 3-amino acid linker (Gly-Ile-Pro). Myc-tagged *cdc25B2* (Lammer et al., 1998) was cloned into the pCDNA3 vector for expression in HeLa cells. All constructs were sequenced using an Applied Biosystems DNA sequencer. Cyclin B1-*cdc2*^{K33R} complexes were expressed in and purified from baculovirus-infected cells as described (Hagting et al., 1998). Cyclin B1^{F146A} was expressed in *Escherichia coli* and purified as described (Hagting et al., 1999).

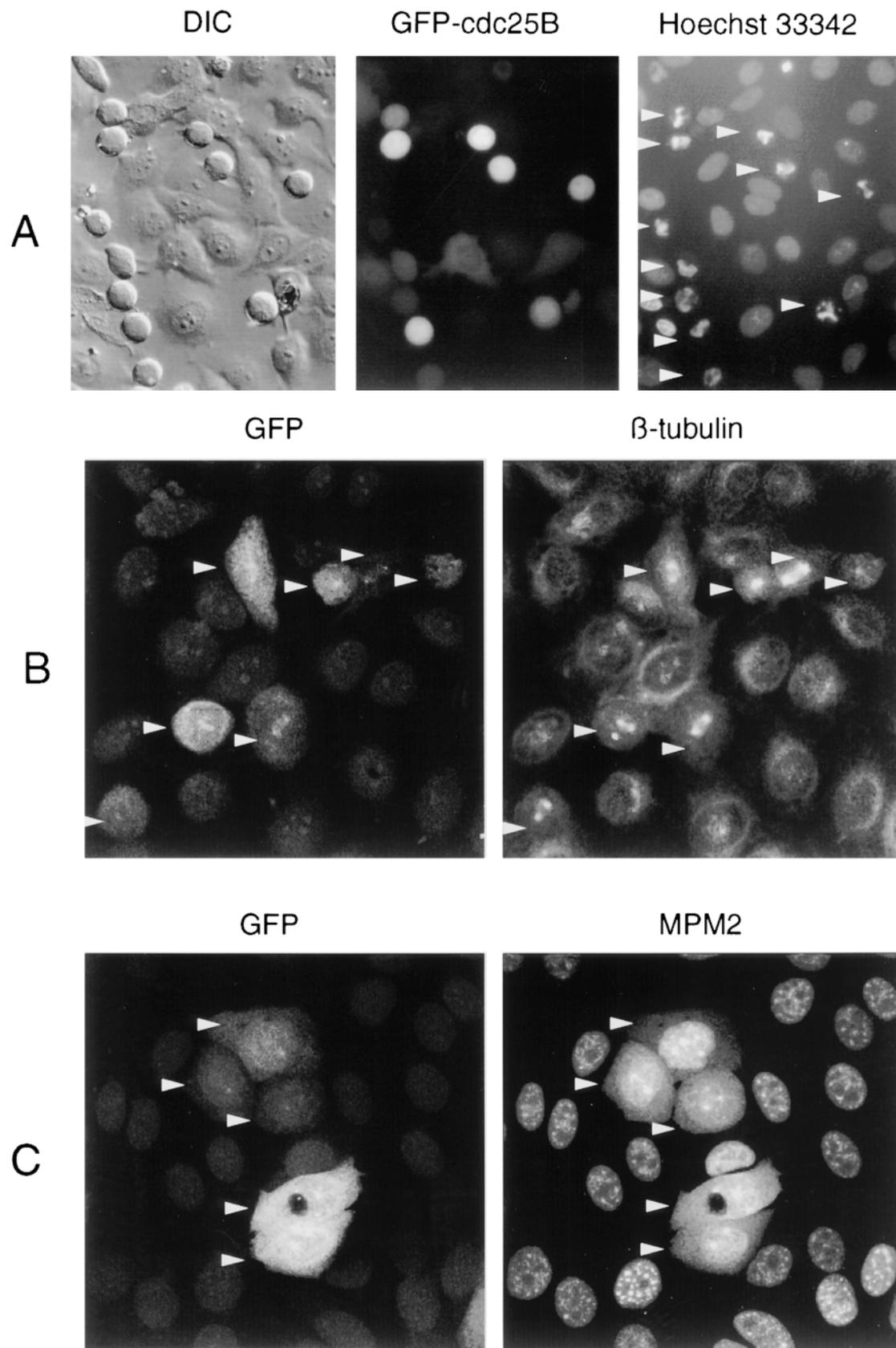


Figure 1. Overexpressing *cdc25B* induces premature mitosis and not apoptosis. (A) HeLa cells in S phase were microinjected with GFP-*cdc25B3* cDNA at a concentration of $0.1 \mu\text{g}/\mu\text{l}$ and cells were followed by time-lapse DIC microscopy. After 5 h cells were analyzed. Arrows indicate GFP-*cdc25B*-expressing cells. DIC (left), GFP fluorescence (center), and Hoechst 33342 staining (right). (B) GFP fluorescence (left) and β -tubulin immunofluorescence (right). (C) GFP fluorescence (left) and MPM2 immunofluorescence (right).

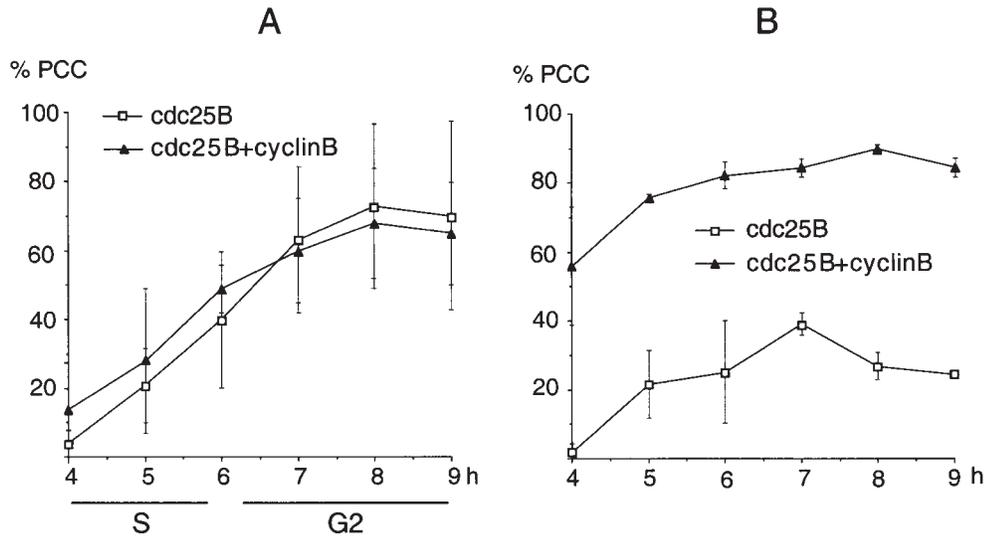


Figure 2. Frequency of PCC induced by overexpressing *cdc25B* in the S phase. Constructs encoding Cdc25B were microinjected with or without constructs encoding cyclin B1 into S phase HeLa cells, ~1 h after release from a thymidine/aphidicolin block in the absence (A) or presence (B) of 2.5 mM hydroxyurea. One of the constructs was expressed as a GFP-chimera to detect injected cells. (Similar results were obtained whether Cdc25B or cyclin B1 was linked to GFP.) Cells were followed by DIC microscopy and cells that rounded up were counted at the indicated time points after release. At the end of the experiment, Hoechst

33342 was added to detect abnormally condensed chromatin and the cells expressing the GFP-chimera were counted and the frequency of PCC was estimated. (A) Frequency of PCC in cells overexpressing *cdc25B* +/- cyclin B1. (B) Frequency of PCC in cells overexpressing *cdc25B* +/- cyclin B1 in the presence of hydroxyurea. All constructs were injected at a concentration of 0.02 $\mu\text{g}/\mu\text{l}$. For cells in the absence of hydroxyurea, the duration of S and G2 phases in the control cells are indicated. Data are the mean of three different experiments.

Microinjection and PCC Detection

Constructs expressing cDNAs under the control of the cytomegalovirus promoter were microinjected into cell nuclei using an Eppendorf semi-automatic microinjection apparatus. To assay for condensed chromatin, Hoechst 33342 was added to cells at a concentration of 1 $\mu\text{g}/\text{ml}$ at the end of the experiment. Injected cells were identified by green fluorescent protein (GFP) fluorescence and those that had rounded up with abnormally condensed chromatin were scored. At least 50 cells were scored for each injected construct and experiment. Apoptotic cells were assayed using the apoptosis detection kit (R&D Systems, Inc.) and HeLa cells treated with cycloheximide plus tumor necrosis factor α were used as positive controls.

Time-lapse Differential Interference Contrast (DIC) and Fluorescence Imaging

To visualize GFP-chimeras in living cells, cells were cultured on an inverted Leica DMIRB/E microscope using the ΔTC3 system (Bioprotechs) to

maintain cells at 37°C. Images were captured with a PentaMax CCD camera (Princeton Instruments) fitted to the lateral photo port. GFP- and yellow fluorescent protein (YFP)-chimeras were detected with custom filter sets JP1 and JP2 (Chroma Technology Corp.) and two Lambda 10-2 filter wheels (Sutter Instrument) controlled by a PowerWave computer (Power-Computing). One filter wheel was used to control the wavelength of the excitation light. The other filter wheel controlled the wavelength of the emission light and also the polarizer for DIC images. To distinguish between GFP and YFP we used the JP3 filter set as described (Hagting et al., 1999). Images were collected and processed using IP Lab Spectrum software (Scanalytics Inc.) and exported to Adobe Photoshop for printing.

Immunofluorescence and Confocal Imaging

For β -tubulin and MPM2 staining, cells were fixed with 3% PFA/Triton X-100 and stained as described (Pines, 1997) 3-4 h after microinjection. Tubulin was detected using an anti- β -tubulin mAb (Nycomed Amer-

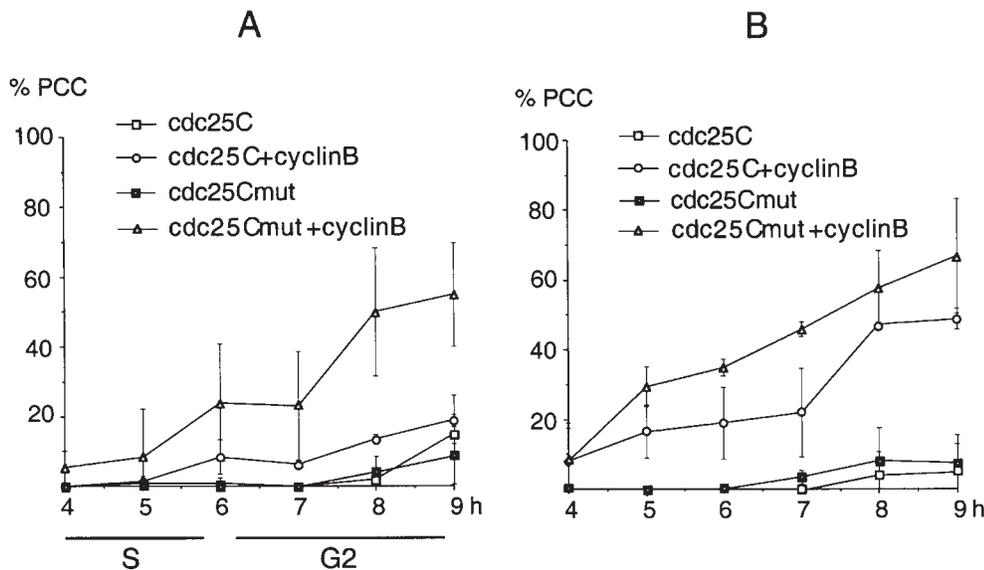


Figure 3. Frequency of PCC induced by overexpressing *cdc25C* and *cdc25C(S216G)* in the S phase. Constructs encoding Cdc25C or a Cdc25C(S216G) mutant were microinjected with or without cyclin B1 into S phase HeLa cells in the absence (A) or presence (B) of 2.5 mM hydroxyurea and followed by DIC microscopy as described in Fig. 2. (A) Frequency of PCC in cells after release from an aphidicolin block. (B) Frequency of PCC in cells after release from an aphidicolin block in the presence of hydroxyurea. Data are the mean of three different experiments.

sham) and mitotic epitopes were detected using the MPM2 mAb (Upstate Biotechnology, Inc.). To detect myc-cdc25B2, pCDNA3/myc-cdc25B2 was microinjected (0.1 $\mu\text{g}/\mu\text{l}$) and cells were fixed with methanol/acetone (1:1) 3 h after injection and stained with the mAb 9E10 (gift of Erich Nigg, University of Geneva, Geneva). A Cy5 conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.) was used as the secondary antibody. Cells were analyzed by confocal laser scanning microscopy using a Bio-Rad 1024 confocal microscope set on 10% laser power and Kalman averaging. Stacks of images were projected using Lasershar software (Bio-Rad Laboratories) and exported to Adobe Photoshop for processing and printing.

Glutathione-S-transferase (GST) Pulldowns

Human cdc25B2, cdc25B3, cdc25C, and cyclin B1 were in vitro translated from pBSK/cdc25B cDNA using the TNT-coupled reticulocyte system (Promega Corp.). GST-cyclin B1, GST-Cdc25B2, GST-Cdc25B3, and GST were expressed in BL21(DE3) cells using the pGEX-4T expression vector and purified on GSH-Sepharose.

Results

Overexpression of cdc25B3 Causes Premature Mitosis in the Presence of Unreplicated DNA

To assay the role of the two types of mitotic Cdc25 phosphatases in the initiation of mitosis, expression constructs encoding Cdc25B or Cdc25C, or the equivalent GFP-chimera were introduced into synchronized HeLa cells by microinjection. The GFP-chimeras became visible about 3 h after microinjection, and comparing the fluorescence between GFP-Cdc25B- and GFP-Cdc25C-expressing cells showed that these proteins are expressed to similar levels (data not shown). The behavior of the cells was followed by DIC microscopy and the cell cycle phase was determined by flow cytometry and by incorporation of BrdU. In these experiments, we primarily used the full-length

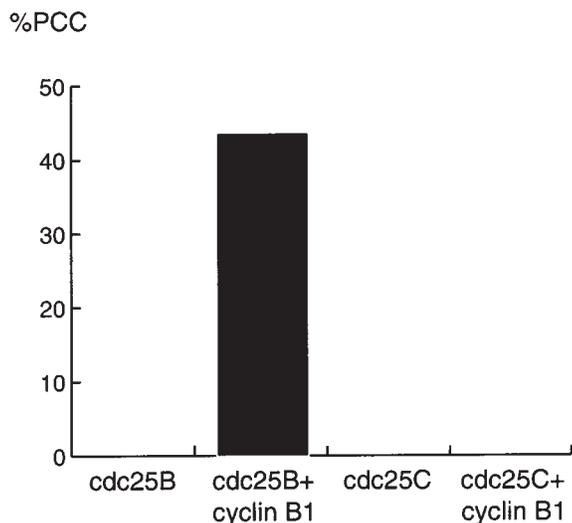


Figure 4. Overexpression of cdc25B together with cyclin B1 can force G1 cells into premature mitosis. Cells in the G1 phase were recognized by virtue of being attached after cytokinesis. Cells were injected with constructs expressing GFP-cdc25B or GFP-Cdc25C with or without constructs expressing a nondestructible cyclin B1 (R42A) mutant. The frequency of PCC was determined 6 h after microinjection as described in Fig. 2. Data are representative of three different experiments.

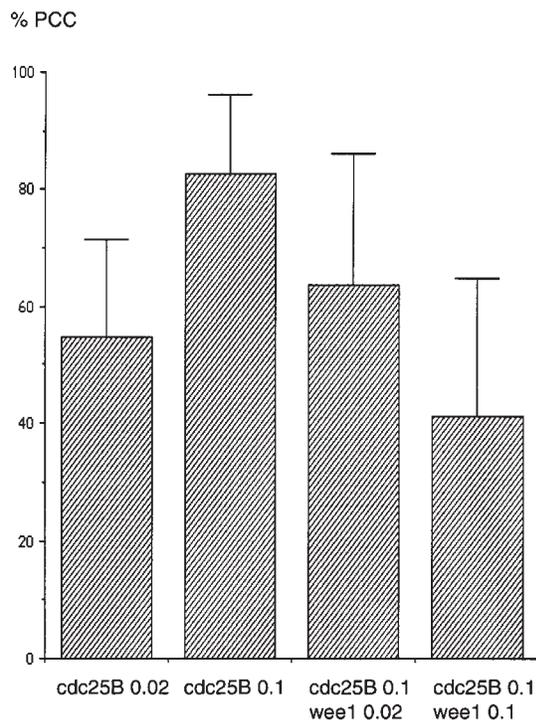


Figure 5. Wee1 can rescue cells overexpressing cdc25B from PCC. Cells were microinjected with expression constructs encoding cdc25B with or without constructs expressing Wee1 at the indicated concentrations (in $\mu\text{g}/\mu\text{l}$). 5 h later, Hoechst 33342 was added and the frequency of PCC was determined as described in Fig. 2. Data are the mean of four different experiments.

Cdc25B3 clone that is found in all cells (Baldin et al., 1997b), but we detected only minor differences between the behavior of this and the other two forms of Cdc25B (data not shown). Using the GFP-chimeras we were able to estimate the half-life of the proteins in cycloheximide-chase experiments and found that there was little or no difference in their turnover rate compared with the endogenous proteins (data not shown).

We found that overexpressing GFP-Cdc25B in early S phase rapidly caused cells to round up and break down their nuclear envelope (Figs. 1 A and 2 A). Staining the cells with Hoechst 33342 showed that the chromatin had condensed abnormally (Fig. 1 A) and anti- β -tubulin immunofluorescence staining revealed the presence of minispindles as previously described (Gabrielli et al., 1996). This suggested that the cells had entered mitosis prematurely rather than undergone apoptosis. To confirm this, we stained cells with the MPM2 mAb that recognizes mitosis-specific phospho-epitopes and for the apoptosis-specific surface marker annexin V. Cells expressing Cdc25B with abnormally condensed chromatin stained very strongly with the MPM2 antibody (Fig. 1 C) but not for annexin V (data not shown). Thus, we concluded that Cdc25B caused cells to enter mitosis prematurely (premature chromosome condensation, PCC) rather than undergo apoptosis. The GFP tag was not responsible for this effect because the untagged Cdc25B cDNA induced premature mitosis with a similar frequency to GFP-Cdc25B

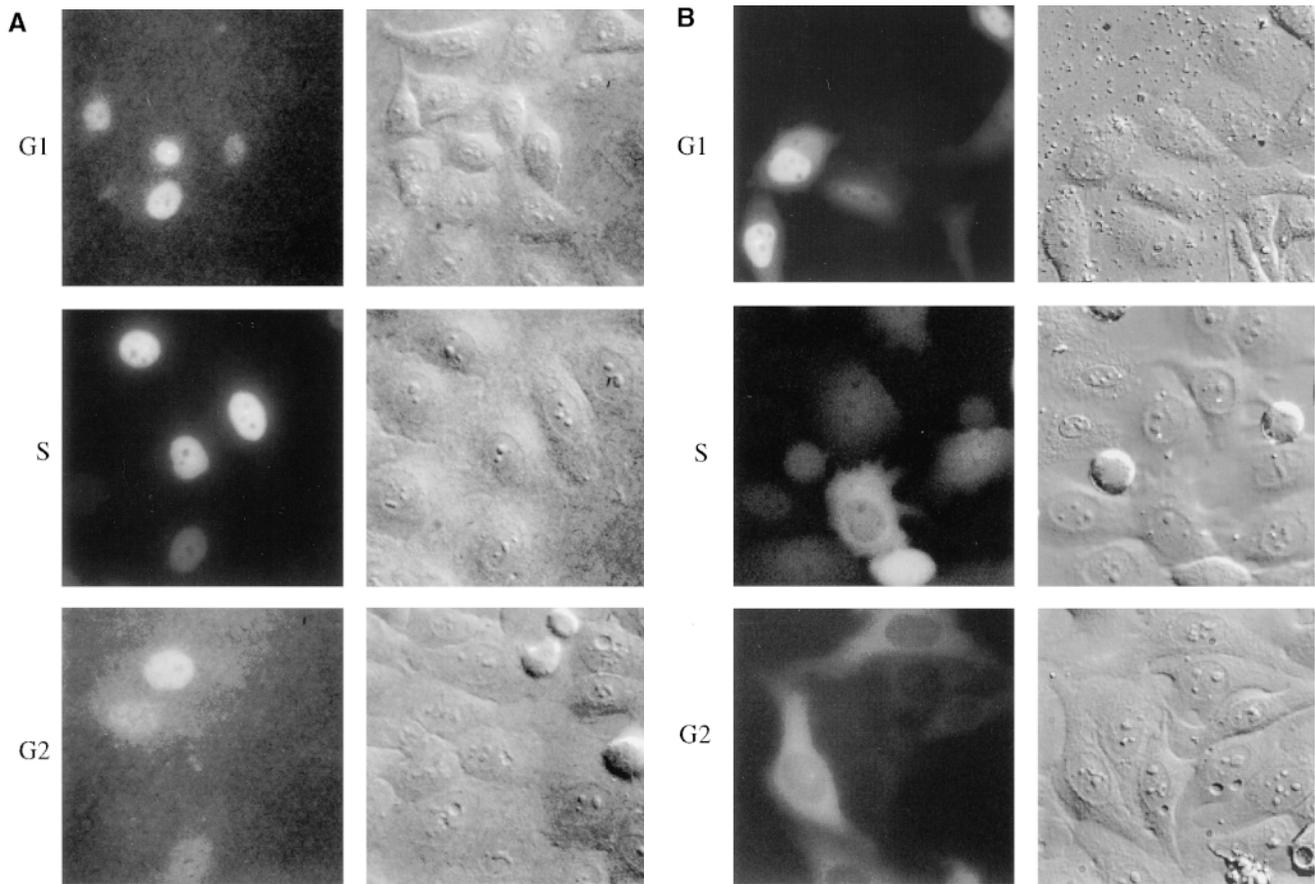


Figure 6.

(not shown) and an inactive mutant of Cdc25B that lacked the catalytic cysteine residue (C488S) could not induce premature mitosis (not shown). Coinjecting a cyclin B1 expression vector with Cdc25B had only a minor effect on the frequency of premature mitosis (Fig. 2 A).

In these experiments, cells were released from an aphidicolin block and, therefore, progressed through S phase in the course of the experiment. We also wished to assay the effect of overexpressing cdc25B in cells in which the unreplicated DNA checkpoint was strongly activated. Therefore, we microinjected cells with the Cdc25B expression construct and released them from the aphidicolin block in the presence of hydroxyurea. Under these conditions cells progressed only very slowly through S phase as determined by flow cytometry (not shown). We found that Cdc25B efficiently induced PCC in these cells, and, in this case, coexpressing cyclin B1 greatly increased the frequency of PCC (Fig. 2 B).

Overexpression of cdc25C Can Only Cause Premature Mitosis at the End of S Phase, and Only When Coexpressed with Cyclin B1

The effect of overexpressing Cdc25C in HeLa cells was markedly different from that of overexpressing Cdc25B; Cdc25C could not induce PCC when overexpressed alone. Cdc25C was only able to cause a significant fraction of cells to enter mitosis prematurely when cyclin B1 was also

overexpressed. Furthermore, overexpressing Cdc25C and cyclin B1 could only cause PCC after cells had entered the G2 phase (Fig. 3 A), not in the S phase cells. The unreplicated DNA and G2 phase DNA damage checkpoints prevent mitosis, at least in part, by acting on Cdc25C. Unreplicated DNA activates the Cds1 and Chk1 kinases; Chk1 is also activated by DNA damage. Both kinases phosphorylate Cdc25C on serine 216 to create a binding site for a 14-3-3 protein that inactivates Cdc25C. Therefore, to test whether Cdc25C was less potent than Cdc25B because it was downregulated by Cds1 and/or Chk1, we used a Cdc25C mutant that cannot be phosphorylated on serine 216 (S216G). Cdc25C^{S216G} was more efficient than wild-type Cdc25C in causing PCC, but again this was only when cyclin B1 was coexpressed (Fig. 3, A and B). Therefore, downregulation by Cds1 and/or Chk1 is not sufficient to explain the difference in potency between Cdc25B and Cdc25C.

Overexpressing cdc25B, but Not Cdc25C, Together with Cyclin B1 Can Force G1 Cells into Mitosis

The potent ability of cdc25B to activate cyclin B1 and induce premature mitosis raised the possibility that cdc25B might be able to force cells into mitosis before they had begun to replicate their DNA. At this stage in the cell cycle, the proteolysis machinery that degrades the mitotic cyclins is still active (Amon et al., 1994; Brandeis and Hunt,

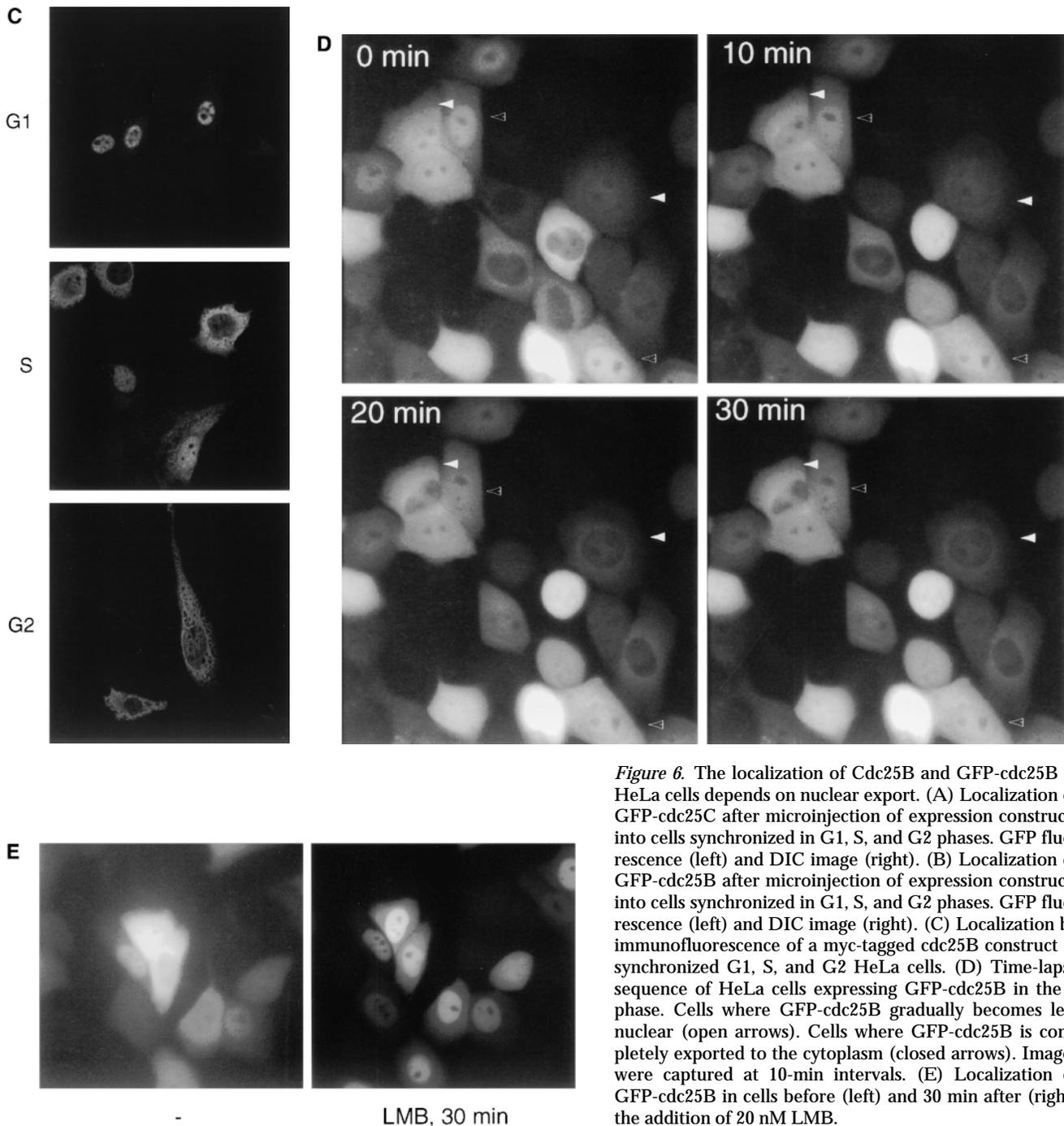


Figure 6. The localization of Cdc25B and GFP-cdc25B in HeLa cells depends on nuclear export. (A) Localization of GFP-cdc25C after microinjection of expression constructs into cells synchronized in G1, S, and G2 phases. GFP fluorescence (left) and DIC image (right). (B) Localization of GFP-cdc25B after microinjection of expression constructs into cells synchronized in G1, S, and G2 phases. GFP fluorescence (left) and DIC image (right). (C) Localization by immunofluorescence of a myc-tagged cdc25B construct in synchronized G1, S, and G2 HeLa cells. (D) Time-lapse sequence of HeLa cells expressing GFP-cdc25B in the S phase. Cells where GFP-cdc25B gradually becomes less nuclear (open arrows). Cells where GFP-cdc25B is completely exported to the cytoplasm (closed arrows). Images were captured at 10-min intervals. (E) Localization of GFP-cdc25B in cells before (left) and 30 min after (right) the addition of 20 nM LMB.

1996), therefore, we microinjected Cdc25B expression constructs with and without a cyclin B1 mutant (R42A) that was resistant to degradation. We injected the constructs into early G1 cells that had just flattened out after cytokinesis. We found that overexpressing GFP-cdc25B alone could not force cells into mitosis, but coexpressing the nondegradable form of cyclin B1 caused PCC in ~50% of the injected cells 6 h after injection. This would normally correspond to the middle of G1 phase (Fig. 4). Furthermore, overexpressing Cdc25C, with or without cyclin B1, or cyclin B1 alone did not force G1 phase cells into premature mitosis (Fig. 4 and data not shown).

Wee1 Rescues Cells from PCC in a Dose-dependent Manner

The balance between the inhibitory kinases of cdc2, Wee1, and Myt1, and Cdc25C phosphatase is crucial to the timing of mitosis (Smythe and Newport, 1992; Heald et al., 1993; Hagting et al., 1998). Therefore, we coinjected varying amounts of expression constructs for Cdc25B and for Wee1 kinase to see whether Wee1 could counteract the ability of Cdc25B to cause PCC. We found that Cdc25B induced PCC in a dose-dependent manner and that Wee1 could rescue Cdc25B-expressing cells, again in a dose-dependent fashion (Fig. 5).

Cdc25B Localization Changes Because of Nuclear Export

We considered the possibility that the differences in the abilities of Cdc25B and Cdc25C to force cells into mitosis might be related to the differences in their localization. Human Cdc25C is usually found to be a nuclear protein (Millar et al., 1991a; Girard et al., 1992) and our Cdc25C chimera is also nuclear throughout interphase (Fig. 6 A). Cdc25B has been previously described as a cytoplasmic protein that colocalizes with centrosomes and becomes nuclear at the end of the G2 phase (Gabrielli et al., 1996, 1997b). To verify that the GFP-Cdc25B chimeras localized correctly, we compared their localization with a myc epitope-tagged Cdc25B, reasoning that the nine amino acids of the myc epitope would be unlikely to perturb the localization of Cdc25B. HeLa cells were synchronized in the G1, S, and G2 phases and the myc-tagged Cdc25B protein localization was determined by immunofluorescence after microinjection of a myc-cdc25B expression construct (Fig. 6 C). Cells expressing the GFP-cdc25B chimera were followed by time-lapse fluorescence microscopy (Fig. 6, B and D) at different stages of the cell cycle.

We found that Cdc25B was nuclear in the G1 phase and gradually moved from the nucleus to the cytoplasm as cells progressed through S phase. Unfortunately, we were unable to visualize the endogenous Cdc25B by immunofluorescence, but we were able to confirm that Cdc25B was substantially nuclear in G1/S phase cells and accumulated in the cytoplasm by immunoblotting nuclear and cytoplasmic fractions through the cell cycle (not shown). These results suggested that Cdc25B might be exported from the nuclei of S and G2 phase cells. Most nuclear export pathways reported so far depended on exportin 1/Crm1, which can be inactivated by leptomycin B (LMB) (Nishi et al., 1994; Fornerod et al., 1997; Kudo et al., 1997; Wolff et al., 1997). Therefore, we added LMB to cells expressing GFP-cdc25B in G2 phase and found that LMB caused GFP-cdc25B to accumulate in the nucleus within 30 min (Fig. 6 E), indicating that the localization of Cdc25B is regulated by nuclear export.

The Cytoplasmic Localization of cdc25B Is Dependent on Cyclin B1

Although the cytoplasmic localization of Cdc25B in S and G2 phases depended on Crm1-mediated nuclear export, Cdc25B was nuclear in the G1 phase. The cytoplasmic localization of Cdc25B correlated with the point in the cell cycle when cyclin B1 started to accumulate and cyclin B1 has been shown to be exported from nuclei in an LMB-sensitive manner (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). Taken together these observations suggested that cyclin B1 might be involved in mediating the cytoplasmic localization of Cdc25B. To test this hypothesis, we expressed GFP-cdc25B in early G1 phase or early S phase cells (i.e., cells that lack any endogenous cyclin B1), and then microinjected purified cyclin B1 or cyclin B1/CDK1^{K33R} protein into the nucleus. GFP-cdc25B was immediately and rapidly exported from G1 (not shown) or S phase nuclei (Fig. 7 A) after injecting cyclin B1/CDK1^{K33R} and reentered the nuclei when LMB was added to inhibit export (Fig. 7 A). This effect was ob-

served using cyclin B1 in a complex with a kinase-dead mutant of CDK1, which strongly suggested that cyclin B1 itself and not CDK1 kinase activity was required for cdc25B export. In support of this, we found that GFP-cdc25B still accumulated in the cytoplasm of G2 phase cells in the presence of the CDK1 inhibitor, roscovitine (data not shown). Furthermore, a cyclin B1 mutant with a defective nuclear export signal, cyclin B1^{F146A}, did not cause nuclear Cdc25B to be exported when microinjected into G1 (Fig. 7 B) or S phase nuclei (not shown). These observations suggested that Cdc25B export could be due to a direct interaction between cyclin B1/CDK1 and Cdc25B. In support of this, we were able to detect an *in vitro* interaction between Cdc25B and cyclin B1 that appeared to be slightly stronger than an association between cyclin B1 and Cdc25C (Fig. 7 C).

Discussion

In this paper, we have used time-lapse microscopy to show that Cdc25B and Cdc25C differ markedly in their abilities to induce mitosis. Our assay allowed us to determine the effects on progress through the cell cycle of overexpressing specific proteins at defined points in the cell cycle. In this way, we were able to avoid the problems of trying to analyze the effects of overexpressing proteins by transfection in a heterogeneous population of cells that must be fixed for immunofluorescence at arbitrary time points. We have shown that simply overexpressing Cdc25B is sufficient to induce premature mitosis when there is cyclin B1 in the cell regardless of the replication state of the DNA. In contrast, Cdc25C will only cause premature mitosis when it is overexpressed with cyclin B1 and this does not override the negative signal generated by unreplicated DNA. We have also shown that the subcellular localization of Cdc25B changes through the cell cycle and is regulated by nuclear export, most likely by binding to cyclin B1.

Our demonstration that overexpressing Cdc25C alone cannot overcome the unreplicated DNA checkpoint, but that Cdc25B can, suggests that Cdc25C and Cdc25B are regulated in different ways. Cdc25C appears to require a further activation step that can be partially supplied by increasing the level of cyclin B (Heald et al., 1993). Indeed, an extra copy of cyclin B (NIME) can partially suppress a mutation in *cdc25* (NIMT) in *Aspergillus nidulans* (O'Connell et al., 1992). This activation step is most likely to be phosphorylation of the amino terminus of Cdc25C, which activates its phosphatase activity ~10-fold *in vitro*, and cyclin B/Cdk1 is able to phosphorylate and activate Cdc25C (Izumi et al., 1992; Kumagai and Dunphy, 1992; Hoffmann et al., 1993; Izumi and Maller, 1995; Gabrielli et al., 1997a). More recently, members of the polo-like family of kinases have been shown to phosphorylate and activate Cdc25C (Kumagai and Dunphy, 1996), but it is unclear whether they initiate Cdc25C activation at the end of G2 phase. In this regard, we were unable to activate prematurely Cdc25C by coexpressing human plk1 (data not shown), although this may be because plk1 itself needs to be activated by phosphorylation (Qian et al., 1998). Our data show that Cdc25C activation is either prevented, or rapidly reversed, in the presence of unreplicated DNA. The inhibition of Cdc25C may be partially effected through

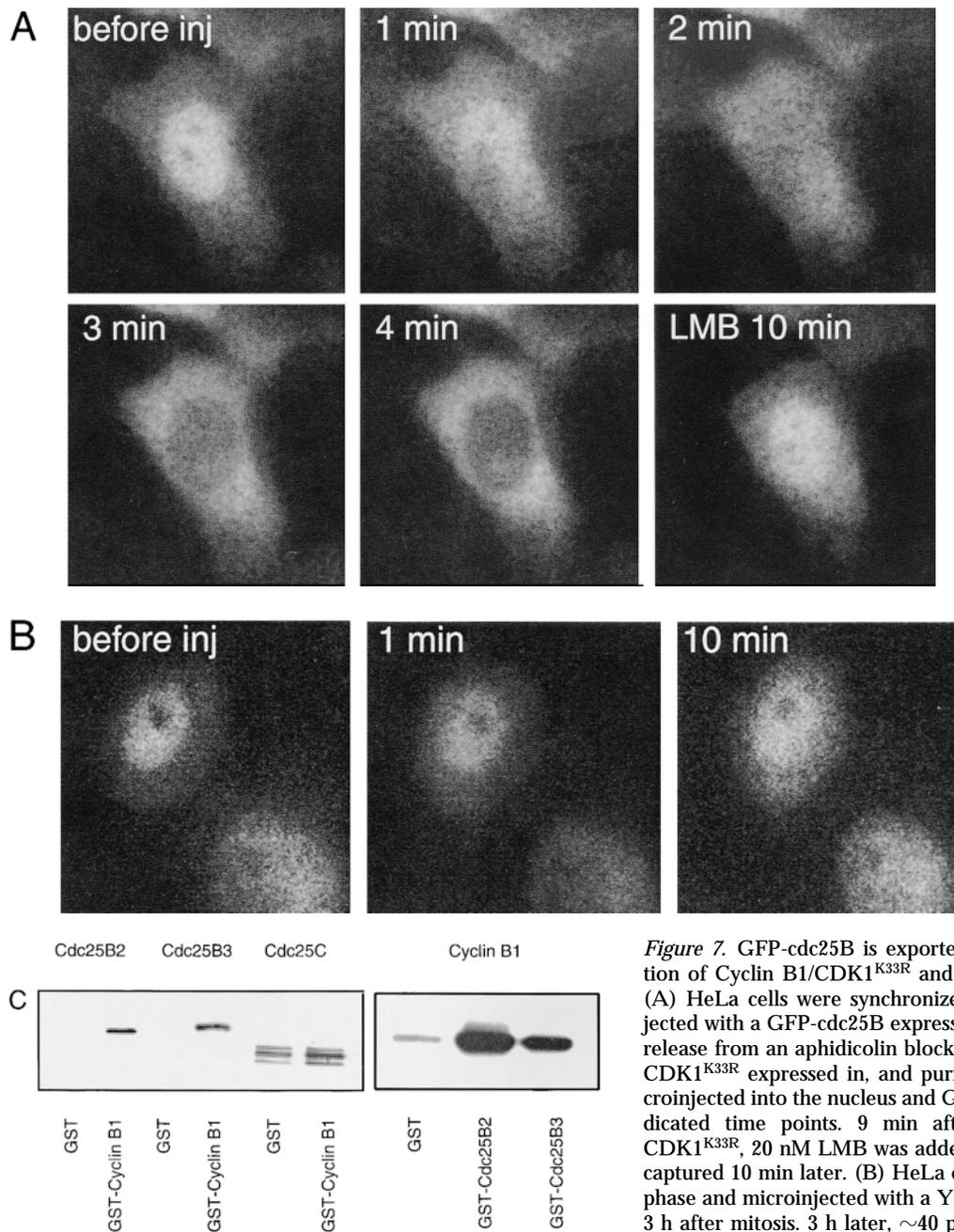


Figure 7. GFP-cdc25B is exported from the nucleus after injection of Cyclin B1/CDK1^{K33R} and can bind to cyclin B1 in vitro. (A) HeLa cells were synchronized in the S phase and microinjected with a GFP-cdc25B expression construct immediately after release from an aphidicolin block. 3 h later, ~40 pg of cyclin B1/CDK1^{K33R} expressed in, and purified from, baculovirus was microinjected into the nucleus and GFP images were taken at the indicated time points. 9 min after the injection of cyclin B1/CDK1^{K33R}, 20 nM LMB was added to the cells and a final image captured 10 min later. (B) HeLa cells were synchronized in the S phase and microinjected with a YFP-cdc25B expression construct 3 h after mitosis. 3 h later, ~40 pg of a nuclear export-defective cyclin B1 (F146A)-GFP was microinjected into the nucleus and

YFP images were taken at the indicated time points using a filter set that distinguishes between GFP and YFP (Hagting et al., 1999). (C) Physical interaction between cdc25B and cyclin B1. (Left) ³⁵S-labeled, in vitro translated cdc25B2 and cdc25B3 and Cdc25C were incubated with GST alone or GST-cyclin B1. (Right) ³⁵S-labeled in vitro translated cyclin B1 was incubated with GST alone, or GST-Cdc25B2, or GST-Cdc25B3. The complexes were bound to glutathione-Sepharose, washed extensively, separated by SDS-PAGE, and exposed to film.

phosphorylation on S216 and the consequent binding of a 14-3-3 protein (Boddy et al., 1998; Rhind and Russell, 1998). However, this cannot provide a full explanation, because we found that although an S216G mutant form of Cdc25C is able to promote premature mitosis more rapidly than the wild-type Cdc25C, it is unable to overcome the DNA replication checkpoint.

In contrast, it appears that Cdc25B either does not need an activation step or that the activator is present whenever cyclin B1 is present. Furthermore, cyclin B/Cdk1 phosphor-

ylates Cdc25B in vitro and this correlates with a fourfold increase in Cdc25B activity (Gabrielli et al., 1996, 1997a; Lammer et al., 1998). One explanation could be that cyclin B/CDK1 activates Cdc25B and vice versa in a positive feedback loop, and that this is more potent than the positive feedback loop between Cdc25C and cyclin B1/CDK1 because Cdc25B and cyclin B1/CDK1 are both cytoplasmic. If Cdc25B and cyclin B do activate one another, then the resulting positive feedback loop must be carefully regulated because overexpressing Cdc25B is sufficient to cause

premature mitosis, regardless of whether the DNA has been replicated. One way in which Cdc25B might be regulated is by protein turnover. Cdc25B is an unstable protein, with a half-life of <30 min in hamster and HeLa cells (Nishijima et al., 1997), which can be targeted for degradation in vitro by cyclin A-CDK2 (Baldin et al., 1997a). Thus, Cdc25B may be primarily regulated by ubiquitin-mediated proteolysis in an analogous fashion to cdc25 in fission yeast (Nefsky and Beach, 1996).

Remarkably, we found that when Cdc25B and cyclin B1 were coexpressed in G1 phase cells the cells attempted to enter mitosis, showing that cell division and DNA replication can be uncoupled in human cells. This may also be relevant to how the cell causes two sequential M phases in meiosis and it will be interesting to determine whether Cdc25B is required for gametogenesis.

Despite the differences in their regulation, both Cdc25B and Cdc25C are antagonized by Wee1. Wee1 and Cdc25C are both nuclear proteins and, therefore, their relative activities will determine whether the nucleus will activate or inhibit cyclin B/CDK1 when it is imported into the nucleus. However, Cdc25B is primarily a cytoplasmic protein in the G2 phase and so might act on the predominantly cytoplasmic pool of cyclin B/CDK1. Therefore, Wee1 may only counteract the effects of Cdc25B when active cyclin B1/Cdk1 shuttles into the nucleus (Hagting et al., 1998).

Human Cdc25B was originally described as being a cytoplasmic protein that translocated to the nucleus at mitosis in concert with cyclin B (Gabielli et al., 1996, 1997b). However, these studies were performed by immunofluorescence in which the exact cell cycle stage of an individual cell was often difficult to judge, except by the appearance of condensed chromosomes. We have shown here that Cdc25B is nuclear in G1 cells, but gradually accumulates in the cytoplasm as cells progress through the S and G2 phases. Furthermore, Cdc25B will accumulate in the nucleus when S or G2 phase cells are treated with LMB, a specific inhibitor of crm1/exportin1-mediated nuclear export (Nishi et al., 1994; Fornerod et al., 1997; Wolff et al., 1997). This suggests that the localization of Cdc25B, like that of cyclin B1 (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998), is primarily determined by nuclear export. However, Cdc25B does not have a recognizable nuclear export signal (Bogerd et al., 1996) and is nuclear in the G1 phase. When we microinjected wild-type cyclin B1 into the nuclei of G1 cells, we observed that cdc25B was immediately exported, but not when we microinjected an export-defective cyclin B1. Furthermore, we found that cyclin B1 binds to Cdc25B in an in vitro binding assay. These results suggest that the cytoplasmic localization of cdc25B depends upon cyclin B1, and that the two proteins are likely to be exported together from the nucleus. This is reminiscent of recent results showing that fission yeast cdc25C is exported from the nucleus after DNA damage by binding to the rad24 14-3-3 protein (Lopez-Girona et al., 1999). In both cases, the Cdc25 protein does not have a nuclear export signal itself, but is exported by virtue of its association with another exportable protein.

In conclusion, we have demonstrated marked differences in the abilities of Cdc25B and Cdc25C to promote mitosis and in their cell cycle behavior. Our results suggest

that controlling the level of Cdc25B is crucial to prevent premature mitosis, whereas Cdc25C can be regulated at a subsequent step, most likely by phosphorylation. The activity of Wee1 in the nucleus is very important in counteracting the effects of both the nuclear Cdc25C and the primarily cytoplasmic Cdc25B, emphasizing the dynamic nature of the interactions between cell cycle components that shuttle between the nucleus and the cytoplasm.

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