INTRODUCTION

A major regulator of entry into mitosis is the cdc2 gene product. Its role is highly conserved, homologues of this gene having been identified in organisms as widely divergent as yeast, mammals and plants (Nurse and Bisset, 1981; Lee and Nurse, 1987; Feiler and Jacobs, 1990). It functions through its protein kinase activity to regulate a vast array of cellular protein substrates, some of which produce the dramatic cytoskeletal and structural rearrangements observed at mitosis.

In yeast, the cdc2 gene product regulates both entry into the cell cycle or START and progression into mitosis (Nurse and Bisset, 1981). In vertebrates these functions are carried out by distinct members of a highly homologous family of proteins known as cyclin dependent kinases (cdk). Cdc2 is the prototypic cdk and retains its central role in regulating entry into mitosis in mammalian cells (Hamaguchi et al., 1992; van den Heuvel and Harlow, 1993).

The activity of cdc2 is itself regulated in a complex manner. The cdc2 monomer is catalytically inactive and requires the association of a regulatory cyclin subunit for activity. In vertebrate systems, cyclin B is synthesised during S and G2 phases and immediately complexes with cdc2 in the cytoplasm. The kinase complex is maintained in an inactive form until the G2/M transition through the inhibitory phosphorylations of the threonine 14 and tyrosine 15 residues of cdc2 (Krek and Nigg, 1991a,b; Norbury et al., 1991). In fission yeast only tyrosine 15 is phosphorylated by the wee1 gene product (Featherstone and Russell, 1991; Parker et al., 1991). In higher eukaryotes, a wee1-like activity has been identified which phosphorylates both threonine 14 and tyrosine 15 in vitro (Atherton-Fessler et al., 1994).

The inactive cdc2/cyclin B complex is activated in late prophase by the cdc25 gene product. Overexpression of wild type and dominant negative mutants of cdc2B and cdc25C shows that prophase Mt nucleation is a consequence of cytoplasmic cdc25B activity, and that cdc25C regulates nuclear G2/M events. Our data also suggest that the functional status of the centrosome can regulate nuclear mitotic events.

SUMMARY

The formation of the mitotic spindle is an essential prerequisite for successful mitosis. The dramatic changes in the level of microtubule (Mt) nucleation at the centrosomes and Mt dynamics that occur in prophase are presumed to be initiated through the activity of cdc2/cyclin B. Here we present data that the cdc25B isofrom functions to activate the cytoplasmic pool of cdc2/cyclin B responsible for these events. In contrast to cdc25C, cdc25B is present at low levels in HeLa cells during interphase, but sharply increases in prophase, when cdc25B accumulation in the cytoplasm correlates with prophase spindle formation. Overexpression of wild type and dominant negative mutants of cdc25B and cdc25C shows that prophase Mt nucleation is a consequence of cytoplasmic cdc25B activity, and that cdc25C regulates nuclear G2/M events. Our data also suggest that the functional status of the centrosome can regulate nuclear mitotic events.

Key words: cdc25, Mitosis, Microtubule
and the number of microtubules (Mt) nucleating from the centrosomes increases up to 5-fold that of the interphase centrosome (Kuriyama and Borisy, 1981). The Mt dynamics also change dramatically, from long, slow growing interphase Mt to shorter, more rapidly turning over mitotic Mt. Both the increased Mt nucleation and changes in the Mt dynamics have been shown to be under the control of cdc2/cyclin B, although the direct targets of this kinase in this function are unclear (Verde et al., 1990, 1992; Masuda et al., 1992; Ohta et al., 1993). Cdc2 and cyclin B both accumulate at the centrosome during interphase (Riabowol et al., 1989; Bailly et al., 1989, 1992; Pines and Hunter, 1991; Gallant and Nigg, 1992), suggesting that the localised kinase functions to phosphorylate the centrosomal targets. Therefore the regulation of the cdc2/cyclin B kinase complex at the centrosome is of central importance for the proper functioning of the centrosome at mitosis.

In this study we have investigated the expression and function of the cdc25B isoform. Using antibodies specific for cdc25B, we demonstrate that cdc25B is expressed in late G2 and metaphase in HeLa cells, that this expression is cytoplasmic and that it correlates with increased Mt nucleation on the centrosomes. By overexpressing catalytically inactive mutants we have demonstrated that cdc25B is a regulator of the G2/M transition, and that this is distinct from the cdc25C regulatory function at G2/M. Finally, we present evidence that cdc25B regulates the Mt nucleating activity of mitotic centrosomes and the dynamics of the Mt arrays at prophase.

**MATERIALS AND METHODS**

**Construction of the GST-cdc25B and N-terminal cdc25B plasmids**

We independently isolated a 3 kb cDNA encoding a 79 bp 5'UTR, full length cdc25B ORF and 3'UTR, which was subcloned at the EcoRI site of pBluescript KS(+) (Stratagene) to yield cdc25B/KS(+). The insert was orientated such that the cdc25B 5'UTR was proximal to the polylinker's BamHI site. The major difference between our cDNA and the published sequence was a 42 bp insert also found in the mouse and rat cdc25B sequences (Nagata et al., 1991; Jinno et al., 1994; I. D. Tonks et al., unpublished data). The 5'UTR and first 354 bp of the cdc25B ORF was isolated from cdc25B/KS(+) as a distinct 469 bp BstXI fragment which was then partially digested with BamHI to remove the entire 5'UTR and first 6 bp of the ORF. The resultant 348 bp BamHI/BstXI product was end filled with dNTPs using the Klenow fragment of DNA polymerase I and subcloned at the SmaI site of pBluescript KS(+) such that the 5' end of the insert was proximal to the polylinker's BamHI site. The full length cDNA minus the 5'UTR and first 6 bp of the ORF was obtained by subcloning the 2.7 kb BstII/HindIII fragment from cdc25B/KS(+) at the corresponding sites of the plasmid containing the 348 bp BamHI/BstXI fragment of cdc25B. To produce a GST-fusion protein of the full length cdc25B, the 5'UTR truncated clone was completely digested with BamHI and Dral, a 3.94 kb fragment isolated and subjected to partial EcoRI digestion. The resultant 2.9 kb BamHI/EcoRI fragment was subcloned in the correct reading frame, at the corresponding sites of pGEX-2T (Pharmacia). This expressed a GST-fusion of cdc25B from the third amino acid onwards.

To produce the GST-N-terminal cdc25B fusion, the 5.8 kb BstGI/EcoRI fragment of the pGEX-2T-cdc25B plasmid was end filled with dNTPs using the Klenow fragment of DNA polymerase I and self ligated. This expressed a GST-fusion of the N-terminal 311 amino acids of cdc25B.

**Expression constructs and mutants**

To transiently overexpress cdc25B in HeLa cells, the 3 kb Xhol/XbaI fragment from cdc25B/KS(+) was subcloned into the corresponding sites of both pSVK3 (Pharmacia) and pUCS8R (Takebe et al., 1988) mammalian expression vectors to yield pSVK3 cdc25B and pUCS8R cdc25B. Dominant negative mutants of cdc25B were produced by mutating the essential catalytic Cys487 and Arg493 residues of the cdc25B/KS(+) construct using the USE mutagenesis kit (Pharmacia) based on the unique site elimination strategy of Deng and Nickoloff (1992). The mutant oligonucleotide primers used eliminated a unique BamHI site in the Bluescript plasmid and introduced a BglII site (KS Bam/BglI) while mutations were introduced into the cdc25B as described above along with a silent mutation of the EcoRI site immediately downstream of the Cys codon. Two mutants were produced with either the single Cys to Ser mutation or the double Cys to Ser and Arg to Lys (25B C/S and 25B CR/SK, respectively). The oligonucleotides used were:

KS Bam/BglI: 5' CT CTC TAG AAT CAG TAG ATC TTG TCG GCT GC G 3';
25B C/S: 5'CCT CAT TTT CCA CAG TGA GTT CTC ATC TGA GCG TGG GCC CCC C G 3';
25B CR/SK: 5'CCT CAT TTT CCA CAG TGA GTT CTC ATC TGA GAA AGG GCC CCC C 3'.

The mutations are underlined. The mutations were introduced into pGEX cdc25B by subcloning the 0.7 kb AvrII-Apat fragment from the appropriate cdc25B/KS(+) mutant into the corresponding sites in pGEX cdc25B. The mutant versions of cdc25B were subcloned into pUCS8R as a XhoI-XbaI full length cDNA from the mutant cdc25B/KS(+).

Dominant negative versions of cdc25C were produced by mutation of the catalytic Cys377 and Arg383 in pML25 (Lee et al., 1992). The oligonucleotides used in the USE strategy mutated the unique EcoRI site in pGEX and introduced an NdeI site (pGEX RV/NdeI), and mutated the catalytic Cys and Arg, and the unique EcoRI site 3' to the Cys codon as described for cdc25B (25C C/S and 25C CR/SK). The mutations are underlined:

pGEX RV/NdeI: 5'GCG TTG GTG CTA GTA TGT CCG TAG TGG G 3';
25C C/S: 5'CCA CTG TGA GTT CTC CTC AGA GAG GGG CCC C G 3';
25C CR/SK: 5'CCA CTG TGA GTT CTC CTC AGA GA A GGG CCC C 3'.

The wild type and mutant cdc25C were subcloned into pUCS8R by digesting pML25 with BamHI and subcloning the resultant 1.8 kb fragment into the corresponding site in pUCS8R.

**Bacterial expression and protein purification**

The GST-cdc25B and GST-cdc25C fusion protein expression was carried out in _E. coli_ DH5a cells transformed with the appropriate pGEX-2T cdc25 construct by induction with 0.5 mM IPTG for 3-4 hours at 37°C. The cell pellet was freeze/thawed and lysed using the Sarkosyl method of Fran猩ioni and Neel (1993). Human cyclin B1 was expressed as a GST-fusion from a recombinant baculovirus in _Sf9_ cells (Parker et al., 1991). To affinity purify GST-fusion proteins, lysate was diluted in 2-4 volumes of NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40) buffer containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A and 5 μg/ml leupeptin) and mixed with glutathione agarose (GTA) beads (Sigma) for 40 minutes at 4°C. The GTA beads were then washed three times in NETN at 4°C, once in PBS containing 2 mM ATP for 30 minutes at 25°C, then twice in NETN containing 1 M NaCl followed by an additional wash in NETN. The GST-fusion proteins were eluted twice with the addition of an equal volume of 50 mM sodium borate, pH 8.2, 0.1% SDS and recovered by acetone precipitation (Fran猩ioni and Neel, 1993). Precipitated protein was redissovled in the same borate buffer and either loaded onto preparative 10% SDS-PAGE for immunogen preparation or coupled to activated...
In vitro 35S-labelling and immunoprecipitation

The nuclear pellet was collected by centrifugation at 500 g for 5 minutes at 4°C then passed through a 26 gauge needle repeatedly. The lysate was prepared with protease inhibitors as for the other lysis buffers above, the eluted proteins was assayed by adding 25 µl of 0.5 M p-nitrophenol phosphate to 225 µl of eluted GST-cdc25 (3 µg of GST-cdc25B and 4 µg of the GST-cdc25C) and incubating for 1 hour at 30°C. The reaction was stopped by the addition of 250 µl of 0.5 M NaOH and the p-nitrophenol produced was quantitated by its absorbance at 410 nm. The wild type and mutants of each cdc25 were treated in an identical fashion.

Antibody preparation and immunoblotting

Polyclonal rabbit antisera were raised against purified, bacterially expressed human full length cdc25B, cdc25C and Sf9 expressed cyclin B1 GST-fusion proteins, obtained as described above. Purified GST-fusion proteins were separated using SDS-PAGE and bands visualised by briefly staining with Coomassie Blue. Gel slices of the appropriately sized fusion protein were excised and used as the immunogen. GST-reactive antibodies were depleted from the crude sera using a GST-Sepharose affinity column prior to affinity purification using either the GST-cdc25B N-terminal fusion, GST-cdc25C or GST-cyclin B1 affinity columns for the respective antibodies. The eluted antibodies were concentrated by precipitation in 50% ammonium sulphate followed by dialysis into 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl. The mouse monoclonal anti-α-tubulin antibody was purchased from Amersham. For immunoblotting, proteins were electrotransferred to nitrocellulose membrane, blocked with 5% Blotto (non fat skim milk in PBS containing 0.05% Tween-20). The membranes were probed overnight at 4°C with a 1/100 dilution of the affinity purified cdc25B antibodies, or 1/1,000 dilutions of the affinity purified cdc25C and cyclin B1 antibodies diluted in 1% Blotto. Blots were developed using horseradish peroxidase conjugated secondary antibodies and the ECL detection system (Du Pont NEN). For the affinity purified cdc25B antibodies, incubation with secondary antibody and all washes were performed at 4°C.

To demonstrate specificity of staining primary antibodies were incubated for 1 hour at room temperature in the presence of the appropriate GST-fusion protein linked to GTA beads. The GTA beads were removed by centrifugation and supernatants used for immunoblotting or cell staining (see below).

HeLa cell culture and synchrony

HeLa cells were grown in 5% FBS/RPMI 1640. Cells were synchronised by a double thymidine block/release as described by Atherton-Fessler et al. (1994). At each time point cells were fixed with 70% ethanol at –20°C and stained with propidium iodide (50 µg/ml) for FACS analysis of their DNA content. The remaining of the cells were lysed in NETN/0.4 M NaCl in the presence of protease inhibitors (as used for bacterial lysates), and the supernatants used for either anti-cyclin B1 immunoprecipitate H1 kinase assays (Atherton-Fesseler et al., 1994) or separated on 10% SDS-PAGE and immunoblotted. To prepare cytoplasmic and nuclear fractions, 2x10⁶ cells were incubated in 0.4 ml lysis buffer (5 mM Tris-HCl, pH 7.4, 150 mM sucrose, 50 mM NaCl, 2 mM DTT, 5 mM KCl, 0.5 mM CaCl₂, 1 mM MgCl₂, 0.1 % NP-40) with protease inhibitors as for the other lysis buffers above, for 5 minutes at 4°C then passed through a 26 gauge needle repeatedly to remove residual cytoplasm from the nuclei. The integrity of the nuclei was monitored by observation using phase contrast microscopy. The nuclear pellet was collected by centrifugation at 500 g for 2 minutes and solubilised by boiling in 0.4 ml of SDS sample buffer. In vitro 35S-labelling and immunoprecipitation

In vitro transcription and translation of cdc25B off the pSVK3 cdc25B construct using T7 polymerase was carried out in rabbit reticularcyte lysate (Promega) and labelled with 50 µCi ³⁵S Met (NEA) in 50 µl of media for 1 hour at 37°C. The lysates were diluted to 400 µl with NETN and protease inhibitors then precleared with 50 µl of a 50% suspension of Protein A-Sepharose (Pharmacia). The supernatants were then incubated with either preimmune or 0.5 µg affinity purified cdc25B N-terminal antibody for 2 hours at 4°C followed by a further hour after adding 40 µl of a 50% suspension of Protein A-Sepharose. The immunoprecipitates were washed three times with RIPA buffer, then twice with NETN containing 1 M NaCl and finally with PBS, prior to analysis on 10% SDS-PAGE.

Transient transfection in HeLa cells and FACS analysis

The wild type and mutant cdc25s were expressed in HeLa cells by transient transfection of the pUCSRtx versions of the appropriate cDNA. 10⁷ cells were transfected with 20 µg plasmid by electroporation in 10% FCS/RPMI 1640. Cell samples were collected at several time points after transfection, fixed with 70% ethanol at –20°C overnight and then incubated with either anti-N-terminal cdc25B (1/50 dilution) or anti-cdc25C (1/250 dilution) antibodies in TBS containing 0.1% skim milk powder for 1 hour. The cell pellets were then stained with anti-rabbit FITC-labelled secondary antibody (1/20 dilution; Amersham) and 50 µg/ml propidium iodide, 1 mg/ml RNase A. The DNA content of the transfected cells was analysed by FACS, gating for cells expressing the transfected cdc25 at levels >20% above the peak mitotic levels in cells transfected with the control vector; 2,000-10,000 overexpressing cells were counted in each experiment. The percentage of cells in G₁, S and G₂/M phases was simply determined by partitioning the DNA histogram based on the G₁ and G₂/M peaks from control transfections performed in each experiment. In some experiments, transfected cells were also seeded onto glass coverslips, fixed at the indicated time after transfection and then analysed by indirect immunofluorescence microscopy.

Immunofluorescence microscopy

Both transfected and non-transfected HeLa cells were grown on glass coverslips for immunofluorescence microscopy. Cells were washed in PHEM buffer (45 mM Pipes + 45 mM Hepes, pH 6.9, 10 mM EGTA, 5 mM MgCl₂ containing 1 mM PMSF) prior to rapid fixation in methanol at –20°C for 6 minutes (Bailly et al., 1992). Coverslips were affixed to slides and air dried before being rehydrated in TBS containing 0.1% skim milk powder. Primary antibodies were incubated on the coverslips overnight at 4°C in PBS containing 0.1% Tween-20 and 3% BSA at the following dilutions; α-tubulin, 1/1,000; N-terminal cdc25B, 1/25 for untransformed cells or 1/100 for transfected cells; cdc25C, 1/200 for untransformed cells or 1/500 for transfected cells; cyclin B, 1/500. The secondary antibodies were either TRITC-labelled anti-mouse Ig (1/50; Sigma) or FITC-labelled anti-mouse Ig (1/20; Amersham) or FITC-labelled anti-rabbit Ig (1/20; Amersham). DNA staining was carried out with 2.5 µg/ml propidium iodide (Sigma) or 0.05 µg/ml Hoechst 33342 (Calbiochem-Behring Corp.). Immunofluorescent preparations were mounted in Vectashield mounting medium (Vector Laboratories). All figures were photographed with a Zeiss Plan Apochromat oil immersion objective (×100) or a Zeiss Plan-Neoilu oil immersion objective (×100) using EPL-400 Kodak film.

RESULTS

To investigate the function of the cdc25B isoform in HeLa cells, we produced polyclonal antibodies against the bacterially expressed GST-fusion product of cdc25B. The antisera was initially depleted of antibodies directed against the GST moiety and the cdc25B directed antibodies were affinity purified against GST-fusion product of the N-terminal
truncated form of cdc25B. This was used to produce an antibody specific for cdc25B, as the three human cdc25 isoforms have a high degree of sequence identity in their catalytic C-terminal domains (Galaktionov and Beach, 1991). The affinity purified N-terminal cdc25B antibody displayed no cross-reactivity to bacterially expressed GST nor to a truncated GST-C-terminal cdc25B fusion protein, but detected both the GST-N-terminal and GST-cdc25B full length proteins (Fig. 1A). This N-terminal cdc25B antibody showed no crossreactivity with bacterially expressed GST-cdc25C, and did not detect bands of between 55 and 65 kDa in HeLa cell lysates, corresponding to the cdc25A and cdc25C isoforms (Fig. 1B). The unmodified full length cdc25B expressed in bacteria was also specifically detected by the antibody, but a similar size band was difficult to detect in immunoblots of asynchronous HeLa cell lysates. A slightly higher molecular mass band of 85 kDa was clearly evident in HeLa cells synchronised with a double thymidine block and then allowed to progress to G2/M (Fig. 1B). The antibody also specifically immunoprecipitated the 85 kDa cdc25B from 35S Met-labelled, in vitro translation of the cdc25B cDNA (Fig. 1C).

Cell cycle dependent expression of cdc25B

The inability to detect cdc25B in asynchronous HeLa cells, and its relative abundance in synchronised G2/M HeLa cells, suggested that the protein may be expressed in a cell cycle dependent manner. Immunoblotting of lysates prepared from equivalent numbers of HeLa cells progressing synchronously from G1/S following release from a double thymidine block, showed that the expression of cdc25B protein rapidly increased in late G2 (9 hours after release from the thymidine block) immediately prior to the activation of the major pool of cdc2/cyclin B (Fig. 2A and B). The level of cdc25B protein increased in metaphase (10 hours after release) where it resolved into a doublet, with the slower migrating form probably representing a phosphorylated form. The protein was then rapidly degraded with only a low level detectable by 12 hours, when the majority of cells had progressed into the next G1. In some experiments, very low levels of the faster migrating form were detected in the other phases of the cell cycle. The expression of cdc25B is reminiscent of cyclin B, although the accumulation of cdc25B protein appeared to be more abrupt than that seen for cyclin B1 (Fig. 2C), and strongly suggested a mitotic function for cdc25B.

cdc25B accumulates in the cytoplasm in a cell cycle dependent manner

The cellular localisation of cdc25B was investigated by preparing cytoplasmic and nuclear fractions of G2/M phase cells from a double thymidine synchrony. The late G2 phase expression of cdc25B was almost exclusively in the cytoplas...
cdc25B regulates spindle formation

Fig. 2. Cell cycle specific expression of cdc25B in HeLa cells. HeLa cells synchronised by a double thymidine block were released and samples taken at the indicated times. (A) Samples were analysed by FACS and the percentage of cells in G1 (squares), S phase (diamonds) and G2/M (circles) quantitated. The histone H1 kinase activity in cyclin B1 immunoprecipitates was also quantitated (solid triangles). (B) Whole cell lysates (60 μg protein) of the indicated time points from the same experiment as in (A) were immunoblotted with cdc25B N-terminal antibody. (C) Aliquots of the same samples as in B (10 μg protein) were immunoblotted with cyclin B1 antibody.

Fig. 3. Cdc25B is expressed in the cytoplasm. Cytoplasmic (Cyto.) and nuclear (Nuc.) fractions were prepared from cells in G2/M phase at 10 hours after release from a double thymidine block synchronisation. Fractions were immunoblotted with the indicated affinity purified antibodies.

Fig. 4. Immunofluorescent localisation of cdc25B in untransfected HeLa cells. (A) N-terminal cdc25B antibody staining of HeLa cells showing the cytoplasmic accumulation of cdc25B correlates with prophase Mt nucleation, shown in B with α-tubulin staining of the same cells. Preincubation of the antibody with the cdc25B N-terminal GST fusion protein abolished the staining in cells displaying the same level of Mt nucleation (D and E). (C and F) Hoechst 33342 staining shows the DNA of the same cells. Exposure times for A and D were identical. Bar, 10 μm.
We next examined the cell cycle dependent expression and localisation of cdc25B by indirect immunofluorescent staining of HeLa cells. Only a low level of granular, intranuclear staining was observed in interphase cells (Fig. 4A), which was specifically blocked by preincubating the antibody with the GST-cdc25B N-terminal fusion protein (Fig. 4D). However, a small number of cells also displayed either a perinuclear or more evenly distributed cytoplasmic staining of the cdc25B protein (Figs 4A and 5A). These cells with cytoplasmic staining of cdc25B were in prophase, as the duplicated centrosomes have begun migration to their mitotic poles, and displayed increased levels of Mt nucleation at the centrosomes and intact nuclei (Kochanski and Borisy, 1990; Fig. 4B and C). Cytoplasmic staining in these prophase cells was higher than those of the surrounding interphase cells and these levels remained high through metaphase, decreasing in anaphase and telophase back to interphase levels (Fig. 5A). The rapid increase in prophase and subsequent decrease in anaphase of cdc25B protein levels is in agreement with the immunoblotting data (Fig. 2B). Both the cytoplasmic and nuclear staining were abolished by prior incubation of the antibody with recombinant N-terminal cdc25B (Fig. 4D).

Inspection of the cell doubly stained for cdc25B and tubulin revealed that cells with only background staining for cdc25B had a basket-like Mt organisation surrounding their nucleus, consistent with that of interphase cells (Figs 4A and B, 5A). However, when cells which had a perinuclear or cytoplasmic accumulation of cdc25B were examined, prophase Mt nucleating activity at the centrosome was found in every case. Likewise, when cells were identified on the basis of having prophase Mt nucleation and an intact nuclear membrane, cdc25B was always observed to be cytoplasmically localised, establishing a strong correlation between the cytoplasmic accumulation of cdc25B and the conversion of the interphase to mitotic centrosome.

Indirect immunofluorescent staining with a human cdc25C antibody revealed a strong granular staining in intact nuclei throughout the cell cycle, as reported previously (Millar et al., 1991). Further, cdc25C remained localised to the nucleus when prophase Mt nucleation was clearly evident (Fig. 5B). This is in contrast to the perinuclear and cytoplasmic localisation of cdc25B at this point in the cell cycle.

cdc25B functions as a G2/M regulator
The restricted expression of the cdc25B protein to the late

![Fig. 5. Conversion from interphase to prophase Mt organisation at the centrosome correlates with the accumulation of cdc25B in the cytoplasm of prophase cells while cdc25C is retained within the nucleus. Untransfected HeLa cells were stained with N-terminal cdc25B (A), cdc25C (B) and α-tubulin (Mt; C and D) antibodies. (A) Cdc25B levels increase at prophase (p) and peak at metaphase (m; two metaphase cells displaying mitotic spindles) before decreasing in anaphase (a) and telophase (t) to the interphase levels of the surrounding cells. Accumulation of cdc25B in the cytoplasm is evident in the prophase cells (p and insert) whereas the interphase cells (unlabelled) display only weak nuclear staining. (C) α-Tubulin staining of the same cells as in A indicates that Mt nucleation has commenced at the centrosomes of the prophase cells (p and insert). Separation of duplicated centrosomes in the prophase cells is also evident. (B) Cells showing only nuclear cdc25C even though the centrosomes of the prophase cell (centre field) clearly display prophase Mt nucleation (D). Bar, 10 μm.
G2 and M phases suggested that the protein functions at the G2/M transition. To investigate the function of cdc25B directly, we have attempted to produce a dominant negative mutant of cdc25B by mutating the essential catalytic Cys and Arg residues in cdc25B (Gautier et al., 1991), and transiently overexpressing either the wild-type or the mutant proteins in HeLa cells. Assay of bacterially expressed GST fusions of the wild-type and mutant cdc25B proteins for their ability to dephosphorylate p-nitrophenyl phosphate confirmed that both a Cys to Ser (C/S) mutation and a Cys to Ser, Arg to Lys double mutation (CR/SK) resulted in catalytically inactive proteins (Fig. 6A). HeLa cells were transiently transfected with either wild-type or mutant cdc25B, then analysed at 24 and 48 hours post transfection. The cells were stained for cdc25B, and the cell cycle status of overexpressing cells analysed by gating for high FITC fluorescence intensity (for cdc25B) and assessing their DNA content (propidium iodide staining) by FACS. Both wild-type and mutant proteins were strongly overexpressed, although the full length wild-type cdc25B accumulated to lower levels than the mutants (Fig. 6B). The lower molecular mass proteins detected are presumably proteolytic breakdown products of the overexpressed cdc25B as similar size fragments were often detected in the bacterially expressed cdc25B proteins (Fig. 1A). Overexpression of either cdc25B mutant resulted in an accumulation of the G2/M population of cells with a concomitant decrease in the G1 population at 24 and 48 hours post transfection compared to the control vector transfections (Fig. 6C and D). Little change was observed in the percentage of cells in S phase. The accumulation of cells in G2/M phase could be seen as early as 10 hours post transfection, and increased by 24 hours post transfection (Fig. 6C) reaching a maximum of 50-60% by 48 hours (Fig. 6D; Table 1). The CR/SK double mutant produced a slightly greater increase in the G2/M population than the C/S mutant although both mutants were catalytically inactive. The declining proportion of G1 cells and accumulation of G2/M cells indicates either a delay in transit through G2 and M phases, or cell cycle arrest in G2 or M.

An increase in the percentage of cells in S and G2/M phases was observed with wild type cdc25B overexpression (Table 1). Unlike the mutants, however, the accumulation was not prominent until 48 hours post transfection, although a small effect was evident as early as 24 hours post transfection (Fig. 6).

**Fig. 6.** Overexpression of wild type and mutant cdc25B in HeLa cells. (A) Bacterially expressed GST or the GST fusions of cdc25B and mutant cdc25B were assayed for their activity towards p-nitrophenyl phosphate. The data are the mean ± s.d. of triplicate determinations. (B) Equal numbers of cells transfected with either the control vector (con), wild-type cdc25B (WT), or the mutants (C/S, CR/SK), were immunoblotted for cdc25B 24 hours post transfection. The full length protein is indicated (arrowhead). (C) Control vector, wild-type cdc25B or catalytically inactive mutants in the pUCSRa expression plasmid were transiently overexpressed in HeLa cells and the cell cycle status of the overexpressing cells were analysed by FACS. The DNA histograms from cells harvested 24 hours post transfection are shown. (D) In a similar experiment to that in C, the DNA histogram from cells harvested 48 hours post transfection are shown.
Cell cycle Control Wild-type nucleators (e.g. Fig. 9B and D, arrows). The percentage of nucleators that were transfected was determined by microscopic examination. The data represents the mean ± s.d. of three separate experiments.

6C and D), perhaps simply reflecting the lower level of accumulation of the wild type at 24 hours post transfection.

The cdc25C isoform has also been assigned a function in regulating the G2/M transition so it was necessary to test whether the cdc25B mutants were producing a specific effect in competing out the normal cdc25B activity, or whether it was simply the effect of overexpression of a catalytically inactive cdc25. To this end, catalytically inactive mutants of cdc25C similar to those of cdc25B, were produced (Fig. 7A).

Table 1. The dominant negative effect of overexpressing mutants of cdc25B

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Control vector</th>
<th>Wild-type cdc25B</th>
<th>Mutant cdc25B</th>
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<tr>
<td>G1</td>
<td>52±1.8</td>
<td>33±3.6</td>
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<td>48±6.4</td>
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</table>

The percentage of cells in each phase of the cell cycle was quantitated by FACS analysis of the overexpressing cells 48 hours post transfection. The data represents the mean ± s.d. of three separate experiments.

The cdc25C isoform has also been assigned a function in regulating the G2/M transition so it was necessary to test whether the cdc25B mutants were producing a specific effect in competing out the normal cdc25B activity, or whether it was simply the effect of overexpression of a catalytically inactive cdc25. To this end, catalytically inactive mutants of cdc25C similar to those of cdc25B, were produced (Fig. 7A). FACS analysis of wild-type cdc25C overexpressed in HeLa cells at 48 hours post transfection showed that in contrast to overexpression of wild-type cdc25C, overexpression of wild-type cdc25C had little effect on cell cycle distribution, with only a small increase in the G2/M population (Fig. 7C) compared with the strong accumulation in S and G2/M phases seen with wild-type cdc25B (Fig. 6D). The cdc25C (both wild type and mutants) was overexpressed greater than 10-fold at this time (Fig. 7B). The cdc25C mutants produced a G2/M arrest, but to a lesser degree than that seen with the cdc25B mutants at 48 hours post transfection (40% compared to 50-60% for cdc25B), although this increased to 60% by 72 hours post transfection. This effect differed from that observed with overexpression of the cdc25B mutants in that it was not evident at 24 hours post transfection (data not shown), at which stage the cdc25B mutants had already produced a significant accumulation of G2/M cells (Fig. 6C). As seen with the cdc25B mutants, the cdc25C CR/SK mutant produced a more marked G2/M accumulation than the C mutant.

Cytoplasmic expression of cdc25B regulates prophase spindle assembly

We have shown that the normal cell cycle dependent expression of endogenous cdc25B in the cytoplasm prior to nuclear envelope breakdown correlated with increased Mt nucleation at the centrosome and prophase spindle formation. Overexpressed cdc25B (both wild type and mutant) was found in the cytoplasm in all cases, but in cells heavily overexpressing cdc25B the majority of transfected cells displayed strong nuclear staining was also observed (Fig. 8a and c). The nuclear accumulation was unexpected, but was also observed with cdc25B transfection into neonatal fibroblasts and COS cells, and was, therefore, not an artefact of overexpression of this protein in HeLa cells (unpublished results).

Overexpression of inactive double mutant cdc25B has a dominant negative effect on prophase microtubule nucleation

Examination of the Mt staining patterns of cells heavily overexpressing mutant cdc25B revealed predominantly interphase Mt networks (Fig. 8D, with very few mitotic spindles observed even though FACS data showed >50% of these cells to be in G2 phase (Fig. 6D; Table 1). Detailed examination of the low level overexpressing cells which displayed increased cdc25B staining in the perinuclear region or the cytoplasm but little nuclear staining, also revealed an almost complete absence of normal prophase Mt nucleation or mitotic spindle formation, although in many cases they possessed centrosomes which had migrated to the mitotic poles (Fig. 8B and D; data not shown). This effect was observed for both C/S and CR/SK mutants. The low level

Table 2. Transfection of HeLa cells with mutant cdc25B results in a loss of prophase Mt nucleation when mutant cdc25B is overexpressed in the cytoplasm

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells counted</th>
<th>Level of Mt nucleation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>26</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>19</td>
</tr>
</tbody>
</table>

HeLa cells were transfected with mutant cdc25B (CR/SK) in a pUCS5Ra vector and seeded onto coverslips. Cells were examined 48 hours post transfection by indirect immunofluorescence using antibodies to the N-terminal region of cdc25B and α-tubulin. Cells with a higher level of overexpressed mutant cdc25B in the cytoplasm than the nucleus (e.g. Fig. 8A and C) were counted and the level of Mt nucleation in these cells was evaluated as follows: 0, an interphase basket-like arrangement of Mt (e.g. Fig. 8B); ±, intermediate Mt arrangement i.e. Mt gathering at the centrosome but with no focus of nucleation (e.g. Fig. 8D and insert); +, weak Mt nucleation i.e. some centrosomal focus of Mt.

Table 3. Overexpression of wild type or mutant cdc25C in HeLa cells does not affect prophase Mt nucleation

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Wild type transfected cells</th>
<th>Mutant transfected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transfection rate</td>
<td>Nucleators counted</td>
</tr>
<tr>
<td>1</td>
<td>41%</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>51%</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>31%</td>
<td>29</td>
</tr>
</tbody>
</table>

HeLa cells were transiently transfected with wild-type or mutant cdc25C (CR/SK) in a pUC5Ra vector and seeded onto coverslips. Cells were examined at 48 hours post transfection by indirect immunofluorescence using antibodies to cdc25C and α-tubulin. The overall percentage of cells transfected in each experiment was determined by microscopic examination. Cells with intact nuclear membranes and centrosomes displaying prophase Mt nucleation were defined as nucleators (e.g. Fig. 9B and D, arrows). The percentage of nucleators that were transfected was determined by microscopic examination.
cdc25B regulates spindle formation

CR/SK expressing cells 48 hours post transfection were quantified for loss of mitotic spindle formation, as this mutant produced a stronger G2/M accumulation (Fig. 6D). Of the 39 cdc25B CR/SK mutant overexpressing cells which displayed low level cytoplasmic cdc25B staining, half showed normal interphase Mt organisation, denoted as ‘0’ nucleation (Table 2; Fig. 8A and B). An almost equal number displayed an area of Mt concentration in the cytoplasm adjacent to the nucleus although no focus of Mt nucleation was apparent (Table 2, ‘+/-’ nucleation; Fig. 8C and D and insert). In only 2 of the 39 cells counted, a low degree of Mt nucleation and focus formation (although still less than observed normally) was detected (Table 2, ‘+’ nucleation). Interestingly, no chromosome condensation or nuclear envelope breakdown was observed in the cells where the overexpressed mutant cdc25B blocked the cytoplasmic Mt nucleation at the centrosomes. The almost total absence of Mt nucleation and prophase spindle formation observed in any of the mutant cdc25B overexpressors is consistent with the G2 accumulation observed with FACS analysis, as these mutant expressing cells are unable to undergo cytokinesis and thus accumulate with a G2 complement of DNA.

Overexpression of wild-type cdc25B causes the formation of abnormal ‘mini-spindles’

Cells overexpressing wild-type cdc25B in the cytoplasm often displayed bizarre ‘mini-spindles’ which were located to one side of the cell in which nuclei were observed on the side of the cell opposite to the spindle (Fig. 8E-H). These nuclei were smaller and more brightly stained than normal nuclei and appeared to have an intact nuclear membrane. Small amounts of DNA staining material were seen in the spindle (Fig. 8G), but it is unclear whether this DNA represented the chromosomes of micronuclei and/or whether these cells were in early apoptosis. Few ‘mini-spindles’ were evident at 10 and 24 hours post transfection, but the number increased at longer periods of time post transfection (48 hours) to a point where few normal prophase Mt arrays were observed in overexpressing cells. This is consistent with the increase in the accumulation of cells in G2 at 48 hours post transfection observed with FACS analysis (Fig. 6D). The overexpression of wild-type cdc25B in the cytoplasm appears to cause inappropriate activation of Mt nucleation without the normally synchronised events of the nucleus, resulting in the formation of abnormal ‘mini-spindles’ in the cytoplasm.

G2/M arrest in cells overexpressing wild-type or mutant cdc25C is not due to an effect on prophase microtubule nucleation

Immunofluorescent staining of cells transfected with wild-type or mutant cdc25C showed that in most cells the overexpressed protein was predominantly localised to the cytoplasm (Fig. 9A and C) in contrast to the nuclear site of normally expressed cdc25C (Fig. 5B). A small number of cells also had increased staining in the nucleus, presumably due to the translocation of the cdc25C to the nuclei in late prophase as reported previously for the human cdc25C overexpressed in BHK cells (Heald et al., 1993). No abnormal ‘mini-spindles’, such as were seen with overexpression of wild-type cdc25B, were observed in cells transfected with wild-type cdc25C, despite the fact that the overexpressed wild-type cdc25C localised in the cytoplasm (Fig. 9B). Also the percentage of cdc25C overexpressing cells showing prophase Mt nucleation at centrosomal foci was the same as the overall rate of transfection, indicating that wild-type cdc25C overexpression has no effect on prophase Mt nucleation (Table 3).

Cells overexpressing mutant cdc25C also displayed normal prophase Mt nucleation (Fig. 9D), and the percentage of nucleating cells overexpressing mutant cdc25C in the cytoplasm was
at least as high as the overall rate of transfection (Table 3). Clearly, the dominant negative effect on prophase Mt nucleation which is observed with overexpression of mutant cdc25B is due to specific blocking of endogenous cdc25B function, while the overexpression of mutant cdc25C must inhibit distinct, cdc25C catalysed G2/M functions.

**DISCUSSION**

One of the striking aspects of cdc25B is its expression pattern, notably its rapid accumulation at prophase and turnover upon the completion of mitosis. This is in contrast to the cdc25C isoform which is present at constant levels throughout the HeLa cell cycle even though its primary function is at mitosis. Likewise cdc25A, which has a G1/S function, is also constant throughout the HeLa cell cycle (Hoffmann et al., 1994). Although cdc25B is the only cdc25 isofrom regulated at the level of protein expression in HeLa cells, cdc25A protein levels oscillate through the cell cycle in untransformed rat fibroblasts (Jinno et al., 1994) and the single S. pombe cdc25 is also regulated at the protein level (Moreno et al., 1990; Docummun et al., 1990). In both these cases, the expression of the respective cdc25 protein corresponds to the point in the cell cycle which is regulated by these proteins. The constant expression of cdc25A and cdc25C isoforms possibly confers a growth advantage, facilitating the continuous cycling of HeLa cells, but unregulated expression of cdc25B is detrimental to cell growth, as we have observed with overexpression of wild-type cdc25B.

We have found that the increased expression of cdc25B at prophase resulted in its accumulation in the perinuclear region and cytoplasm prior to nuclear envelope breakdown. The overexpression of catalytically inactive mutants of cdc25B produced an effective G2/M delay, evidence for a G2/M function for cdc25B. The lack of prophase Mt nucleation in the mutant overexpressing cells, even in those with low levels of overexpression and which displayed predominantly cytoplasmic accumulation of cdc25B, suggests that the cytoplasmically localised mutants blocked this late G2 cytoplasmic event.
overexpression of mutant cdc25C also produced a G2/M delay, but with much slower onset than that seen with mutant cdc25B overexpression and without any detectable effect on the prophase MTOC activity. The delayed effect of mutant cdc25C overexpression may be due to a higher endogenous level of cdc25C compared to cdc25B expressed in HeLa cells requiring much higher levels of mutant protein expression to produce an effect, or possibly could be due to the unexpected cytoplasmic localisation of the overexpressed cdc25C.

Further evidence for a role for cdc25B in the regulation of Mt nucleation and dynamics comes from the unexpected and striking results produced with the overexpression of the wild-type cdc25B. FACS analysis indicated that overexpression of wild-type cdc25B produced an accumulation of G2/M phase cells, albeit at a much slower rate than that observed for the catalytically inactive mutants, and some disturbance of S phase. Overexpression of wild-type cdc25C did not similarly affect the cell cycle, with only a small increase in the G2/M population observed. This in itself was unexpected, as overexpression of cdc25 in fission yeast produces the wee phenotype due to the shortening of G2 (Russell and Nurse, 1986) rather than the small delay observed here. Most remarkable was the appearance of ‘mini-spindles’ in cells overexpressing wild-type cdc25B in the cytoplasm. These cells had sharply delineated nuclei which appeared to have intact nuclear membranes and were brightly stained with Hoechst 33342 perhaps suggestive of early apoptosis (Sun et al., 1992; Cohen et al., 1993). The inappropriate cdc25B activity in the cytoplasm may cause the premature activation of Mt nucleation at the centrosome and aberrant spindle formation, but without normal chromosome condensation. This appeared to be an irreversible effect, witnessed by the slow accumulation of the G2/M population seen in the FACS analysis. The potentially lethal phenotype resulting from the wild-type overexpression could explain why the expression of cdc25B is normally so tightly regulated. Similar ‘mini-spindles’ have been reported in BHK cells co-transfected with cdc25C and cyclin B1, but were not seen with transfection of cdc25C or cyclin B1 separately (Heald et al., 1993). This supports our findings that cdc25C overexpression did not effect Mt dynamic or spindle formation, but indicates that co-transfection of a cyclin may compromise this specificity. These observations, together with

**Cyclin B**

![A](image1.png)  
**DNA**

![C](image2.png)  

**Fig. 9.** Transfection of wild type or mutant cdc25C into HeLa cells results in cytoplasmic overexpression which does not affect prophase Mt nucleation. Coverslip cultures of HeLa cells transiently transfected with wild type or mutant cdc25C in the pUCSCRα vector were examined by indirect immunofluorescence using cdc25C and α-tubulin (Mt) antibodies. Transfected cells overexpressed wild type or mutant cdc25C in the cytoplasm (A and C). Normal prophase Mt nucleation was observed in those cells which were overexpressing either wild type or mutant cdc25C (B and D). Bar, 10 μm.

**Fig. 10.** A prophase cell stained for cyclin B1 (A), α-tubulin (B) and DNA with Hoechst 33342 (C) is shown. The nuclear accumulation of cyclin B1 is clearly evident and the centrosomal localisation of cyclin B1 can be seen as the two brightly stained dots diagonally opposed across the horizontal axis of the nucleus. Cyclin B1 associated with the spindle Mt can also be discerned. The nuclear membrane is still intact, and chromatin condensation has not begun, with the nucleoli still visible as negatively staining areas within the nucleus (C). Bar, 8 μm.
the mutant cdc25B and cdc25C overexpression results, are strong evidence that the dominant negative effect produced by cdc25B mutants is due to the specific inhibition of the endogenous cdc25B activity, and that cdc25B functions at prophase to regulate the conversion of the interphase centrosome to a prophase MT-organiser and the change in MT dynamics. However, the fact that cdc25B does localise to the nucleus in heavily overexpressing cells points to a mechanism for cdc25B to accumulate in that compartment, so that an additional, nuclear function for cdc25B cannot be dismissed.

The normal prophase expression of cdc25B was restricted to the soluble cytoplasmic fraction; no evidence of cdc25B associating with either the MT network or centrosomes was found by either indirect immunofluorescence or subcellular fractionation, suggesting that cdc25B regulates the changes in MT nucleation and dynamics via an indirect mechanism. This is probably through its ability to dephosphorylate and activate cdc2/cyclin B (Sebastian et al., 1993), and this kinase complex is probably a major substrate in vivo. Evidence from a number of laboratories has pointed to the active cdc2/cyclin B having a major role in producing the prophase changes in MT dynamics and mitotic spindle formation (VeIve et al., 1992; Masuda et al., 1992; Ohta et al., 1993), and cyclin B1 has been shown to associate with MT fibres and centrosomes (Fig. 10A; Pines and Hunter, 1991; Bailly et al., 1992).

What is the rationale for separate isoforms of cdc25 regulating the same cell cycle transition? In fission yeast, cdc2 and its cyclin partner cdc13 are localised strictly to the nucleus (Alpha et al., 1990). The mitotic spindle itself forms within the nucleus and thus the single cdc25 is sufficient for the regulation of nuclear rearrangements and spindle formation at mitosis in S. pombe. The diversity of cellular structure and shape apparent in mammalian cells in comparison to yeast has necessitated the development of an extensive cytoskeletal network underpinned by MT fibres. The prophase rearrangement of the interphase MT network produces the mitotic scaffold to catch condensed chromosomes by their kinetochores after nuclear envelope breakdown, allowing the metaphase plate to assemble efficiently and quickly. Thus the formation of a perinuclear array of prophase MT would be expected to occur earlier than nuclear envelope breakdown as is clearly evident in a typical mid-prophase cell such as that seen in Fig. 10B and C. The economy of reassembling the tubulin component of this cytoplasmic scaffold by forming cytoplasmic (rather than intranuclear) mitotic spindle gives meaning to the evolution of separate cytoplasmic and nuclear isoforms of cdc25. From our data, we propose that cdc25B is responsible for the activation of the cytoplasmic (and centrosomal) cdc2/cyclin B at prophase, and that one of the consequences is the change in MT dynamics and spindle formation. Cdc25C does not appear to effect this cytoplasmic function, and we would propose that it activates the large nuclear pool of cdc2/cyclin B, suggested by its localisation to that compartment.

Centrosomal MT nucleation and prophase spindle formation occurs prior to nuclear envelope breakdown, when cdc25C is still intranuclear (Fig. 5B). At prophase, the majority of the cdc2/cyclin B1 complex has been translocated to the nucleus, but small pools remain in the cytoplasm and associated with the centrosomes and MT arrays (Fig. 10A). The nuclear pool of cdc2/cyclin B1 is presumably not yet active, evidenced by the absence of events initiated by cdc2/cyclin B activity such as chromosome condensation or nucleolar disintegration (Fig. 10C; Lamb et al., 1990; Peter et al., 1990; Belenguer et al., 1990), whereas the cytoplasmic (and MT and centrosomal associated) cyclin B1 complex is presumably not yet active, evidenced by the increased MT nucleation at the centrosomes and change in MT dynamics (Fig. 10B). Thus, the presence of a preformed mitotic spindle for rapid capture of condensed chromosomes after nuclear envelope breakdown requires that the cytoplasmic mitotic events precede the nuclear rearrangements in a strictly ordered manner. Two further observations support the existence of a sequential mechanism. First, the cytoplasmic overexpression of cdc25B mutants not only inhibited the formation of the mitotic spindle in cells showing prophase separation of centrosomes, but also blocked chromosomal condensation and nucleolar disintegration (Fig. 8A and C). We failed to find any cdc25B mutant expressing cells with condensed chromatin but lacking some form of a mitotic spindle. Second, the higher percentage of cells displaying both prophase MT nucleation and uncondensed DNA in the mutant cdc25C overexpressing group compared to the untransfected cdc25 is sufficient for the regulation of nuclear rearrangements and spindle formation at mitosis with the resultant accumulation of cells with prophase spindles.

The data presented here provide evidence that the activation of the mitotic kinase, presumably cdc2/cyclin B, in separate cellular compartments is controlled by distinct isoforms of cdc25 phosphatase, and clarify a mechanism by which essential cytoplasmic functions, such as prophase MT nucleation and spindle formation, can occur prior to nuclear envelope breakdown.

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