

Characterisation of Cdc25B localisation and nuclear export during the cell cycle and in response to stress

Arne Lindqvist, Helena Källström and Christina Karlsson Rosenthal*

Department of Cell and Molecular Biology, Karolinska Institutet, S-171 77 Stockholm, Sweden

*Author for correspondence (e-mail: christina.karlsson@cmb.ki.se)

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Summary

Cdc25 phosphatases are essential regulators of the cell cycle. In mammalian cells, the Cdc25B isoform activates cyclin A- and cyclin B1-containing complexes and is necessary for entry into mitosis. In this report, we characterise the subcellular localisation of Cdc25B by immunofluorescence in combination with RNA interference to identify specific antibody staining. We find that endogenous Cdc25B is mainly nuclear, but a fraction resides in the cytoplasm during the G2 phase of the cell cycle. Cdc25B starts to appear in S-phase cells and accumulates until prophase, after which the protein disappears. We characterise a nuclear export sequence in the N-terminus of Cdc25B (amino acids 54-67) that, when mutated, greatly reduces the ability of Cdc25B to shuttle in a fluorescence loss in photobleaching assay. Mutation of the nuclear export sequence makes Cdc25B less efficient in

inducing mitosis, suggesting that an important mitotic function of Cdc25B occurs in the cytoplasm. Furthermore, we find that when cells are exposed to cycloheximide or ultraviolet irradiation, Cdc25B partially translocates to the cytoplasm. The dependence of this translocation event on a functional nuclear export sequence, an intact serine 323 residue (a 14-3-3 binding site) and p38 mitogen-activated protein kinase activity indicates that the p38 pathway regulates Cdc25B localisation in different situations of cellular stress.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/117/21/4979/DC1>

Key words: Cell cycle, cyclin B1, Cdc25B, Localisation, Nuclear export, MAPK

Introduction

Progression through the cell cycle is mediated by cyclin dependent kinases (CDKs) in complex with cyclins. The activity of cyclin-CDK complexes is regulated at multiple levels: expression and proteolysis of cyclins, phosphorylation, binding of inhibitors, and subcellular localisation (for a review, see Obaya and Sedivy, 2002). In mammalian cells, the kinase activities of CDK1 and CDK2 are inhibited by the phosphorylations of Thr14 and Tyr15 in their catalytic cleft by Myt1 and Wee1 kinases (Liu et al., 1997; Mueller et al., 1995; Parker and Piwnicka-Worms, 1992). The cell division cycle 25 (Cdc25) dual specificity phosphatases remove these phosphates and are therefore important determinants of cyclin-CDK activity (Gautier et al., 1991; Kumagai and Dunphy, 1991; Millar et al., 1991b; Sebastian et al., 1993; Strausfeld et al., 1991). Whereas yeast has one *cdc25* gene, there are three different Cdc25 isoforms in mammalian cells: Cdc25A, B and C (Galaktionov and Beach, 1991; Nagata et al., 1991; Sadhu et al., 1990). The C-terminal catalytic domain is well conserved between the isoforms, but the N-terminal regulatory part shows more variation in sequence. In accordance with their key roles in cell cycle control, Cdc25 phosphatases are also involved in tumour development. Cdc25A and Cdc25B but not Cdc25C are overexpressed in several human cancers (Ito et al., 2002; Kudo et al., 1997; Nishioka et al., 2001; Takemasa et al., 2000). Cdc25B has also been reported to be a steroid-receptor coactivator, a function distinct from its phosphatase activity

(Ma et al., 2001; Ngan et al., 2003), which may contribute to Cdc25B's oncogenic potential.

Entry into mitosis is largely orchestrated by the cyclin B1-CDK1 complex, whose activity is regulated both temporally and spatially. During interphase, cyclin B1-CDK1 is continuously exported from the nucleus and accumulates in the cytoplasm (Hagting et al., 1998; Pines and Hunter, 1991; Toyoshima et al., 1998; Yang et al., 1998). In early prophase, the initial activation of the complex occurs in the cytoplasm and at centrosomes (De Souza et al., 2000; Jackman et al., 2003), but in late prophase cyclin B1-CDK1 rapidly moves into the nucleus where it reaches full activity (Li et al., 1997).

It is unclear which Cdc25 isoforms are responsible for the activation of the cyclin B1-CDK1 complex. At present, all three Cdc25 isoforms have been shown to play a role in mitosis. Cdc25A was originally thought to function only at the G1-S transition but an additional role in mitosis has recently been reported (Mailand et al., 2002; Molinari et al., 2000). Cdc25B and Cdc25C were found necessary for the G2-M transition because overexpression of dominant negative mutants or microinjection of antibodies blocked cells in G2 (Gabielli et al., 1996; Lammer et al., 1998; Millar et al., 1991a; Seki et al., 1992). Both Cdc25B and Cdc25C can induce premature mitosis when overexpressed but Cdc25B is more efficient in doing so (Karlsson et al., 1999). The phosphatase activity of Cdc25C increases after phosphorylation of its N-terminus by cyclin B1-CDK1, thus a positive feedback loop is

created at the G2-M transition which leads to the rapid activation of cyclin B1-CDK1 (Gabrielli et al., 1997a; Hoffmann et al., 1993). It has been proposed that Cdc25B functions to initiate activation of cyclin B1-CDK, which then phosphorylates and activates Cdc25C, leading to full activation of the cyclin B1-CDK1 complex (Gabrielli et al., 1996; Lammer et al., 1998; Nilsson and Hoffmann, 2000).

Checkpoints are surveillance mechanisms that ensure cell cycle arrest in response to internal or external damage to allow time for repair or apoptosis. All three human Cdc25 isoforms are suggested targets of checkpoint pathways: Cdc25A is degraded at the S-phase DNA-replication checkpoint via Chk1- and Chk2-mediated phosphorylation (Falck et al., 2001; Falck et al., 2002; Zhao et al., 2002). Cdc25C is phosphorylated and inactivated by Chk1 in the G2 phase following unreplicated DNA and irradiation-induced damage (Peng et al., 1997). This checkpoint-induced phosphorylation of Cdc25C mediates binding to the 14-3-3 family of proteins. 14-3-3 proteins are abundant scaffolding or chaperone proteins that bind to phosphoserine or phosphothreonine containing motifs. Cdc25 phosphatases were found to bind 14-3-3 proteins in interphase but not in mitosis (Peng et al., 1997). The 14-3-3 binding to Cdc25C after DNA damage occurs at a conserved phosphoserine binding site, leading to cytoplasmic accumulation of Cdc25C (Graves et al., 2001). In response to ultraviolet (UV) irradiation, the analogous 14-3-3 binding site in Cdc25B is phosphorylated by p38 mitogen-activated protein kinase (MAPK), initiating a UV-induced G2-phase delay (Bulavin et al., 2001).

The reported spatial distribution of Cdc25B is consistent with a role in activating cyclin B1-CDK1 in the cytoplasm. Gabrielli and co-workers found that endogenous Cdc25B was nuclear in early G2 phase but exclusively in the cytoplasm just before centrosomal nucleation in prophase (Gabrielli et al., 1996; Gabrielli et al., 1997b). Multiple factors seem to be involved in regulating Cdc25B localisation. We previously found that Cdc25B shuttles between the nucleus and the cytoplasm in an exportin-1 dependent manner (Karlsson et al., 1999). A nuclear localisation signal (NLS) is present between amino acid residues 335 and 353 and one nuclear export sequence (NES) has been characterised in the N-terminus (residues 28-40) (Davezac et al., 2000). Cyclin B1 might influence the localisation of Cdc25B because microinjection of cyclin B1-CDK1 protein into the nucleus results in immediate export of Cdc25B (Karlsson et al., 1999). Furthermore, the 14-3-3 family of proteins are important regulators of Cdc25B localisation. A conserved 14-3-3 binding site in *Xenopus* Cdc25 is located close to the NLS, and 14-3-3 binding was found to interfere with nuclear import (Kumagai et al., 1998; Yang et al., 1999). In analogy with this finding, mutation of the high affinity 14-3-3 binding site in Cdc25B (Mils et al., 2000), leads to nuclear localisation of the phosphatase (Davezac et al., 2000).

Despite the importance of Cdc25 phosphatases in the cell cycle, a complete understanding of their localisation and spatio-temporal regulation is missing. Here, we present the first study that establishes the localisation of the Cdc25B cell cycle phosphatase by controlling antibody specificity using RNA interference. We observed that Cdc25B is mainly nuclear but is also present in the cytoplasm in G2 phase. The cytoplasmic localisation of Cdc25B depends on a previously uncharacterised NES, whose mutation causes Cdc25B to

partially lose its activity as a mitotic inducer. Our results imply that Cdc25B needs to be exported to exert its normal function in mitosis. In addition, we have observed a stress-induced cytoplasmic translocation of Cdc25B which depends on a functional NES, an intact 14-3-3 binding site and p38 MAPK activity. These findings suggest that a change in Cdc25B localisation is part of a G2 checkpoint response.

Materials and Methods

Antibodies and RNA interference

Two guinea pigs were immunised with six peptides corresponding to different Cdc25B epitopes coupled to ovalbumin by Peptide Specialty Laboratories (Heidelberg, Germany). The sera of both animals were incubated with the peptides coupled to sulpho-link coupling gel. The gel was washed with PBS and 10 mM sodium phosphate pH 6.8 and the antibodies were eluted with 1 M glycine pH 2.85. The B1:5 antibody was purified with the peptide LVKTLEK-EEKDLVMYSKC corresponding to amino acids 297-315 in Cdc25B3. Mouse anti-cyclin B1 (GNS1) and rabbit anti-GST (sc-459) antibodies were from Santa Cruz Biotechnology. Mouse anti-GFP (8362-1) was from Clontech and rabbit anti-GFP (210-199-R100) was from Alexis Biochemicals. The specificity of the anti-Cdc25B antibodies was assessed by RNA interference, with small interfering RNAs (siRNAs) designed as described by Elbashir and co-workers (Elbashir et al., 2001), directed against AAUCCUCCC-UGUCGUCUGAAU corresponding to bases 361-381 in Cdc25B mRNA and AACCGACCGUCACUAUGGAC corresponding to bases 127-347 in Cdc25A mRNA (Dharmacon research). As a control, siRNA directed against lamin A/C was used (Elbashir et al., 2001).

Cell culture and microinjection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax, and human telomerase immortalised fibroblast (hTERT-BJ1) cells (Clontech) were cultured in minimal essential medium α (MEM α ; with ribonucleosides and deoxyribonucleosides). During microinjections, cells were cultivated on the microscope stage in HEPES-buffered DMEM on a heated dish (Bioprotech). All media were from Gibco and, except for serum starvation, supplemented with 10% foetal calf serum and antibiotics. Non-cycling (G0) hTERT-BJ1 cells were generated by serum-starving them in MEM α complemented with 0.2% foetal calf serum for four days. Microinjection was performed using an Eppendorf Femtojet in combination with an Eppendorf Injectman (Eppendorf). Plasmids were injected at the concentration of 0.1 μ g/ μ l, siRNA at 10 μ M, Texas Red-coupled dextran at 1 μ g/ μ l and protein at 2 μ g/ μ l. Transfection of plasmids and siRNA was performed using Lipofectamine 2000 or Oligofectamine (both from Invitrogen), respectively, according to the manufacturer's recommendations. Leptomycin B was used at 20 nM. Cycloheximide (Labora) was used at 100 μ g/ml. MG132 (proteasome inhibitor) and SB202190 (specific inhibitor of p38 isoforms α and β) were obtained from Calbiochem, and used at 2 μ g/ml and 10 μ M, respectively. LY294002 (specific inhibitor of PI3-kinase) and PD98059 (specific inhibitor of MEK1) were from Cell Signalling and Calbiochem, respectively and used at 50 μ M. When reagents were supplied in DMSO, an equal amount of DMSO was added to control cells. Cells without medium were exposed to UV irradiation of 50 J/m² using a Hoefer UV-C 500 crosslinker (Pharmacia); the medium was added again after exposure.

Plasmids

The pECFP (enhanced cyan fluorescent protein)-Golgi and pEYFP (enhanced yellow fluorescent protein)-C1 plasmids were purchased from Clontech. GST-Cdc25B(1-67) was constructed by amplifying

the 201 first base pairs of Cdc25B3 with PCR followed by cloning into the pGEX-1 λ T vector (Pharmacia) using *Bam*HI and *Eco*RI. EYFP-Cdc25B3 N-terminal constructs were made by PCR amplification of the first 81, 138, 162, 231 and 273 base pairs of Cdc25B3, followed by cloning into the pEYFP-C1 vector using *Bam*HI and *Eco*RI. By cloning the N-terminal part of Cdc25B3 into pEYFP-C1/EYFP constructs with two YFP molecules were made. The vector pEYFP-C1/EYFP was made by amplifying EYFP by PCR using primers 5'-CGCAGATCTATGGTGAGCAAGGG-3' and 5'-GCAAGCTTTCTTGTACAGCTCGTC-3' and then cloning EYFP into pEYFP-C1 using *Bgl*III and *Hind*III. The L62A and NLS(KRR/AGA)-mutants were constructed using the GeneEditorTM in vitro site-directed mutagenesis system (Promega) on pCMX/GFP-Cdc25B3 (Karlsson et al., 1999) as a template. The primers used were 5'-ACCATGCACGACGCCGCCGGGGCCGGCAGCGAG-3' (L62A) and 5'-ACGCCCCGTGCAGAAATGCCGGGGCGCGGAGC-GTGACCCCTC-3' (KRR/AGA). The part of Cdc25B3 containing the mutations was cloned into pEYFP-C1/Cdc25B3 by digestion with *Bst*XI and *Eco*RI.

Protein purification

GST-Cdc25B(1-67) was transformed into *Escherichia coli* BL21 cells. The cells were grown at 30°C and expression was induced at OD₆₀₀ 0.5 using 1 mM Isopropyl-beta-D-thiogalactopyranoside (IPTG). After 2 hours, cells were sonicated and recombinant protein was purified on GSTrap columns (Pharmacia) according to the manufacturer's recommendations.

Immunofluorescence and fluorescence imaging

Cells were grown on hexametaphosphate-metasilicate coated coverslips and fixed 2-3 hours after microinjection with PBS containing 3% paraformaldehyde and 2% sucrose for 5 minutes, followed by permeabilisation for 5 minutes in 0.5% Triton X100. Antibody incubations were performed as described (Karlsson et al., 1999). Images were captured with a Deltavision Spectris system (for deconvolution, see below), with Openlab software using a fluorescence microscope (Leica) connected to a camera (Hamamatsu), or using a Zeiss LSM 510 confocal microscope. Images were processed using ImageJ software (NIH) and exported to Adobe Photoshop and Adobe Illustrator for printing.

Deconvolution and quantification of fluorescence

For quantification of fluorescence, six fields with cyclin B1-expressing cells were chosen and a z-stack of 42 images with 200 nm spacing was acquired for each field using a Deltavision Spectris system with a 60 \times objective (Applied Precision). After deconvolution, using Softworx software (Applied Precision), a 2D-projection was made from the z-stack. The cytoplasm or nucleus of each cell was marked and the average signal calculated using ImageJ. The background was calculated for each experiment as the mean signal of cells not expressing Cdc25B.

Fluorescence loss in photobleaching (FLIP)

HeLa cells were arrested in 300 ng/ml nocodazole for 15 hours. Rounded cells were then microinjected with expression vectors and subsequently released from the nocodazole block by washing them three times with DMEM. Six hours later, cells were moved to a Zeiss LSM 510 microscope. An image was acquired, followed by a 45-seconds bleach (100% laser power, 514 nm) in the cytoplasm of one of the separated daughter cells. This was repeated 15 times. Measurement of the average intensity of the YFP signal in the nucleus and background subtraction was performed using ImageJ. The initial fluorescence for each cell was set to 1, and the data for the

fluorescence loss in photobleaching (FLIP) were displayed as the ratio between the bleached cell and the control cell.

Premature chromosome condensation assay

HeLa cells were synchronised by a double thymidine-block (Rao and Johnson, 1970) and transfected with indicated constructs immediately after release from the second block. At the indicated time points, cells were fixed with 3.7% formaldehyde and the DNA was stained with Hoechst 33342. Cells expressing YFP-tagged constructs were counted and scored for chromatin condensation.

Results

Cdc25B immunofluorescence

We sought to investigate the subcellular localisation of Cdc25B and therefore produced peptide antibodies against different Cdc25B epitopes in guinea pig. After affinity purification against the peptides, one of the antibodies (named B1:5) strongly recognised overexpressed Cdc25B, but not Cdc25A, on a western blot (Fig. 1A). In whole cell lysates of untransfected cells, the B1:5 antibody recognised two weak bands, one of which was significantly reduced by siRNA-mediated targeting of Cdc25B but not Cdc25A (Fig. 1B). This band is approximately 70 kDa, the size equivalent of in vitro translated Cdc25B (data not shown). To assess the specificity in immunofluorescence of the B1:5 antibody, we either transfected or microinjected siRNA against Cdc25B into HeLa cells and then stained the cells with the B1:5 antibody. Microinjected cells were identified by co-injecting a plasmid encoding a CFP-tagged Golgi marker. To obtain a correlation with cell cycle phase we co-stained cells with cyclin B1, which starts to accumulate in late S phase (Pines and Hunter, 1989). We found that all cells expressing cyclin B1 were stained by the B1:5 antibody and that this staining was significantly reduced in cells treated with Cdc25B siRNA (Fig. 1C, upper panel). In cells microinjected with a control siRNA targeting lamin A/C no reduction of B1:5 staining was observed, which shows that the disappearance of Cdc25B staining was specific for cells treated with siRNA towards Cdc25B (Fig. 1C, lower panel). We also correlated Cdc25B immunofluorescence with the expression of cyclin A, which appears at the G1-S transition (Erlandsson et al., 2000), and found that Cdc25B appeared weakly in some cells that were cyclin-A-positive but cyclin-B1-negative (data not shown). Specific Cdc25B staining was easily distinguished from weak, non-specific cytoplasmic and nuclear staining observed in all cells, because the RNA interference (RNAi)-sensitive staining was observed only in cells expressing cyclins A and B (Fig. 1C, Fig. 2A and data not shown).

Using the B1:5 antibody in combination with Cdc25B siRNA we determined that Cdc25B is expressed mainly in the nucleus but that in G2 cells it is also present in the cytoplasm. The highest Cdc25B levels were found in cells where cyclin B1 had just started to move to the nucleus (late prophase). This localisation pattern was observed in HeLa cells (Fig. 2A) and also in untransformed fibroblasts (not shown). However, we never observed Cdc25B exclusively in the cytoplasm which is in contrast to a previous report that describes Cdc25B as being cytoplasmic in G2 (Gabielli et al., 1996). To confirm our observed Cdc25B localisation we also quantified the Cdc25B distribution in HeLa cells. Nuclear and cytoplasmic Cdc25B

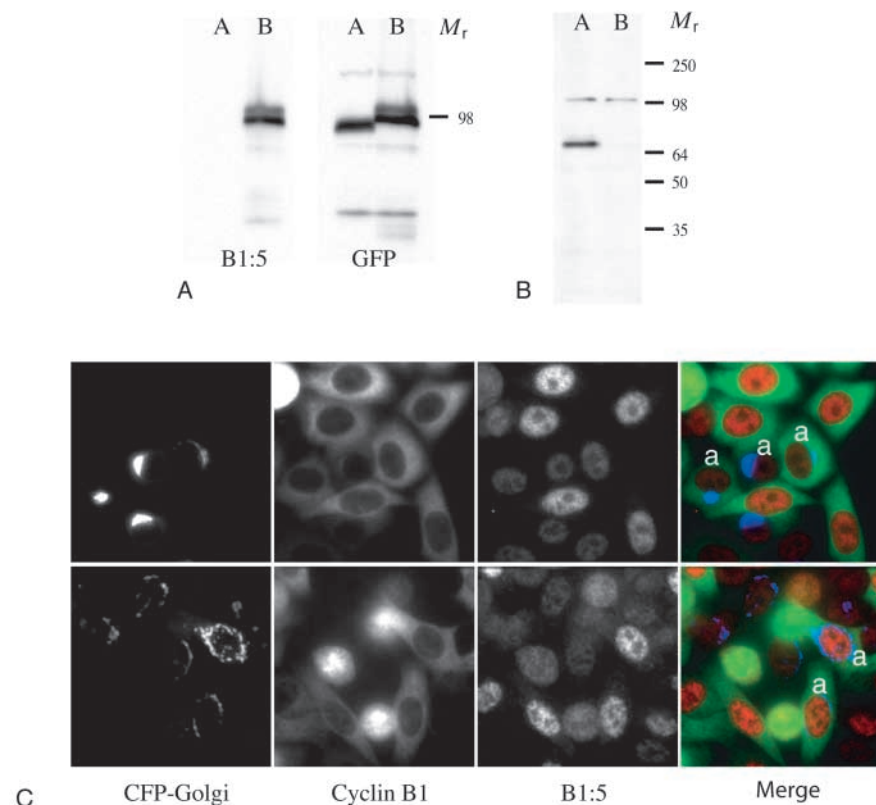


Fig. 1. Characterisation of a Cdc25B peptide antibody. (A) The guinea pig anti-Cdc25B antibody B1:5 recognises YFP-Cdc25B, but not YFP-Cdc25A. Cell lysates from HeLa cells transfected with YFP-Cdc25A (lane A) and YFP-Cdc25B (lane B) were subjected to immunoblotting using B1:5 (anti-Cdc25B) and anti-GFP antibodies as indicated in the figure. (B) The B1:5 antibody recognises Cdc25B in whole cell lysates. HeLa cells were transfected with siRNA against Cdc25A (lane A) or Cdc25B (lane B). Cell lysates were harvested 48 hours after transfection and analysed by immunoblotting using the B1:5 antibody. (C) Assessment of antibody specificity by RNA interference. HeLa cells were microinjected with siRNA against Cdc25B (upper panel) or lamin A/C (lower panel) together with a pCFP-Golgi plasmid to mark injected cells. Thirty-five hours later, cells were fixed and stained with B1:5 and cyclin B1 antibodies. The B1:5 antibody stained all cells expressing cyclin B1. In cells microinjected with Cdc25B siRNA, but not with lamin A/C siRNA, this staining is diminished. Cells expressing cyclin B1 that also are microinjected with siRNA are indicated by the letter a.

staining in deconvolved images was quantified and the average non-specific background was then subtracted (for details, see Materials and Methods). As shown in Fig. 2B, the levels of both nuclear and cytoplasmic Cdc25B increased with increasing cyclin B1 levels but the ratio between cytoplasmic and nuclear staining did not change dramatically. Thus, Cdc25B does not change its localisation from nuclear in S phase to cytoplasmic in G2 phase. When observing cells in different stages of mitosis, we found that Cdc25B levels started to decrease in cells with visibly condensed chromatin and Cdc25B was completely absent at the time of metaphase (Fig. 2C). This agrees with earlier observations that Cdc25B is degraded in mitosis (Baldin et al., 1997a) and specifies the stage where Cdc25B proteolysis is initiated to be mitotic prophase.

In summary, our results show that Cdc25B starts to accumulate in the nucleus in mid S phase, just before cyclin B1 appears. Cdc25B protein levels increase during S and G2 and are visible also in the cytoplasm in late G2 phase. After a peak in prophase, Cdc25B levels decrease and become undetectable in metaphase.

The N-terminus of Cdc25B mediates nuclear export

We have previously demonstrated that Cdc25B is a shuttling protein, and that its cytoplasmic localisation is mediated by nuclear export because the export-inhibitor leptomycin B caused nuclear accumulation of Cdc25B (Karlsson et al., 1999). To further analyse the mechanism behind the nuclear export of Cdc25B we made expression constructs of N-terminal deletions of Cdc25B coupled to EYFP. Of the three

splice forms of Cdc25B we chose to study Cdc25B3 because it is the full-length isoform, is more abundantly expressed and is the only isoform that is present in all human cell lines tested (Baldin et al., 1997b; Forrest et al., 1999). In agreement with a previous study (Davezac et al., 2000), a YFP-Cdc25B3 construct lacking the first 91 amino acids localised to the nucleus only, when expressed in HeLa cells (data not shown). Reasoning that this deleted region contains sequence(s) important for regulating the cytoplasmic localisation of Cdc25B, we expressed smaller parts of this region of Cdc25B3 coupled to YFP in HeLa cells or hTERT immortalised fibroblasts. YFP fusions of the first 27, 46 or 54 amino acids of Cdc25B3 were distributed both in the nucleus and the cytoplasm. By contrast, YFP fusions of the first 77 or 91 amino acids of Cdc25B3 were exclusively cytoplasmic (Fig. 3B). All localisation patterns were independent of cell cycle phase (data not shown). To demonstrate that the localisation of our smaller constructs was not because of passive diffusion, we also tagged all constructs with an additional 27 kDa YFP molecule. These fusion proteins showed the same localisation pattern as the single YFP-fusions (data not shown).

To investigate whether the cytoplasmic localisation of YFP-Cdc25B3(1-77) and YFP-Cdc25B3(1-91) was owing to nuclear export, leptomycin B was added to cells expressing these constructs. As shown in Fig. 3B, leptomycin B treatment caused nuclear accumulation of YFP-Cdc25B3(1-77) and YFP-Cdc25B3(1-91), whereas shorter constructs or YFP alone were unaffected. The nuclear accumulation of the longer constructs after leptomycin B treatment was not an effect of new transcription because it occurred in the presence of the transcription inhibitor α -amanitin (data not shown). Thus, a

region between amino acids 54 and 77 mediates nuclear export.

Two leucine-rich sequences that could act as putative nuclear export sequences occur in the N-terminus of Cdc25B (Davezac et al., 2000). These sequences are indicated as NES1 (residues

29-40) and NES2 (residues 55-67) in Fig. 3A. We wanted to assess the ability of a region containing these sequences to export a protein from the nucleus of a cell. To this end we expressed the first 67 amino acids of Cdc25B3 fused to GST in bacteria and purified the protein. The GST-Cdc25B(1-67)

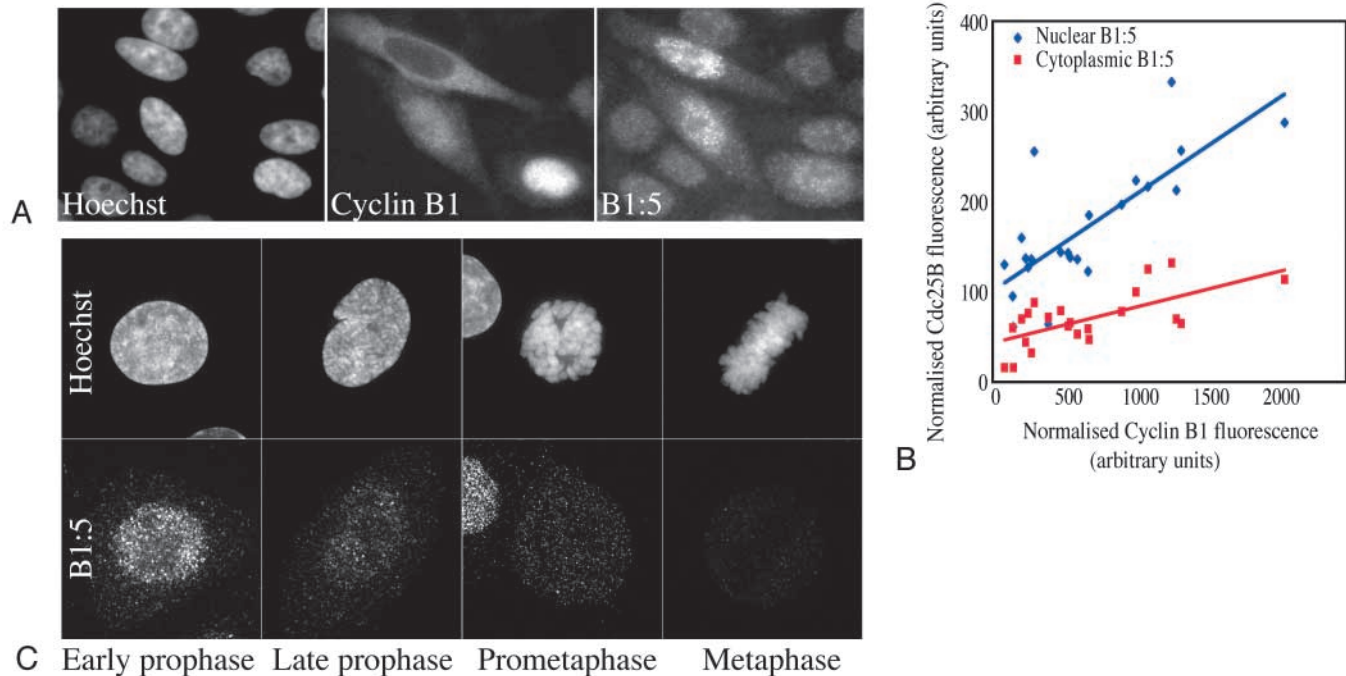


Fig. 2. Subcellular localisation of Cdc25B. (A) Representative images of Cdc25B-staining using the B1:5 antibody. Cells were co-stained with cyclin B1 antibodies and Hoechst 33342 was used to stain nuclei. (B) Nuclear and cytoplasmic Cdc25B levels increase as cyclin B1 accumulates. The fluorescence signal from a deconvoluted image was quantified, the average background from cells not expressing Cdc25B was subtracted and the quantified B1:5 signal (y-axis) was plotted against the quantified cyclin B1 signal (x-axis). Each point in the graph corresponds to one cell. (C) Cdc25B immunofluorescence of cells at different stages of mitosis.

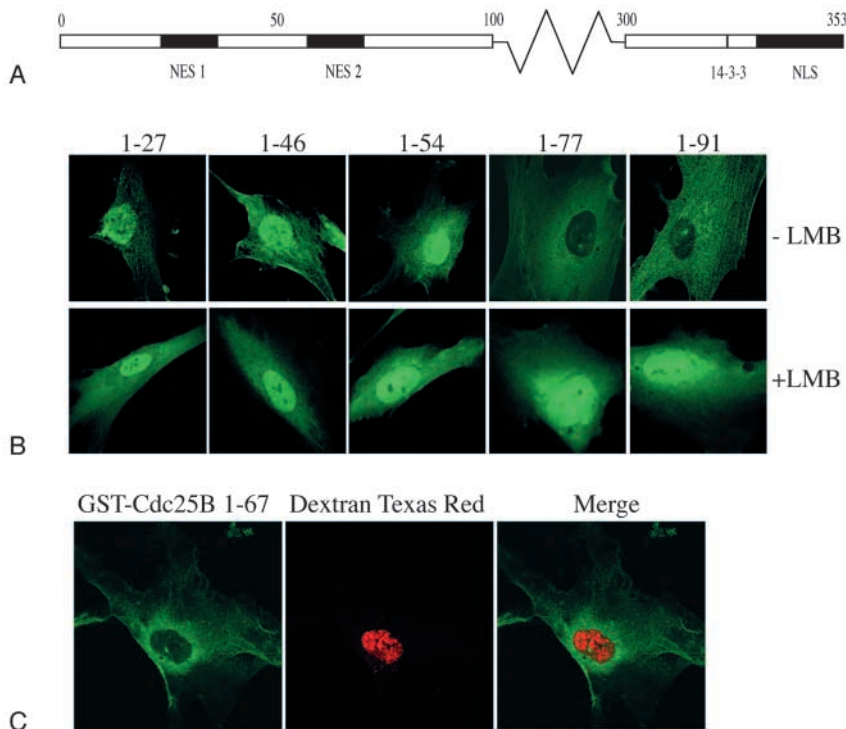


Fig. 3. Cdc25B nuclear export is mediated by a region between amino acids 54 and 67. (A) Schematic presentation of the N-terminus of Cdc25B. (B) Localisation patterns of EYFP fusion proteins containing parts of the Cdc25B N-terminus as indicated, in the presence or absence of leptomycin B (LMB). hTERT immortalized human fibroblasts were microinjected with constructs expressing parts of the Cdc25B N-terminus fused to YFP. During the last 4 hours before fixation half of the cells were incubated with leptomycin B. (C) Nuclear export of GST Cdc25B 1-67. Purified GST-Cdc25B was microinjected together with Texas Red-conjugated dextran into the nucleus of hTERT immortalized human fibroblasts. One hour later, cells were fixed and stained with anti-GST antibodies (green).

fusion protein was then microinjected together with Texas Red-coupled dextran into fibroblast nuclei. After 1 hour, GST-Cdc25B(1-67) had migrated to the cytoplasm as shown in Fig. 3C. The dextran remained in the nucleus, showing that the nuclear membrane remained intact. This demonstrates that residues 1-67 of Cdc25B3 can export GST from the nucleus.

Characterisation of NES2 in the N-terminus of Cdc25B

As described above we found that constructs containing NES1 tagged to one or two YFP molecules were distributed evenly in the nucleus and cytoplasm. By contrast, constructs containing NES1 and NES2 were cytoplasmic and accumulated in the nucleus with leptomycin B. This suggested that NES2 was the primary determinant of Cdc25B export and that NES1 may constitute a weaker nuclear export sequence. Therefore, the NES2 region was further characterised. We changed the leucine at position 62 to alanine in the full length YFP-Cdc25B3 (Fig. 4A) and expressed the mutant protein.

The localisation of the putative Cdc25B3 NES-mutant was analysed in HeLa cells, synchronised in G2 because that is the phase during which endogenous Cdc25B is expressed. We previously found that cyclin B1 can mediate export of Cdc25B to the cytoplasm (Karlsson et al., 1999). Therefore, to avoid the influence of cyclins, we also introduced the construct into G0 fibroblasts (which do not express cyclins). We found that in both cell types, the YFP-tagged wild-type Cdc25B3 localised in a variable manner, from mostly in the cytoplasm to mostly in the nucleus (Fig. 4B and C). By contrast, YFP-Cdc25B3(L62A) localised mostly to the nucleus in both cell types. Thus, the mutation of a single leucine in NES2 makes Cdc25B significantly more nuclear in fibroblasts and HeLa cells.

For comparison, we also analysed the localisation of the mutants YFP-Cdc25B3(S323G) and YFP-Cdc25B3(NLS-) (see below). The change of serine 323 to glycine prevents phosphorylation of serine 323 and disrupts binding of 14-3-3 proteins to this site (Mils et al., 2000). This mutation was

previously described as rendering the protein more nuclear (Davezac et al., 2000) which is also seen in Fig. 4C. An NLS was previously identified in Cdc25B (Davezac et al., 2000) and in accordance with that study we find that when mutating KRR to AGA in the NLS, the protein becomes predominantly cytoplasmic. However, 30% of the cells also express YFP-Cdc25B3(NLS-) in the nucleus which suggests that factors other than the NLS can cause Cdc25B to enter the nucleus.

To obtain a relative measurement of the export rate of the L62A mutant compared to wild-type Cdc25B, we performed FLIP (fluorescence loss in photobleaching) assays (reviewed in Lippincott-Schwartz et al., 2001) in living cells. We microinjected mitotic HeLa cells with plasmids encoding YFP-Cdc25B3 or YFP-Cdc25B3(L62A). Six hours later, the cells had gone through mitosis, resulting in two G1-daughter cells with similar amounts of nuclear fluorescence. At that point, we determined the ability of the fusion proteins to be exported by repeatedly bleaching one daughter cell in the cytoplasm with a 514 nm argon laser line, and simultaneously monitoring the nuclear fluorescence of both cells. Because the other daughter cell contained the same initial amount of fluorescence, this cell provided a control for potential new protein synthesis and degradation. If the protein was continuously being exported, nuclear fluorescence should be depleted in the bleached cell. As shown in Fig. 5, the nuclear fluorescence of YFP-Cdc25B3 decreased dramatically after bleaching in the cytoplasm. Approximately half of the initial nuclear fluorescence remained after 5 minutes, and after 11 minutes of bleaching only 30% of initial fluorescence remained. To make sure that the loss of nuclear fluorescence was owing to shuttling and not to degradation of Cdc25B, cells were stained with GFP antibodies after the FLIP assay. Immunofluorescence staining showed that the nucleus of the bleached cell and the adjacent control cell contained a similar amount of YFP-tagged protein, suggesting that no significant YFP-Cdc25B3 proteolysis took place during the experiment (Fig. 5). The YFP-Cdc25B3(L62A) mutant was exported much more slowly than

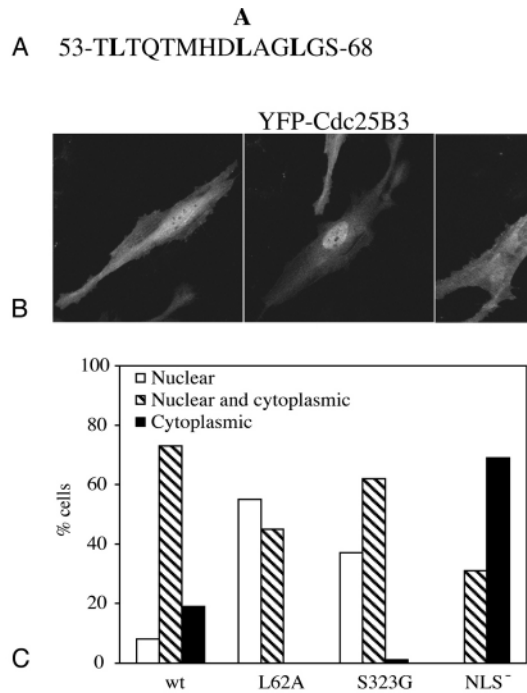
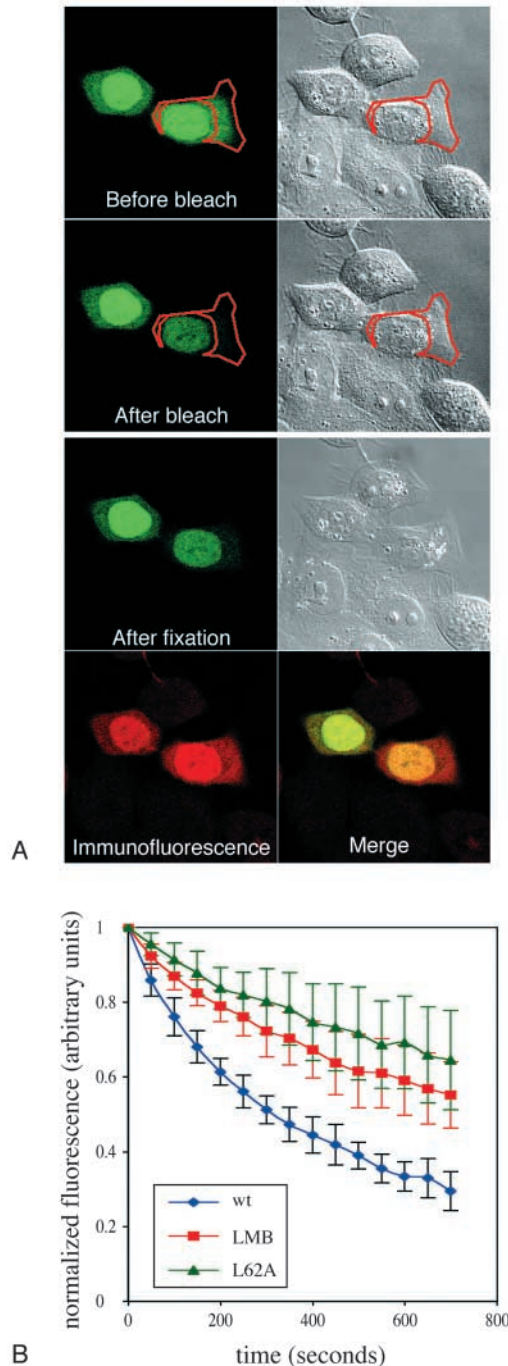


Fig. 4. Mutation of leucine 62 to alanine impairs nuclear export of Cdc25B. (A) The amino acids 54 to 67 of Cdc25B, which constitutes NES2. The three leucines are indicated in bold and the residue subjected to site-directed mutagenesis is indicated above. (B) Localisation of YFP-Cdc25B3 or YFP-Cdc25B3(L62A) 30 hours after transfection in G0 hTERT immortalised human fibroblasts. (C) Localisation patterns of YFP-Cdc25B3, YFP-Cdc25B3 L62A, YFP-Cdc25B3 S323G and YFP-Cdc25B3 NLS- in G2 HeLa cells. Cells were synchronised by a double thymidine-block, transfected immediately after release and fixed after 8 hours. Cells with interphase nuclei were scored for nuclear, both nuclear and cytoplasmic, or cytoplasmic YFP fluorescence.



wild-type YFP-Cdc25B3. At the end of the 11-minute assay 65% of initial nuclear fluorescence was still present. Thus, mutating the NES2 sequence significantly reduces the export rate of Cdc25B in G1. In FLIP assays performed in the G2 phase of the cell cycle, the YFP-Cdc25B(L62A) mutant was also exported more slowly than the wild type, although the export of both proteins was faster than in G1 (see Fig. S1 in supplementary material). A FLIP assay was also carried out in the presence of leptomycin B, which reduced the speed of Cdc25B3 nuclear export, again confirming that Cdc25B export is exportin-1 dependent. The reduction in nuclear export rate seen in the presence of leptomycin B was similar to that seen with the Cdc25B NES mutant (Fig. 5).

Fig. 5. Mutation of NES2 reduces the export rate of Cdc25B3. Fluorescence loss in photobleaching (FLIP) of YFP-Cdc25B3, YFP-Cdc25B3(L62A), and YFP-Cdc25B3 together with leptomycin B (LMB) in G1 HeLa cells. Mitotic nocodazole-arrested HeLa cells were microinjected with expression vectors and released from nocodazole block. Six hours later, differential interference contrast (DIC) and YFP fluorescence images were acquired and cells were bleached in the cytoplasm for 45 seconds. The imaging and bleaching cycle was repeated 15 times. (A) Example of a FLIP experiment with YFP-Cdc25B3. Top four panels: Image before and after bleaching the right of the two daughter cells from the microinjected mitotic cell. The bleach area is circled in red. Bottom four panels: The same cells as above, after fixation and immunofluorescence with anti-GFP antibodies (red). (B) Quantification of FLIP. The nuclear fluorescence of the two daughter cells was quantified. After background subtraction, the value of nuclear fluorescence at the start of the experiment was normalised to 1. Measurements of nuclear fluorescence of the bleached cell relative to the unbleached cell are plotted in the graph. Each curve in the graph shows the average of six cells from three independent experiments (bars indicate standard deviation).

The localisation of Cdc25B affects its ability to induce mitosis

Cdc25B can induce premature mitosis when overexpressed in S- or G2-phase cells (Baldin et al., 2002; Gabrielli et al., 1996; Karlsson et al., 1999). To investigate the importance of nuclear export and import of Cdc25B3 for its ability to induce mitosis, we transfected S-phase HeLa cells with the localisation mutants: HeLa cells were synchronised by a double thymidine-block, transfected with different YFP-Cdc25B3 constructs immediately after release from the second block, and fixed 8 hours after transfection. The 8-hour time point was chosen because almost no untransfected cells reach mitosis by that time. The cells were scored for premature chromosome condensation (PCC) by Hoechst 33342 staining. As shown in Fig. 6, wild-type Cdc25B and the NLS mutant were equally competent in pushing cells into mitosis (35% PCC), whereas the NES mutant Cdc25B3(L62A) showed a significant decrease in its ability to induce PCC, with only 21% of the cells having entered premature mitosis at 8 hours. The highest frequency of PCC – 55% – was observed with the Cdc25B3(S323G) mutant, which indicates that 14-3-3 binding keeps Cdc25B3 inactive, as previously observed (Forrest and Gabrielli, 2001). In cells expressing YFP alone, only 5% entered mitosis.

These experiments demonstrate that Cdc25B3 export is needed for efficient induction of mitosis in HeLa cells. This in turn supports the model that Cdc25B initiates the activation of the cyclin B1-CDK1 complex in the cytoplasm.

Stress induces cytoplasmic translocation of Cdc25B

In the FLIP assays described above, we initially tried to use cycloheximide to shut down protein synthesis to avoid the influence of newly synthesised YFP fusion proteins. However, to our surprise, when we added cycloheximide at 100 µg/ml to cells expressing YFP-Cdc25B3, we noticed that the fusion protein rapidly translocated to the cytoplasm (Fig. 7A). The translocation was independent of cell cycle phase (data not shown) and started approximately 20 minutes after the addition of cycloheximide. Because cycloheximide is found to activate

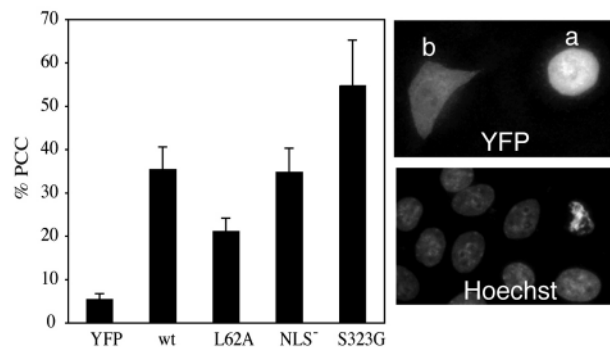


Fig. 6. Induction of premature mitosis by overexpressing Cdc25B mutants. Cells were synchronised by a double thymidine-block, transfected with YFP-Cdc25B3, YFP-Cdc25B3(L62A), YFP-Cdc25B3(S323G), YFP-Cdc25B3(NLS-) or YFP alone immediately after release and fixed after 8 hours. The different constructs were expressed at similar levels as determined by the intensity of YFP fluorescence. Cells expressing the constructs were counted and scored for premature chromatin condensation (% PCC) as judged by Hoechst 33342 staining. Data show the mean of three independent experiments. Images on the right show examples of a typical cell displaying prematurely condensed chromatin (a) and an interphase cell (b).

the p38 MAPK stress signalling pathway (Barros et al., 1997; Itani et al., 2003; Zinck et al., 1995) we decided to investigate whether the Cdc25B translocation occurred in cells treated with another p38-inducing agent, UV irradiation. As shown in Fig. 7B, exogenously expressed Cdc25B translocated to the cytoplasm after exposure of cells to UV-C at 50 J/m². Leptomycin B inhibited the cytoplasmic translocation, and YFP-Cdc25B3(L62A) did not translocate after UV treatment indicating that nuclear export mediated by NES2 was required for this event. YFP-Cdc25B3(S323G) also stayed in the nucleus after UV-C treatment, suggesting that phosphorylation of serine 323 and/or 14-3-3 binding is needed for UV-mediated cytoplasmic translocation of Cdc25B. To confirm that the p38 MAPK pathway was involved in this event, we treated cells with the specific p38 inhibitor SB202190. We found that the UV-mediated cytoplasmic translocation was inhibited (Fig. 7C and D), whereas normal Cdc25B localisation was not altered (data not shown). By contrast, treating cells with inhibitors of MEK1 (Fig. 7C) and PI3-kinase (data not shown) did not influence the localisation of Cdc25B after UV. This demonstrates that p38 kinase is involved in regulating the observed change in Cdc25B localisation. All the above experiments were also performed with cycloheximide, yielding very similar results (data not shown).

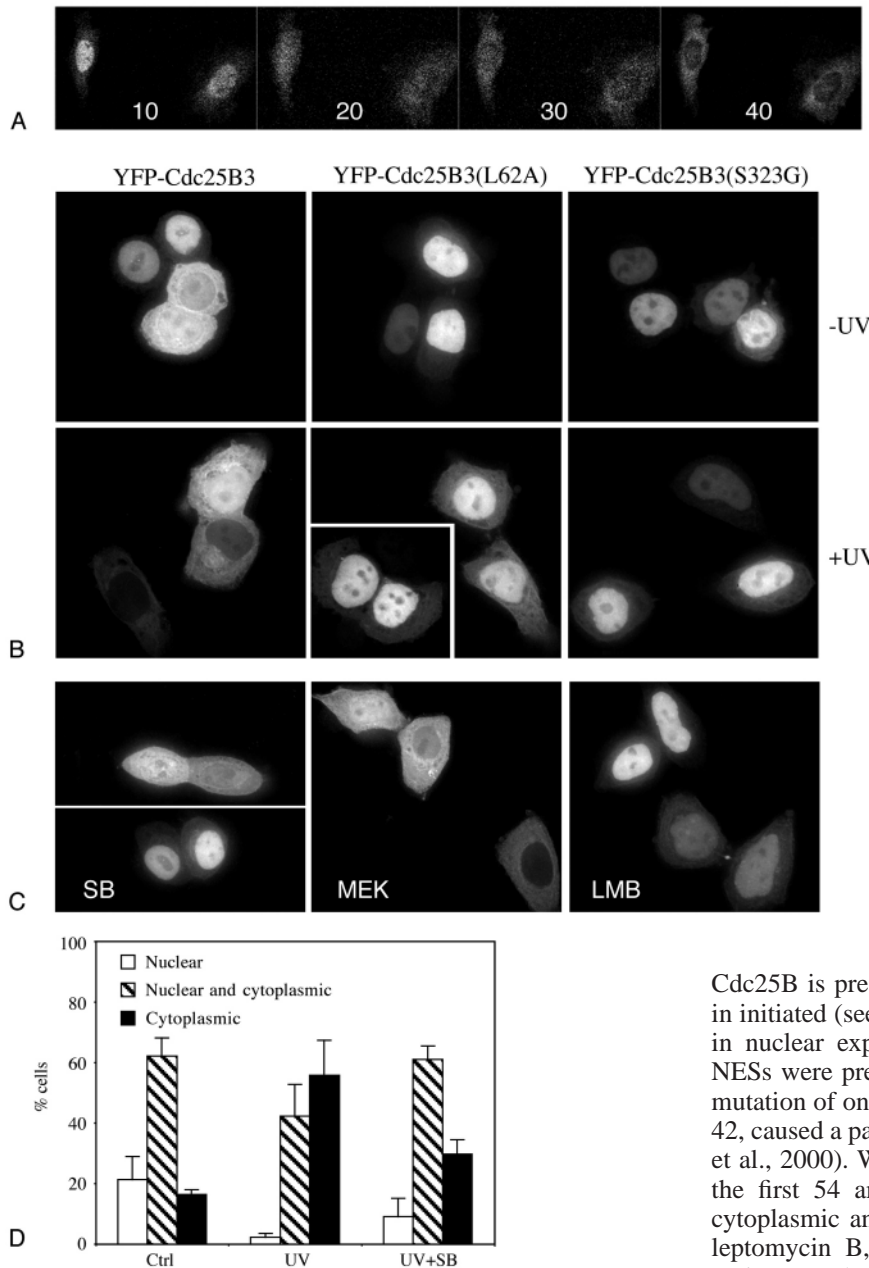
We next demonstrated that the UV- and cycloheximide-mediated export of Cdc25B was seen not only in cells overexpressing Cdc25B, but also for the endogenous protein. Cells were treated with UV-C, leptomycin B and kinase inhibitors as above, fixed 1 hour later and stained with Cdc25B and cyclin B1 antibodies. As shown in Fig. 8, we found that UV-C induced a partial translocation of the endogenous protein to the cytoplasm, although to a lesser extent than observed for the exogenously expressed protein. The change in Cdc25B localisation was most readily observed in cells expressing high levels of cyclin B1 (G2-phase cells). The cytoplasmic

translocation was inhibited by leptomycin B and the p38 inhibitor but not by the MEK1 and PI3-kinase inhibitors (Fig. 8 and data not shown). Fig. S2 shows a quantification of cytoplasmic versus nuclear fluorescence in cells treated with cycloheximide, demonstrating a partial translocation of Cdc25B that is reversed by a p38 inhibitor (Fig. S2 in supplementary material). Thus, UV-C irradiation and cycloheximide induce p38-dependent cytoplasmic translocation of Cdc25B.

Discussion

A general problem in immunofluorescence is how to distinguish the specific binding of antibody to antigen from cross-interactions with other proteins. This problem is particularly obvious when studying low-abundance proteins. RNAi provides a tool to solve this problem. Before initiating this study, we analysed a number of commercially available antibodies to Cdc25B, which gave rise to various localisation patterns, none of which were affected by Cdc25B-siRNA treatment. Here, we make use of RNAi to evaluate a new peptide antibody, B1:5, directed to residues 297-315 in the N-terminus of Cdc25B3. In immunofluorescence, the B1:5 antibody gave rise to a staining that was diminished in cells treated with Cdc25B siRNA. Another peptide antibody, B1:4, directed to residues 201-217 gave an identical, albeit weaker staining (data not shown). Thus, we had the means to analyse the distribution of Cdc25B during the cell cycle. By using antibody staining in combination with RNAi and fluorescence-microscopy, we find that, in HeLa cells, Cdc25B appears just before cyclin B1 in S-phase and the amount of Cdc25B increases until late prophase. From that point, Cdc25B gradually disappears and is completely absent from metaphase cells. The staining is predominantly nuclear, but a fraction of Cdc25B resides in the cytoplasm. This is in contrast to reports (Gabrielli et al., 1997b; Gabrielli et al., 1996) that describe the occurrence of cells with solely cytoplasmic Cdc25B. In previous studies, cross-reactivity with other proteins could not be excluded, whereas here, we have determined the specificity of our antibody using RNAi. The localisation pattern of the endogenous protein described here also differs from the distribution of the overexpressed protein, which is more cytoplasmic (Davezac et al., 2000; Gabrielli et al., 1996; Karlsson et al., 1999) (and this study). Such a discrepancy between endogenous and overexpressed Cdc25B was recently noticed in etoposide-treated cells (Giles et al., 2003). The difference in behaviour between endogenous and exogenous Cdc25B could result from overexpressed Cdc25B titrating a binding partner, or might reflect a stress-response (see below) induced by Cdc25B overexpression.

Cdc25B is expressed from S phase until early mitosis (Gabrielli et al., 1996) (and this study). The temporal expression pattern and the presence of Cdc25B in the nucleus makes the phosphatase a candidate for activating cyclin-A-containing complexes because they, although shuttling, are mainly nuclear (Girard et al., 1991; Jackman et al., 2002). The level of Cdc25B increases during S and G2 and reaches its highest cytoplasmic levels at the time of cyclin B1-CDK1 activation. This observation, together with the finding that a Cdc25B mutant impaired in nuclear export is also less potent in inducing mitosis, suggests that, even though the fraction of cytoplasmic Cdc25B level is low, it is required in the cytoplasm



to induce mitosis. The initial activation of the cyclin B1-CDK1 complex occurs in the cytoplasm in prophase (Jackman et al., 2003) and thus, Cdc25B is a probable candidate for catalysing this activation. One might speculate that activation of cyclin B1-CDK1 occurs when a threshold level of Cdc25B has been reached in the cytoplasm. However, Cdc25B is not probable to sustain the activity of translocated cyclin B1-CDK1 in the nucleus because Cdc25B levels rapidly decrease once cyclin B1 enters the nucleus. In agreement with a dual role for Cdc25B, Cdc25B immunoprecipitated from S-phase cells most efficiently activates cyclin A-CDK2, whereas Cdc25B immunoprecipitated from G2-M cells is more active towards cyclin B1-CDK1 (Lammer et al., 1998).

Given that Cdc25B is shuttling (Davezac et al., 2000; Karlsson et al., 1999), and after establishing that endogenous

Fig. 7. YFP-Cdc25B3 translocates to the cytoplasm after treatment with cycloheximide or exposure to UV-C. (A) Cycloheximide induces cytoplasmic translocation of Cdc25B. HeLa cells were microinjected with YFP-Cdc25B3, treated with 100 µg/ml cycloheximide and followed by time-lapse microscopy. The time after cycloheximide addition is indicated (in minutes).

(B) Representative images of cells expressing YFP-Cdc25B3, YFP-Cdc25B3(L62A) and YFP-Cdc25B3(S323G) with or without UV-C irradiation. The different constructs were transfected into HeLa cells. Five hours after transfection, cells were irradiated with 50 J/m² UV-C and fixed 1 hour later. (C) Representative images of cells expressing YFP-Cdc25B3 after UV-C treatment and addition of kinase inhibitors or leptomycin B. YFP-Cdc25B3 was transfected into HeLa cells. Four hours after transfection, cells were treated with SB202190 (SB, 10 µM), PD98059 (MEK, 50 µM), or leptomycin B (LMB, 20 nM) for 1 hour. At that point, cells were exposed to 50 J/m² UV-C and fixed 1 hour later. (D) The cytoplasmic translocation of YFP-Cdc25B3 after UV-C treatment is abolished by a p38 inhibitor. HeLa cells were transfected with YFP-Cdc25B3 and 3 hours later SB202190 was added. Four hours after transfection cells were exposed to 50 J/m² UV-C and fixed 1 hour later. Cells were scored as in Fig. 4. The graph shows the mean of 200 cells counted in three independent experiments.

Cdc25B is present in the cytoplasm at the time when mitosis is initiated (see above), we analysed the mechanisms involved in nuclear export of the protein. Two putative leucine-rich NESs were previously observed in the Cdc25B sequence and mutation of one of these (NES1), between amino acids 29 and 42, caused a partial nuclear accumulation of Cdc25B (Davezac et al., 2000). We found that a YFP-fusion protein made up of the first 54 amino acids of Cdc25B3 is both nuclear and cytoplasmic and does not become nuclear in the presence of leptomycin B, which argues against a strong NES in this region. We demonstrate that mutating leucine-62 to alanine in the other proposed NES (NES2), which is located between amino acids 54 and 67, leads to nuclear accumulation of a YFP-Cdc25B3 fusion protein. When assessing nuclear export rates by FLIP, we found that the NES2 mutant YFP-Cdc25B3(L62A) was exported much more slowly than wild-type Cdc25B. We also observed that the NES2 mutant was not exported to the cytoplasm after stress-induction (see below), another indication that NES2 is the major export sequence in Cdc25B. We note that Cdc25B retains some ability to traffic between the nucleus and cytoplasm in the presence of leptomycin B in the FLIP assay. Similarly, the Cdc25B-NES2 mutant L62A is exported to a small extent. Therefore we cannot exclude that Cdc25B also can leave the nucleus in an exportin 1-independent manner.

We find that the nuclear fluorescence of wild-type Cdc25B is reduced to half of its initial value after approximately 5 minutes. This occurs on the same time scale as other shuttling

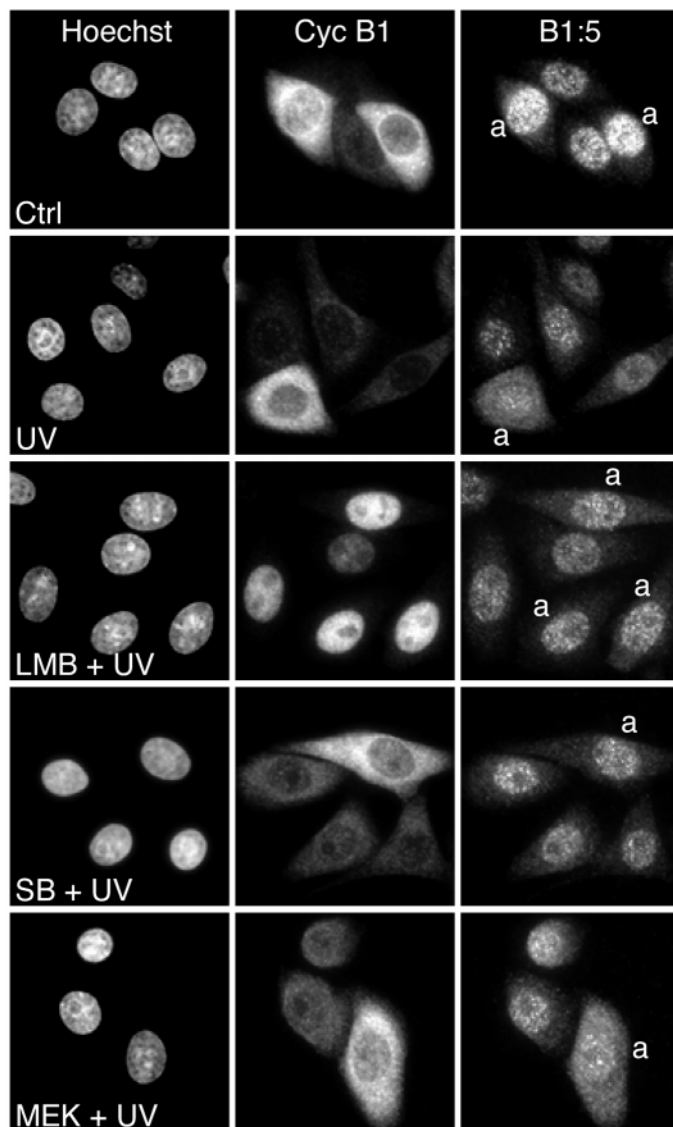


Fig. 8. Endogenous Cdc25B partly translocates to the cytoplasm after exposure to UV-C. One hour before 50 J/m² UV-C treatment, HeLa cells were incubated with the p38 inhibitor SB202190 (SB), leptomycin B (LMB) or the MEK1 inhibitor PD98059 (MEK) as indicated in images on the left. Cells were fixed 1 hour after UV-C exposure. Representative images of the B1:5 and cyclin B1 immunostaining are shown. Cells that express high levels of cyclin B1 (G2 cells) are indicated with the letter a in the images showing Cdc25B staining (B1:5). The cytoplasmic translocation of Cdc25B is observed in cells treated with UV-C and with UV-C in combination with the MEK1 inhibitor but not in untreated cells, or cells treated with UV-C in combination with leptomycin B or the p38 inhibitor.

proteins in the literature (Griffis et al., 2002; Rosin-Arbesfeld et al., 2003). We conclude that the distribution of Cdc25B is potentially very dynamic and sensitive to interactions with binding partners in the nucleus or cytoplasm, which would determine whether the subcellular localisation of the protein is nuclear or cytoplasmic.

We find that interfering with nuclear export reduces the ability of Cdc25B3 protein to induce mitosis, which suggests

that it is needed to activate cyclin B1-CDK1 in the cytoplasm. Interfering with nuclear import, by contrast, does not affect the ability of Cdc25B3 to push cells into mitosis. This result differs from observations reported by Baldin and co-workers, who found that overexpression of an NLS-deficient version of the Cdc25B1 splice form had a reduced ability to induce chromatin condensation (Baldin et al., 2002). This discrepancy might reflect a difference between Cdc25B splice versions because, in contrast to Cdc25B1, we do not find Cdc25B3 to become completely cytoplasmic when mutating the NLS (Fig. 4C). Baldin et al. also found that Cdc25B1 was less potent in inducing mitosis (Baldin et al., 2002), and in contrast to Cdc25B3 (Gabrielli et al., 1996; Karlsson et al., 1999) did not affect mitotic-spindle formation when overexpressed. It is possible that the different splice versions have different functions at the G2-M transition.

In contrast to our expectations, we found that YFP-Cdc25B3 translocates to the cytoplasm when cells expressing the fusion protein are treated with cycloheximide, an inhibitor of protein synthesis that also induces the p38 MAPK signalling pathway. This effect is unique to the Cdc25B isoform, because YFP-fusions of Cdc25A or Cdc25C did not change localisation after similar treatment (data not shown). We found that another p38-inducing agent, UV-C, also caused a cytoplasmic translocation of overexpressed Cdc25B. In the case of both cycloheximide and UV-C, the translocation depended on nuclear export and an intact serine 323 residue (14-3-3 binding site) as well as on p38 activity. Endogenous Cdc25B is also exported from the nucleus when cells are treated with UV or cycloheximide, albeit to a lesser extent. In a recent study, p38 was shown to phosphorylate Cdc25B1 on serine 309 (which is the equivalent of serine 323 in the Cdc25B3 splice version) in response to UV-irradiation (Bulavin et al., 2001). Inactivation of p38 MAPK kinase inhibited this phosphorylation and reduced 14-3-3 binding to Cdc25B1. Thus, our data suggest that the p38 signalling pathway, activated by cycloheximide or UV, leads to phosphorylation of serine 323 in Cdc25B3, and that this phosphorylation mediates 14-3-3 protein binding. Because of the proximity of the 14-3-3 binding site to the NLS, the binding of 14-3-3 proteins probably interferes with nuclear import as has been shown for *Xenopus* Cdc25 (Kumagai et al., 1998; Yang et al., 1999), leading to increased cytoplasmic localisation of the phosphatase. It is possible that a partial translocation of Cdc25B to the cytoplasm might contribute to a p38-mediated cell cycle delay in response to stress. Recently, a cytoplasmic accumulation of Cdc25B was observed in hydrogen-peroxide-treated cells, and the PKB-Akt pathway was implicated in this event (Baldin et al., 2003). Thus different stress- and checkpoint-induced signalling pathways might converge on regulation of the Cdc25B phosphatase. It is not clear how localising Cdc25B to the cytoplasm can inhibit mitosis because cyclin B1 is activated in the cytoplasm and Cdc25B is needed in the cytoplasm for efficient induction of mitosis (see above). However, it might be that access of Cdc25B to its substrate(s) in the cytoplasm is restricted by 14-3-3-binding under these conditions. It was found that mutation of the 14-3-3 binding site makes Cdc25B more efficient in overriding the G2-delay induced by UV irradiation (Bulavin et al., 2001).

The results described in this report demonstrate that, in contrast to what was previously reported, Cdc25B is mainly

present in the cell nucleus, although it is also needed in the cytoplasm to efficiently induce mitosis. We further characterised the nucleo-cytoplasmic shuttling of Cdc25B and evaluated a nuclear export mutant in a live cell shuttling assay. The finding that Cdc25B is exported from the nucleus in a p38-dependent manner when cells are exposed to stress, suggests a link between the p38 signalling pathway and factors regulating Cdc25B localisation.

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Note added in proof

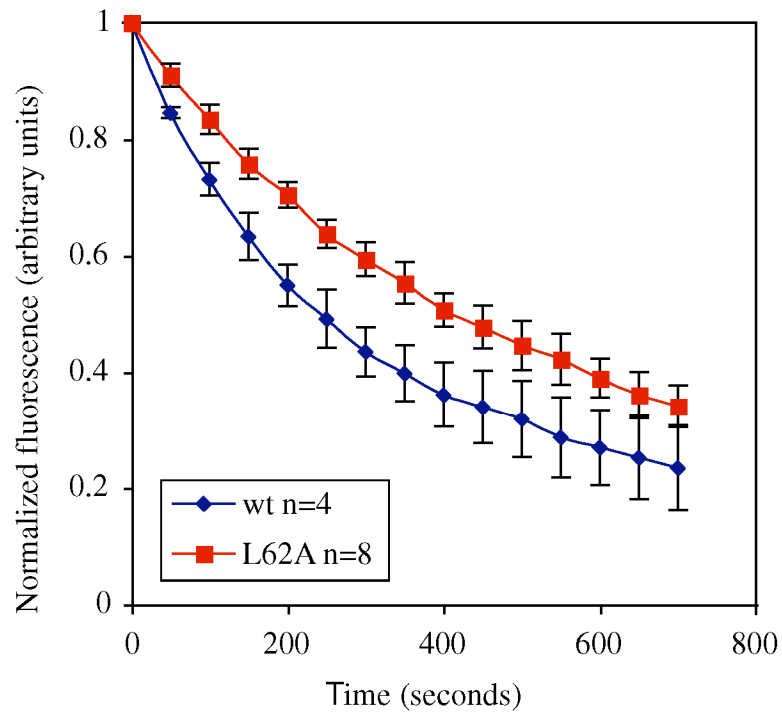
A mutational analysis was recently published confirming that the NES we characterised in this paper is the major NES in Cdc25B (Uchida et al., 2004).

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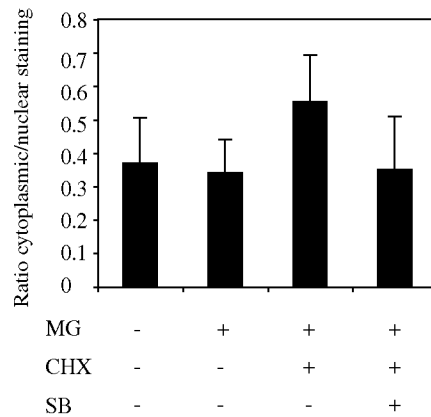
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Supplementary figure 1



Mutation of NES2 reduces the export rate of Cdc25B3 in G2. Fluorescence loss in photobleaching (FLIP) of YFP-Cdc25B3 and YFP-Cdc25B3(L62A) in G2 HeLa cells. Cells were synchronised with thymidine, released and microinjected with expression vectors five hours after release. Three hours later, the FLIP assay was performed as described for G1 cells. The nuclear fluorescence of the bleached cells was quantified. After background subtraction, the value of nuclear fluorescence at the start of the experiment was normalised to 1.

Supplementary figure 2



Endogenous Cdc25B partly translocates to the cytoplasm after treatment with cycloheximide. When treating cells with cycloheximide for 1 hour, both the nuclear and cytoplasmic staining was greatly reduced indicating a short half-life of endogenous Cdc25B. Therefore, to be able to study the localisation pattern changes we added the proteasome inhibitor MG132 to the cells. HeLa cells were treated for two hours with or without MG132 (MG) and SB202190 (SB), and for 1 hour with or without cycloheximide (CHX) as indicated. The nuclear and cytoplasmic B1:5 (Cdc25B) fluorescence of Cyclin B1 positive cells from deconvolution images was quantified, the background was subtracted and the cytoplasmic signal was divided with the nuclear signal. Cycloheximide (Labora) was used at 100 µg/ml. MG132 and SB202190 were used at 2 µg/ml and 10 µM respectively.