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Nuclear Localization of CDC25B1 and Serine 146 Integrity Are Required for Induction of Mitosis*

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CDC25B phosphatases are essential regulators that control cyclin-dependent kinases activities at the entry into mitosis. In this study, we demonstrate that serine 146 is required for two crucial features of CDC25B1. It is essential for CDC25B1 to function as a mitotic inducer and to prevent CDC25B1 export from the nucleus. We also show that serine 146 is phosphorylated *in vitro* by CDK1-cyclin B. However, phosphorylation of CDC25B does not stimulate its phosphatase activity, and mutation of serine 146 had no effect on its catalytic activity. Serine 146 phosphorylation is proposed to be a key event in the regulation of the CDC25B function in the initiation of mammalian mitosis.

The first member of the CDC25 family was identified in the fission yeast *Schizosaccharomyces pombe* as a dose-dependent inducer of mitosis (1). Since then, homologous regulators have been found in every eukaryotic organism examined. Meanwhile, the biochemical properties of CDC25 were deciphered and its essential role in the control of the activation of CDC2 by dephosphorylation was characterized. CDC25 is a dual specificity phosphatase that directly activates the CDC2 kinase at the G₂/M transition by dephosphorylating tyrosine 15 and threonine 14 (2). In yeast, there is only one form of CDC25, whereas in human cells three CDC25 phosphatase genes have been identified (3–5). Although they share about 50% similarity at the protein level, they are involved in specific regulatory processes. CDC25C and CDC25B are thought to activate CDK1-cyclin complexes at the G₂/M transition, and CDC25A regulates G₁/S complexes, respectively (6–8). In fact, the exact role of CDC25B is still controversial (9). On the basis of antisense oligonucleotide studies, it has been proposed that CDC25B is required for progression in S phase (10). Other reports are more in favor of a role for CDC25B in late G₂ as a regulator of centrosomal microtubule nucleation (11) and as a starter of mitosis (9, 12). Ablation of CDC25B by microinjection of specific antibodies blocks cell cycle progression by inhibition of entry into mitosis (13). Since several different isoforms of CDC25B have been detected in human cells (14, 15) and since the above

mentioned studies made no distinction between CDC25B variants, we cannot exclude the possibility that each isoform has a specific function at a particular stage of the cell cycle or in a defined subcellular compartment.

The activity of CDC25 phosphatases is regulated both at the translational and the post-translational levels. CDC25A and CDC25B expression appears to be cell cycle-regulated, whereas CDC25C is fairly constant. Phosphorylation of CDC25 is an important regulator of its phosphatase activity. CDC25C undergoes extensive phosphorylation of its NH₂-terminal regulatory domain at mitosis, and this strongly stimulates its catalytic activity toward CDK1-cyclin B, thus creating a positive feedback loop (16, 17). Similarly, CDC25A is phosphorylated and activated by CDK2-cyclin E at the G₁/S transition and in turn dephosphorylates and fully activates that complex (18). It has been shown that CDC25B is phosphorylated in cell extracts prepared from cells in S-phase to mitosis, and it has been proposed that it is consequently activated (13, 19). CDC25B is an unstable protein, and phosphorylation also participates in the regulation of its degradation (12); it is degraded in a proteasome-dependent manner upon phosphorylation by CDK1-cyclin A (20).

As already mentioned, we and others have identified at least three splice variants of CDC25B (14, 15). These three isoforms differ by the presence or the absence of two peptides, peptide A (14 residues) and peptide B (41 residues), which are both located in the amino terminus regulatory region of the phosphatase. These two peptides lie in domains that are fairly well conserved between evolutionarily distant members of the CDC25 family (14). In this study, we show that serine 146, a phosphorylation site located within domain B, is essential for the mitotic inducer activity associated with the nuclear retention of CDC25B1.

EXPERIMENTAL PROCEDURES

DNA Constructs—pET14b- and pcDNA₃-derived plasmids containing CDC25B and the N-terminal deletion mutants are described elsewhere (14, 20–22). The serine 146 mutant was generated by PCR. pGEX-2T containing the entire open reading frame of human CDC25C (16) was a gift from Dr. G. Draetta (European Institute of Oncology, Milan, Italy).

Cell Culture Conditions—HeLa cells and U2OS cells were grown as previously described (21). To obtain cells arrested in mitosis, HeLa cells were incubated for 16 h in the presence of 250 ng/ml nocodazole (Sigma). Lovastatin and hydroxyurea were used at a concentration of 40 μ M and 10 mM, respectively, to obtain cells either in G₁ or at the G₁ to S transition (14). HeLa cells were transfected using Exgen-500 (Euromedex) following the manufacturer's instructions.

The U2OS cell line conditionally expressing HA-tagged CDC25B1 (under the control of the *tet* promoter) is described elsewhere.² Expression of CDC25B1 was induced by tetracycline removal from the culture medium, and cells were harvested 24 h later.

Cell Lysate and Immunoprecipitation—Exponentially growing cells,

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§ Recipient of a postdoctoral fellowship from the Société de Secours des Amis des Sciences.

¹ The abbreviations used are: CDK, cyclin-dependent kinase; HA, hemagglutinin; GST, glutathione S-transferase; LMB, leptomycin B; NLS, nuclear localization signal.

² N. Theis-Febvre, B. Ducommun, and V. Baldin, submitted for publication.

drug-arrested cells, or transfected HeLa cells were lysed in LB buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM dithiothreitol) in presence of protease inhibitors: leupeptin (5 μ g/ml), aprotinin (5 μ g/ml), trypsin inhibitor (10 μ g/ml), L-1-tosylamido-2-phenylethyl chloromethyl ketone (20 μ g/ml), 1-chloro-3-tosylamido-7-amino-2-heptanone (20 μ g/ml), phenylmethylsulfonyl fluoride (17 μ g/ml), calpain inhibitor I (20 μ g/ml), and phosphatase inhibitors (50 mM NaF and 0.1 mM Na_3VO_4) (except for phosphatase assays) for 30 min on ice and centrifuged at 14,000 rpm for 10 min at 4 °C. Protein concentration was determined. In the case of nocodazole-arrested cells, a total cell extract was directly used for phosphorylation assay, or in some experiments CDK1-cyclin B was immunoprecipitated using anti-cyclin B antibody.³ In the case of transfected cells, HA-tagged CDC25B1 or S146G-CDC25B1 proteins were immunoprecipitated using 12CA5 mouse monoclonal antibody raised against the HA epitope (Roche Molecular Biochemicals) and then used in phosphatase assay or Western blot analysis. Western blot analyses were performed with the CDC25B C-terminal peptide antibody (1:2000).

Production of Recombinant Proteins—*In vitro* transcription and translation of CDC25B and mutant proteins were performed using the TNT-quick system (Promega) in the presence or absence of [³⁵S]methionine.

Spodoptera frugiperda (Sf9) cells were maintained in Insect X-press medium (BioWhittaker) supplemented with 10% heat-inactivated fetal calf serum (Eurobio), gentamicin (250 μ g/ml), amphotericin B (2.5 μ g/ml), penicillin (100 units/ml), and streptomycin (100 μ g/ml) at 27 °C. For CDK-cyclin complex expression, Sf9 cells were seeded at a density of 60,000 cells/cm² 1 day prior to the infection and then co-infected with recombinant baculoviruses encoding human CDK1 (CDC2) and cyclin B. Insect cell extracts were prepared 72 h postinfection as follows. Cells were lysed with lysis buffer (10 mM Tris, pH 7.5, 25 mM NaCl, 50 mM NaF, 0.1 mM sodium orthovanadate) using a Dounce homogenizer and then diluted in 4 volumes of solubilization buffer (25 mM NaH_2PO_4 , pH 7.5, 250 mM NaCl, 10% glycerol, 0.02% Tween 20) and centrifuged at 100,000 $\times g$ for 1 h at 4 °C. GST-CDC25B1, GST-S146G-CDC25B1, and GST-CDC25C fusion proteins produced in bacteria were purified as previously described (16).

Phosphorylation Assays—*In vitro* translated protein or recombinant GST fusion proteins were immunoprecipitated with an anti-CDC25B polyclonal antibody directed against a C-terminal peptide of CDC25B and incubated with 15 μ g of cellular extract from baculovirus-infected Sf9 cells or 200 μ g of different cellular extracts, respectively, in kinase assay buffer (16, 20) containing 5 μ Ci of [γ -³²P]ATP for 60 min at 30 °C or cold ATP. The precipitates were subjected to SDS-8% PAGE electrophoresis. Peptide phosphorylation was performed in the same kinase assay condition except that we used immunoprecipitated CDK1-cyclin B instead of total cell lysate, and the reaction was carried out in 30 min at 30 °C. In the indicated experiments, histone H1 kinase activity was determined on an immunoprecipitation performed with anti-cyclin B antibodies on 200 μ g of cell extracts (23).

Phosphatase Activity Assay—Recombinant GST fusion proteins (CDC25B1, S146G-CDC25B1, and CDC25C) were incubated with 400 μ g of nocodazole-arrested HeLa cells extracts in kinase assay buffer in presence of 1 mM ATP, 10 μ g of creatine kinase, and 10 mM creatine phosphate for 30 min at 30 °C. Recombinant proteins were recovered on glutathione beads, washed three times with LB buffer in the absence of phosphatase inhibitors (NaF and Na_3VO_4), and washed once with phosphatase buffer (30 mM Tris pH 8.2, 75 mM NaCl, 0.67 mM EDTA, 0.033% bovine serum albumin, 1 mM dithiothreitol). CDC25 B phosphatase activity was determined using fluorescein diphosphate (Molecular Probes, Inc., Eugene, OR) as substrate at the final concentration of 20 μ M for 30 min at room temperature as described (24). Fluorescence was monitored using a multiwell plate reader (Fluoroskan Ascent, Labsystems; excitation filter, 485 nm; emission filter, 538 nm). The phosphatase activity of HA-CDC25B1 or HA-S146G-CDC25B1 expressed in HeLa cells was determined as described above after immunoprecipitation from cell lysates with anti-HA antibody.

Immunofluorescence—HeLa cells seeded on glass coverslips were transfected, and cells were fixed 24 h later using 3.7% formaldehyde and then permeabilized with 0.25% Triton X-100 and cold methanol. HA-CDC25B1 and HA-mutants were detected by incubating first the coverslips with 12CA5 mouse monoclonal (1:2500) (Roche Molecular Biochemicals), followed by a second incubation with Alexa-594-conjugated goat anti-mouse antibody (1:500) (Molecular Probes). In all cases, DNA was visualized using Hoechst 33258 dye at 1 μ g/ml (Sigma). The

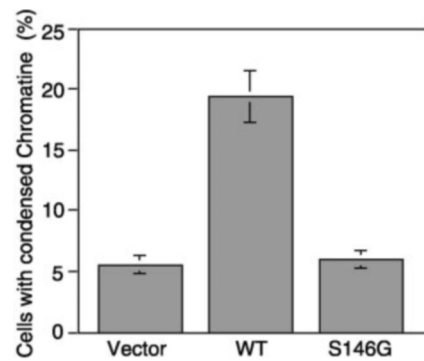


FIG. 1. Serine 146 modulates CDC25B1 activity *in vivo*. Exponentially growing HeLa cells were transfected with pcDNA₃ (vector) or constructs allowing the expression of an HA-tagged version of wild-type CDC25B1 (WT) and S146G-CDC25B1 mutant. The cells were fixed 24 h after transfection and stained to detect the expression of CDC25B1 by immunofluorescence using monoclonal anti-HA antibodies. The number of CDC25B1-positive cells displaying a condensed chromatin aspect was determined. The results presented are the average of three independent experiments.

mitotic index was calculated by monitoring the cells (transfected or not) presenting a condensed chromatin aspect.

RESULTS

Ability of CDC25B1 to Induce Mitosis Is Abolished by the S146G Mutation—In order to study the *in vivo* activity of CDC25B1 phosphatase, we expressed an HA epitope-tagged version of the wild type CDC25B1 in asynchronous HeLa cells under the control of the cytomegalovirus promoter. The transfected cells were identified and monitored by immunofluorescence staining with anti-HA antibodies. As shown in Fig. 1, CDC25B1 acted as an inducer of mitosis, since, 24 h after transfection, about 20% of the cells expressing the CDC25B1 phosphatase displayed a condensed chromatin aspect. In contrast, this mitotic inducing activity was abolished with CDC25B1-S146G, a mutant protein that was tested in the course of a systematic study on putative phosphorylation sites. This serine, located in the alternately spliced B domain, is reminiscent of a CDK consensus phosphorylation site and is conserved in most of the CDC25 sequences from various eukaryotic species (14).

Serine 146 Is a Major Phosphorylation Site—Using a cell line conditionally expressing HA-CDC25B1, we show that, as inferred from its electrophoretic mobility shift, CDC25B1 was strongly phosphorylated in cells that were arrested at mitosis by a nocodazole treatment (Fig. 2A, upper panel). This effect correlated with a high level of CDK1-cyclin B kinase activity (Fig. 2A, lower panel). We next investigated whether serine 146 was a phosphorylation site for the CDK1/CDC2 protein kinase. Using a set of amino-terminally truncated CDC25B1 proteins generated by site-directed mutagenesis (Fig. 2B), we found that deletion of the first 274 residues fully abolished the phosphorylation of the protein, indicating that major phosphorylation sites for CDK1-cyclin B are all located in that amino-terminal region. Deletion of 186 residues of CDC25B already had a dramatic effect on its phosphorylation, but a shorter deletion (Δ 108) had no detectable impact (Fig. 2C). These results indicate that the region ranging from amino acids 108 to 186 comprises one or several residues that is (are) directly phosphorylated. Alternatively, this region may be required for the phosphorylation of a site located elsewhere in the protein.

We next examined whether the synthetic 42-residue peptide containing serine 146 that is located between residues 108 and 186 was phosphorylated *in vitro* by CDK1-cyclin B. As shown in Fig. 3A, CDK1-cyclin B immunoprecipitate readily phosphorylated it (lanes 1 and 2), whereas control immunoprecipitates did

³ V. Baldin, unpublished data.

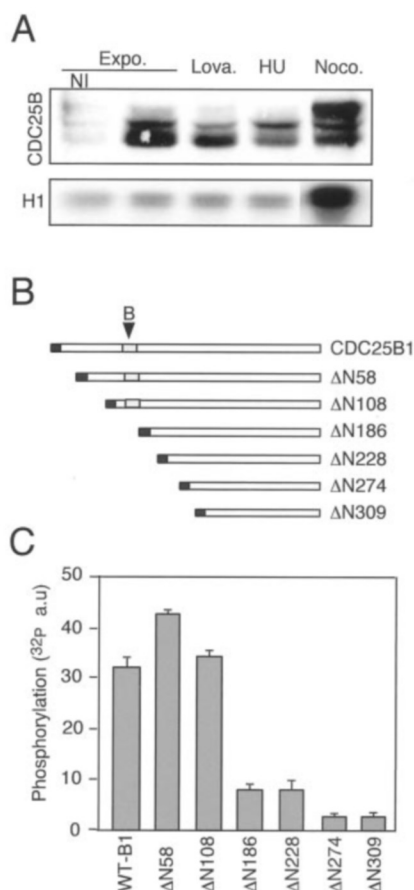


FIG. 2. CDC25B1 is phosphorylated *in vivo* and *in vitro* by CDK1-cyclin B. **A**, upper panel, analysis of CDC25B1 expression and mobility in U2OS cells conditionally expressing CDC25B1. 100 μ g of total cell extract from exponentially growing (*Expo.*), lovastatin (*Lova.*), hydroxyurea (*HU*), and nocodazole (*Noco.*)-treated cells induced or not (*NI*) to produce CDC25B1 were analyzed by Western blot using an anti-CDC25B antibody. Lower panel, CDK1-cyclin B kinase activity. 200 μ g of total cell extract, used in **A**, were immunoprecipitated with an anti-cyclin B antibody, and phosphorylation of histone H1 was monitored. **B**, schematic representation of the CDC25-B1 amino-terminal deletion mutants. The N terminus poly-His tag is indicated (gray box). Numbering indicates the number of residues that have been deleted at the N terminus of the protein. The position of the B domain is indicated with an arrowhead. **C**, *in vitro* translated wild-type CDC25B1 and amino-terminal truncated mutants were incubated for 60 min at 30 °C with CDK1-cyclin B. Proteins were visualized after electrophoresis, and the incorporation of 32 P was quantified. Phosphorylation was normalized to the concentration of each CDC25B protein estimated on a parallel Western blot (data not shown). Background phosphorylation obtained with a control cell lysate was subtracted from the data that are presented. The S.E. from three independent experiments is indicated.

not (lanes 3 and 4). The serine 146 residue was then mutated to a nonphosphorylatable residue, and the kinetics of phosphorylation of both S146G-CDC25B1 mutant and wild type CDC25B1 by CDK1-cyclin B were analyzed. As shown in the autoradiography (Fig. 3B, left panels), the S146G-CDC25B1 mutant did not display the electrophoretic mobility shift that rapidly occurs upon phosphorylation of wild-type CDC25B1 by CDK1-cyclin B and was less efficiently phosphorylated. Consonant with this observation, quantification of the level of 32 P incorporation (Fig. 3B, right panel) indicated that the overall phosphorylation of S146G-CDC25B1 was about 50% of that observed in the wild-type CDC25B1 protein. To confirm this observation, an *in vitro* phosphorylation assay with recombinant GST-CDC25B1 proteins and mitotic HeLa cell extract obtained by nocodazole treatment was performed. As shown in

Fig. 3C, upon incubation, wild type CDC25B1 displayed changes in its electrophoretic mobility that were only partially observed in the case of the S146G mutant protein, the band of highest electrophoretic mobility and the additional smear being absent. When the HeLa cell extract was depleted from CDK1-cyclin B activity by cyclin B immunoprecipitation (Fig. 3E) prior to the incubation with CDC25B1, the electrophoretic mobility shift of the protein was fully abolished (Fig. 3D, lane 3). Taken together, these data indicate that serine 146 is phosphorylated *in vitro* by the CDK1-cyclin B complex.

CDC25B1 Is Not Activated upon Phosphorylation by CDK1-Cyclin B—Several reports have described the activation of CDC25A and CDC25C phosphatases by CDC2-dependent phosphorylation (16, 18). On the basis of indirect evidence, it was also proposed that CDC25B is similarly regulated (13). We therefore examined this hypothesis and the possibility that serine 146 participates in the activation of CDC25B1. To address this question, we prepared both wild-type and S146G mutant recombinant proteins in fusion with GST by expression in *Escherichia coli* and affinity purification on glutathione-Sepharose. A GST-CDC25C protein was also produced in the same way. These purified proteins were incubated in the presence of mitotic cell extracts prepared from nocodazole-treated HeLa cells to allow their phosphorylation by CDK-cyclin complexes (Fig. 4A). The GST fusion proteins were recovered from the incubation mix by affinity purification, and the phosphatase activity of CDC25B was then quantified. As shown in Fig. 4B, we found that the specific activities of unmodified CDC25B and S146G-CDC25B1 recombinant proteins were identical (271 and 259 units, respectively) and about 9-fold higher than the activity of CDC25C (28 units). As expected, the activity of recombinant CDC25C was amplified 6–7-fold upon phosphorylation by a mitotic extract. However, in contrast and in disagreement with what has been proposed by others (13), the phosphatase activity of recombinant CDC25B was not increased following incubation and phosphorylation with a mitotic extract. Thus, CDC25B activity is not up-regulated upon phosphorylation by CDK1, and mutation of serine 146 has no effect on the catalytic activity of this phosphatase.

We also next examined whether a down-regulation of the catalytic activity of CDC25B1 *in vivo* could account for the observation that the S146G mutation abolished the mitotic inducing effect. The phosphatase activity of wild type CDC25B1 and S146G-CDC25B1 was monitored in HeLa cells transfected with the corresponding HA-tagged constructs. After immunoprecipitation with anti-HA antibodies, the phosphatase activity was measured. As shown in Fig. 4C, the phosphatase activities of these two enzymes were found to be very similar. Thus, the role of serine 146 in the mitotic inducer effect of CDC25B1 does not appear to depend on a change of its catalytic activity.

Serine 146 Regulates the Intracellular Localization of CDC25B1—The intracellular localization of CDC25B1 was investigated by immunofluorescence staining in exponentially growing HeLa cells transfected with constructs allowing the expression of the wild-type and the S146G-CDC25B1 mutant proteins. As we previously reported (21), in the case of wild type CDC25B1 expression, the percentage of cells displaying either a strictly nuclear or cytoplasmic localization pattern was 15 and 20%, respectively (Fig. 5A). In contrast, in cells expressing the S146G-CDC25B1 mutant, the percentage of exclusive nuclear localization was only about 5%, whereas in about 45% of the cells, the localization of the protein was entirely cytoplasmic. Thus, it appears that the mutation of serine 146 impairs the ability of CDC25B1 protein to be located within the nucleus and favors its retention in the cytoplasmic compartment.

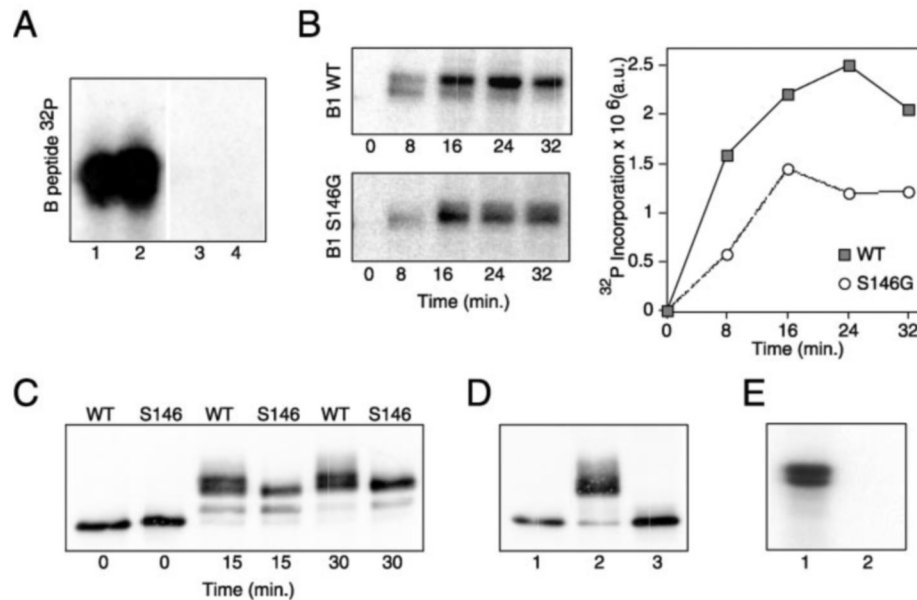


FIG. 3. Serine 146 is phosphorylated by CDK1-cyclin B. A, phosphorylation of domain B peptide (41 residues) by a cyclin B immunoprecipitate obtained from Sf9 insect cell lysates producing (lanes 1 and 2) or not producing (lanes 3 and 4) CDK1-cyclin B complexes. The assay was performed using 2 μ g (lanes 1 and 3) and 4 μ g (lanes 2 and 4) of domain B peptide for 30 min at 30 °C. The samples were run on a 20% gel and autoradiographed. B, kinetics of phosphorylation of identical amounts of unlabeled *in vitro* translated wild-type and S146G CDC25B1 by CDK1-cyclin B-expressing Sf9 cell lysates. The samples were run on an 8% gel and autoradiographed (left panel). The quantification of the incorporation is shown in the right panel. C, recombinant GST-wild type (WT) and GST-S146G mutant CDC25B1 were incubated for 0, 15, or 30 min at 30 °C in the presence of mitotic HeLa cell extract (200 μ g) obtained from nocodazole-treated cells. The samples were run on an 8% acrylamide gel (30:0.2) and transferred, and Western blot detection was performed using anti-CDC25B polyclonal antibody. D, recombinant GST-wild type CDC25B1 was incubated (lanes 2 and 3) or not (lane 1) for 60 min at 30 °C in the presence of mitotic HeLa cell extract (200 μ g) obtained from nocodazole-treated cells. In lane 3, the extract was subjected to a cyclin B immunodepletion prior to the incubation with CDC25B1. E, histone H1 kinase activity was determined by immunoprecipitation using anti-cyclin B antibodies on mitotic HeLa cell extracts (200 μ g) immunodepleted (lane 2) or not (lane 1) in CDK1-cyclin B complexes.

Since it is likely that CDC25 proteins shuttle between cytoplasmic and nuclear compartments, this defect in nuclear localization may be due either to altered nuclear import or to deficient nuclear retention. We further examined this issue using leptomycin B (LMB), a potent CRM1 exportin inhibitor (25). Wild-type CDC25B1 was almost totally retained in the nucleus in HeLa cells treated with LMB (Fig. 5B). Similarly, the mutant protein CDC25B1-S146G was also detected in the nucleus in 86% of the cells, indicating that it was correctly targeted to the nucleus but not exported because of the presence of leptomycin B. In contrast, a CDC25B1 mutant protein with an inactivating mutation of the nuclear localization signal (NLS) (*i.e.* the replacement of the KRR motif by AGA (21)) was incapable of being targeted to the nucleus (Fig. 5B). Thus, we can conclude that the S146G mutation does not impair entry of CDC25B1 into the nucleus but alters its retention.

CDC25B1 Mitotic Inducing Effect Is Dependent on Its Nuclear Localization and Is Abolished by the S146G Mutation—The effect of the subcellular localization of CDC25B1 on its activity as an inducer of mitosis was further examined. This effect was found to be dependent on the nuclear localization of CDC25B1, since when an NLS-mutant was expressed, the percentage of cells in mitosis was decreased to 7.7%, a level similar to that observed in control pcDNA-transfected cells (Fig. 6). Furthermore, in the presence of LMB, about 20% of the cells expressing the wild-type CDC25B1 entered mitosis, whereas only about 8% of the cells expressing the CDC25B1-S146G mutant did so (Fig. 6).

These results indicate that localization of CDC25B1 into the nucleus is required for its mitotic inducer function. However, when CDC25B1-S146G is artificially tethered within the nucleus by LMB, it fails to act as a mitotic inducer, indicating that this function probably requires an additional nuclear event that is also dependent on serine 146 (see "Discussion").

DISCUSSION

The work presented here stems from the observation that a mutation of serine 146 glycine abolishes the mitotic inducing effect of the CDC25B1 phosphatase and reports the experiments that have been undertaken to identify the molecular basis of that observation.

We have found that serine 146 is one of the major sites of CDC25B1 phosphorylation *in vitro* by CDK1-cyclin B. Serine 146 is included in the alternately spliced domain B, a 41-residue peptide that is present in the CDC25B1 and -B3 variants but absent in CDC25B2. As we reported when CDC25B splicing variants were identified (14), this domain is conserved between species. In some cases, a threonine residue replaces the serine, but this phosphorylatable amino acid is always followed by a proline. Furthermore, this residue is conserved between the three human CDC25s, and it has recently been shown that alternative splicing of this domain also exists in the other human CDC25 phosphatase (26). Such a conservation of the genomic organization of the CDC25 locus is likely to reflect a crucial functional role for this region of the protein. However, structural data are still missing to help in clarifying this issue.

We have shown that CDC25B catalytic activity is not up-regulated upon phosphorylation by CDK1-cyclin B, and its specific activity is already high when compared with CDC25C. Furthermore, using recombinant CDK-cyclin complexes produced and purified from baculovirus-infected Sf9 cells (CDK2/E, CDK2/A, CDK1/A), we also did not observe any increase of the catalytic activity of CDC25B1.⁴ Thus, in contrast to what has been reported for CDC25A and CDC25C (16, 18), CDC25B activity appears to be constitutively high and not up-regulated by CDK-cyclin complexes.

⁴ V. Baldin and C. Cans, unpublished data.

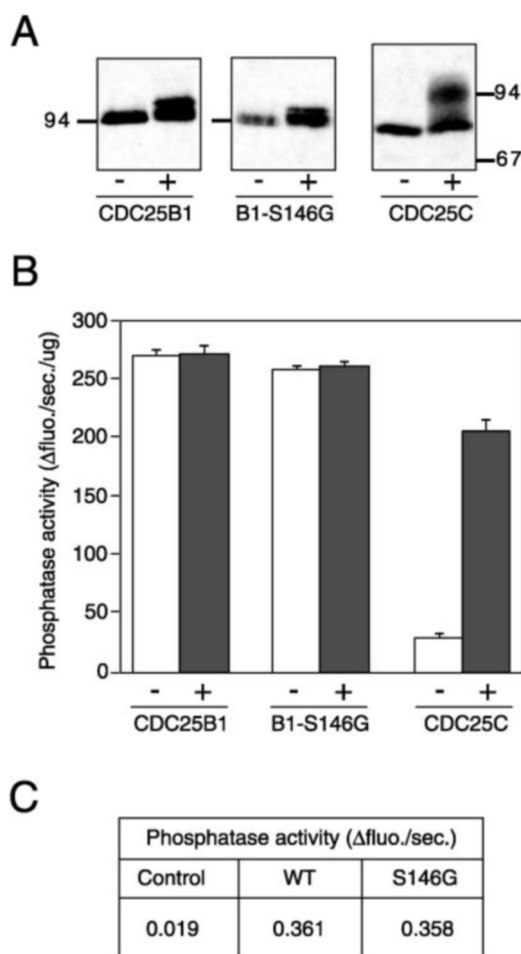


FIG. 4. CDC25B1 is not activated by phosphorylation. *A* and *B*, purified GST-CDC25B1, GST-CDC25B1-S146G, and GST-CDC25C recombinant proteins were incubated (+) or not (-) with 400 μ g of mitotic HeLa cell extract for 30 min at 30 °C obtained from nocodazole-treated cells and then recovered on glutathione-Sepharose beads. The beads were washed as indicated under "Experimental Procedures," and then CDC25 phosphatase activity was determined using fluorescein diphosphate as substrate (*B*). In *A*, an aliquot of each sample was run on 8% SDS-PAGE and then processed for immunodetection of CDC25B or -C to visualize the molecular weight shift induced by phosphorylation. *C*, HeLa cells were transfected as described in the legend to Fig. 1 and harvested after 24 h. The CDC25B1 wild type or mutant phosphatase was immunoprecipitated using monoclonal anti-HA antibodies, and its activity was determined using fluorescein diphosphate as substrate. The phosphatase activity was normalized to the same amount of CDC25B proteins, measured by Western blot analysis after immunoprecipitation. The S.E. from three independent experiments is indicated.

We demonstrate here that nuclear targeting of CDC25B1 is essential to activate entry into mitosis. The loss of function of the S146G-CDC25B1 mutant together with its predominant cytoplasmic localization led us to examine the effect of a nuclear localization signal mutation. We have shown that the NLS-mutant is fully retained in the cytoplasm and that it is not active either as a mitotic inducer. Together, these results indicated that cytoplasmic accumulation of the CDC25B1 phosphatase had no detectable effect on the triggering of mitotic events. At least as far as CDC25B1 is concerned, our data do not support the proposal that cytoplasmic accumulation of CDC25B phosphatase at mitosis triggers centrosomal microtubule nucleation, whereas CDC25C regulates nuclear G₂/M events (11). In this model, the activity of CDC25B is suggested to be required to activate the cytoplasmic pool of CDK1-cyclin B, which is responsible for the earliest changes in microtubule

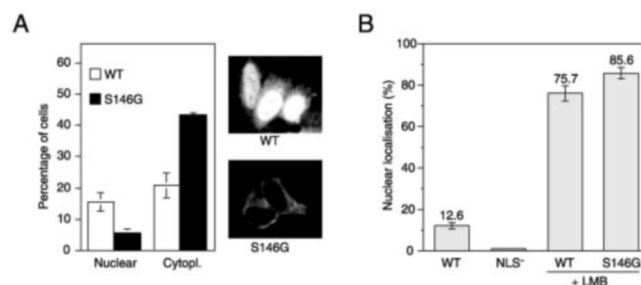


FIG. 5. Serine 146 regulates intracellular localization of CDC25B1. *A*, asynchronous HeLa cells were transiently transfected with pcDNA₃ vector, allowing the expression of an HA epitope-tagged version of wild-type or S146G CDC25B1 proteins. Cells were fixed 24 h after transfection and stained with anti-HA antibodies. Quantification of the intracellular localization is shown. The percentages of transfected cells expressing wild type CDC25B1 (white bars) or S146G CDC25B1 (black bars) and displaying exclusive nuclear staining or cytoplasmic staining were obtained from five independent experiments with at least 200 transfected cells examined per experiment. The S.E. is indicated. Microphotographs of cells displaying typical cytoplasmic or nuclear staining are presented. *B*, asynchronous HeLa cells were transiently transfected with pcDNA₃ vector, allowing the expression of wild type CDC25B1 or NLS-CDC25B1 (mutation of the KRR motif to AGA) or S146G-CDC25B1 mutant proteins. Cells were either untreated or treated with 20 ng/ml LMB for 2 h prior to fixation 24 h after transfection. Cells were stained with anti-HA antibodies, and the intracellular localization of each protein was determined. The percentage of cells displaying an exclusive nuclear staining is indicated. The values indicated are the mean of three independent experiments.

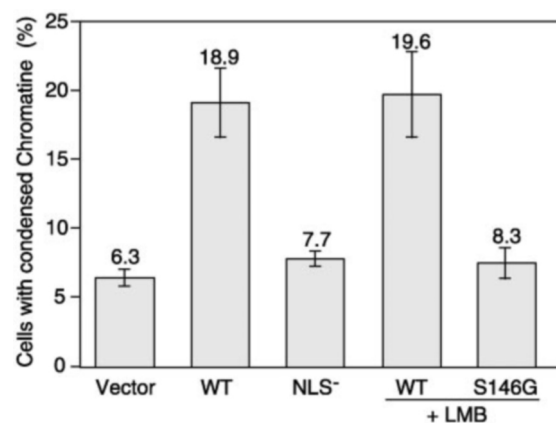
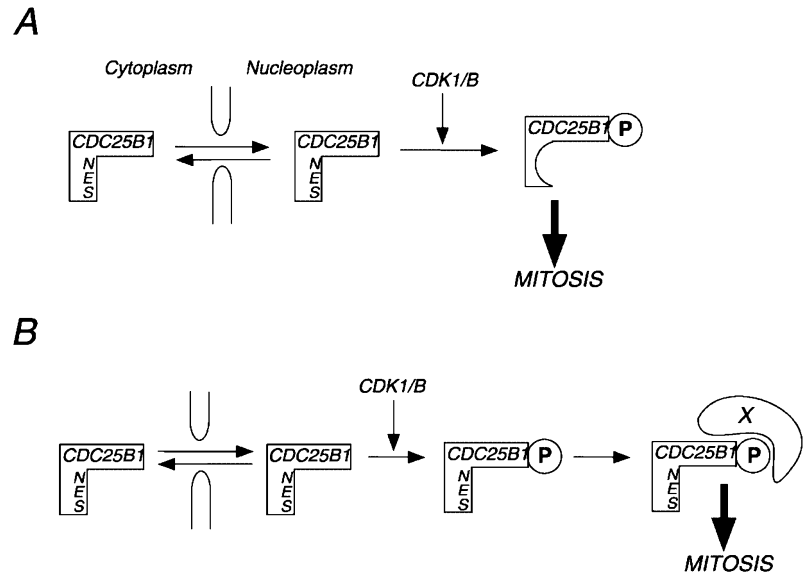


FIG. 6. CDC25B1 activity is dependent on its nuclear localization. Asynchronous HeLa cells were transiently transfected with pcDNA₃ vector, allowing the expression of wild type CDC25B1, NLS-CDC25B1, or S146G-CDC25B1 mutant proteins. Cells were either untreated or treated with 20 ng/ml LMB for 2 h prior to fixation 24 h after transfection and stained with anti-HA antibodies and Hoechst. The percentage of CDC25B1-positive cells (wild type or mutants) displaying condensed chromatin aspect was determined. The data are derived from three independent experiments. The S.E. is indicated.

dynamics. Of course, we cannot exclude the possibility that another variant of CDC25B may be specifically targeted to CDK1-cyclin B complexes associated with the cytoskeleton and therefore involved in the regulation of its dynamics. In fact, the CDC25B3 variant was used in Gabrielli's work, where it was shown to cause the formation of abnormal minispindles upon overexpression (11). Under our experimental conditions (*i.e.* with CDC25B1) we did not observe these minispindles; however, we found that the CDC25B3 variant is a less potent inducer of mitosis than CDC25B1, with only about 10% of cells in mitosis 24 h after transfection (data not shown).

The data reported in this study demonstrate that CDC25B1 must be targeted to the nucleus to exert its mitotic inducer effect. This localization is dependent both on the integrity of its nuclear localization signal and on the integrity of the phospho-

FIG. 7. Current working model. CDC25B shuttling between the cytoplasm and the nucleus is dependent on an NLS and a nuclear export signal (NES) (21). *A*, following phosphorylation of Ser¹⁴⁶ by CDK1-cyclin B, the nuclear export signal sequence is masked, and CDC25B1 is therefore retained in the nucleus. *B*, phosphorylation of Ser¹⁴⁶ by CDK1-cyclin B allows the interaction of CDC25B1 with a regulatory partner that sequesters it in the nucleus.



rylatable serine 146. Nevertheless, the nuclear localization of CDC25B1 is not sufficient in itself to trigger mitosis, as demonstrated by the lack of induction of mitosis when the S146G-CDC25B1 mutant is retained in the nucleus by leptomycin B. This observation indicates the existence of an additional level of regulation that is essential for access to the nuclear substrates and that is likely to be dependent on serine 146 phosphorylation. Furthermore, only about 20% of the cells expressing CDC25B1 entered mitosis even when it was fully retained in the nucleus in the presence of LMB. This observation strongly suggests that only a given population of cells is sensitive to the mitotic inducer effect of the phosphatase. One can suggest that the only cells targeted are those that have reached a late cell cycle window where rate-limiting regulatory partners or substrates are available.

As depicted in Fig. 7, we currently have two working hypotheses to explain our observations. The first is that the nuclear export sequence of CDC25B1 is masked or altered when serine 146 is phosphorylated by CDK1-cyclin B. A change in the steric conformation of CDC25B1 would then prevent its interaction with the export machinery and retain it in the nucleus (Fig. 7A). Phosphorylation-dependent interaction between cyclin D1 and the CRM1 exportin (27) and between Pho4 and the Msn5 exportin (28) have already been reported, suggesting that site-specific phosphorylation events could either positively or negatively regulate nuclear export. In the case of CDC25B1, phosphorylation of serine 146 would prevent the interaction between the nuclear export signal that is located within the first 40 residues and CRM1. Alternatively, phosphorylation of serine 146 may induce an interaction between CDC25B1 and an associated protein (Fig. 7B). This interaction would be required for the phosphatase to remain within the nucleus and would be essential to perform its function as demonstrated by the observation that the nuclear retention of S146G-CDC25B1 by LMB is not sufficient to restore its activity. The identity of the protein that putatively associates with CDC25B1 remains to be established.

This is the first report of a regulatory mechanism that is specific for one of the three CDC25B splice variants. Although the exact function of these variants in cell cycle control remains to be elucidated, one can speculate that specific phosphorylation events may regulate the interactions with partners, modulate the subcellular localization, and therefore have important implications for the regulation of CDC25B activity. Whether

these specific properties are involved in the oncogenic properties of CDC25B remains to be investigated. However, since an association with CDC25B2 overexpression has been reported in several types of tumors (29, 30),⁵ it is tempting to speculate that the alteration of CDC25B variant-specific properties participates in such a process.

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