The Dual Specificity Phosphatase Cdc25B, but Not the Closely Related Cdc25C, Is Capable of Inhibiting Cellular Proliferation in a Manner Dependent upon Its Catalytic Activity*

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Cdc25B and Cdc25C are closely related dual specificity phosphatases that activate cyclin-dependent kinases by removal of inhibitory phosphorylations, thereby triggering entry into mitosis. Cdc25B, but not Cdc25C, has been implicated as an oncogene and been shown to be overexpressed in a variety of human tumors. Surprisingly, ectopic expression of Cdc25B, but not Cdc25C, inhibits cell proliferation in long term assays. Chimeric proteins generated from the two phosphatases show that the anti-proliferative activity is associated with the C-terminal end of Cdc25B. Indeed, the catalytic domain of Cdc25B is sufficient to suppress cell viability in a manner partially dependent upon its C-terminal 26 amino acids that is shown to influence substrate binding. Mutation analysis demonstrates that both the phosphatase activity of Cdc25B as well as its ability to interact with its substrates contribute to the inhibition of cell proliferation. These results demonstrate key differences in the biological activities of Cdc25B and Cdc25C caused by differential substrate affinity and recognition. This also argues that the antiproliferative activity of Cdc25B needs to be overcome for it to act as an oncogene during tumorigenesis.

In mammalian cells, cyclin-dependent kinase 1 (Cdk1) activity regulates entry into mitosis, whereas Cdk2 action primarily determines initiation of the cell cycle and proper progression through the S phase (1). Cdk1 and Cdk2 activities are regulated at several levels. These include binding to different cyclins, activation by phosphorylation of a regulatory threonine (threonine 161 in Cdk1 or threonine 160 in Cdk2), reversible phosphorylation at two inhibitory residues (threonine 14 and tyrosine 15), subcellular localization, and binding to cyclin-dependent kinase inhibitors (1). The last step in activation of Cdk1 and Cdk2 is removal of the inhibitory phosphates from threonine 14 and tyrosine 15. In mammalian cells, this dephosphorylation event is accomplished by three related dual specificity phosphatases: Cdc25A, Cdc25B, and Cdc25C (2–6). Cdc25A is involved in the initiation of DNA replication as well as mitosis by targeting Cdk2 and Cdk1, respectively (7). In contrast, Cdc25B and Cdc25C induce mitosis by activating Cdk1/cyclin B (7, 8). Cdc25B has been implicated as the initiating phosphatase (9, 10). Activated Cdk1/cyclin B then phosphorylates and activates Cdc25C, which in turn keeps Cdk1/cyclin B active, creating a positive feedback loop that drives the cell through mitosis (11). Cdc25B can dephosphorylate pT14pY15Cdk2/cyclin A in vitro (12–15). A role for Cdc25B in activation of the G2 pool of Cdk2/cyclin A has also been suggested (16).

The functions of Cdc25B and Cdc25C are regulated at transcriptional and post-translational levels (7). Modification by phosphorylation and dephosphorylation has been shown to determine their protein stability, activity, substrate specificity, interaction with regulatory proteins, and subcellular localization (7, 9, 14, 17–24). Cdc25B is a relatively unstable protein, which is detected from S phase until the beginning of mitosis (25–27). Its phosphorylation by Cdk1/cyclin A targets it for degradation by the proteasome (17). Cdc25C levels, on the other hand, do not fluctuate during the cell cycle (7). Phosphorylation of Cdc25B has been suggested to be cell cycle-dependent and a determinant for its substrate specificity (9, 14). Cdc25B immunoprecipitated from cells in S phase is active toward pT14pY15Cdk2/cyclin A, whereas it targets pT14pY15Cdk1/cyclin B when immunoprecipitated from extracts of cells in the G2/M phases. Abrupt hyperphosphorylation of Cdc25C at the G2/M transition is believed to activate phosphatase activity toward its substrate pT14pY15Cdk1/cyclin B (11). Phosphorylation on specific residues also transforms these phosphatases into targets for certain members of the 14-3-3 protein family. This in turn regulates their activities and subcellular localization under normal conditions or in response to activation of cell cycle checkpoints (19–23, 28–31). Subcellular localization of Cdc25B and Cdc25C changes during the cell cycle and determines accessibility to their substrates (7, 9, 18, 22, 25, 29–32).

Cdc25B and Cdc25C are of comparable size (~500 amino acids). Their N termini, consisting of approximately 300 residues, are believed to have regulatory functions and show a low degree of sequence homology (20% identity) (7). These regions contain nuclear import and export signals and binding sites for 14-3-3 family members. The majority of the regulatory phosphorylations occur within the N termini of Cdc25B and Cdc25C (11, 14). Their C termini (~200 amino acids in length)
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contain the catalytic domain (starting with a conserved LIDG motif and including a HCX₉R motif) and are quite similar (61% identity).

Several studies have investigated the roles of the N-terminal regulatory domains and C-terminal catalytic regions in the activities of Cdc25B and Cdc25C in vitro. Here chimeric Cdc25B and Cdc25C proteins are used in a cellular context to determine the roles of different regions of Cdc25B and Cdc25C in regulation of cellular proliferation as well as substrate recognition. Moreover, it is demonstrated that overexpression of Cdc25B, but not Cdc25C, inhibits cell proliferation. It is shown that the catalytic activity of Cdc25B as well as its interaction with Cdk5s is important for its ability to suppress cell growth.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human HCT116 (colon carcinoma) and U2OS (osteosarcoma) cells were grown as monolayers in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 10% penicillin/streptomycin (Invitrogen) at 5% CO₂ and 37 °C.

**Construction of Plasmids and Chimeras**—Cdc25B and Cdc25A cDNAs were generated by reverse transcription. Total RNA was extracted from human WI-38 fibroblasts using the RNeasy® mini kit (Qiagen) according to the manufacturer’s instructions. RNA was reverse-transcribed using the Thermoscript™ RT-PCR system (Invitrogen) also following the manufacturer’s instructions. Subsequent PCR amplifications were performed on a Mastercycler Gradient (Eppendorf) in a total volume of 50 μl containing 2 μl of the cDNA reaction, 200 μM dNTPs, 1× PCR buffer (20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 1% TritonX-100 and 1 μg/ml bovine serum albumin), 400 μM of each Cdc25B or Cdc25A-specific primers, and 1 unit of Pfu Turbo DNA polymerase (Stratagene). The following primers were used for PCR amplification. For Cdc25B, the primers were 5’-ATG GAG GTG CTC TAG C (forward) and 5’-GGG CCA TAT CCT ATC ACT ACT GGT CCT GCA GCC G (reverse). For Cdc25A, the primers were 5’-ATG GAA CTG GGC CCG GA (forward) and 5’-GGG CGA TAT CCT TCA GAG CTT CTT CAG ACG (reverse). For Cdc25C, the primers were 5’-ATG TCT ACG GAA CTC TTC (forward) and 5’-GGG CCA TAT CTC ATG GCC TCA TGT C (reverse). Cdc25C was amplified from a plasmid. PCR conditions were as follows: a hot start at 94 °C for 2 min was followed by (for Cdc25B and Cdc25A) 30 cycles of the cycling step (94 °C for 30 s, 60 °C for 30 s, 68 °C for 1 min) or for Cdc25C, 18 cycles of the cycling step (94 °C for 25 s, 61 °C for 20 s, 68 °C for 30 s) and an additional extension time of 10 min at 68 °C.

To generate the 25B/C and 25C/B chimeras, sequences corresponding to the N and C termini of Cdc25B and Cdc25C were PCR-amplified. The following primer sets were used. The C-terminal part of Cdc25B was amplified with 5’-ATGAGGAGGCTGCCAGCCG (forward) and 5’-TCGGTGGTCACTGTCC (reverse). The C terminus of Cdc25B was amplified with 5’-GAGCTGATTGGAGATTTC (forward) and 5’-GGGCTGATTGGAGATTTC (reverse). Underlining indicates the restriction sites for EcoRV. These fragments were digested with BamHI and EcoRV, purified, and blunt end-ligated (Roche Applied Science). ΔN25B and ΔN25C were generated with the primers used to amplify the C termini of Cdc25B and Cdc25C. ΔN25B/C and ΔN25C/B were produced by fusion PCR. For the first round of PCR, the following primers were used. For the Cdc25B parts, the forward primer used to amplify the C terminus of Cdc25B together with 5’-CTGGCTTCAGACACCTCAGTTCATCCTGGAAGGC (forward) and the reverse primer used to amplify the C terminus of Cdc25B together with 5’-GAGGGGCTGGTCTCAGCCAG were used. For the Cdc25C parts the forward primer used to amplify the C terminus of Cdc25C together with 5’-AGGGGGCTGGTCTCAGCCAG were used and the reverse primer for the C terminus of Cdc25C was 5’-GAGGGGCTGGTCTCAGCCAG. ΔN25B/C, they were the forward primer for the C terminus end of Cdc25B and the reverse primer for the C-terminal end of Cdc25C. For ΔN25C/B, they were the forward primer for the C-terminal end of Cdc25C and the reverse primer for the C terminus of Cdc25B. The sequence encoding the FLAG epitope contained a restriction site for BamHI (underlined) (GGGC GGA TCC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG) and was contained within the 5’ end of each forward primer. A restriction site for EcoRV (underlined) was included at the beginning of some of the reverse primers. The PCR products were cleaved with BamHI and EcoRV, purified, and subcloned into the pcDNA3 plasmid. The identity of the plasmids was confirmed by DNA sequencing.

**Site-directed Mutagenesis**—The catalytically inactive mutants of full-length Cdc25B (C488S) and Cdc25C (C377S) and their derivatives were generated using the QuikChange site-directed mutagenesis kit (Stratagene) following protocols provided by the manufacturer. Plasmids carrying the FLAG-tagged full-length Cdc25B, Cdc25C, or their derivatives were used as templates in the PCRs. The following primer sets were used. For mutant Cdc25B, it was 5’-ATT TTTC CAG TCC TGT GAA TTC TCC TCA GTT GAG CTT CCC ACC CCC TCT AAC AGA TG A ACG CTA CTT GGA ACC AAC (forward) and 5’-GGG GCC CCT CTC TCA GAG CTA GGA GAA TTC GAG GTC (reverse). For mutant Cdc25C, it was 5’-AGT TCC TCT CAG TCT CAG TCG TCT GAA ATG TAG GTC CTA AGA TAG CTA GCA GCA GAG GGC CCA TTG CTA GAG AGC CGC (forward) and 5’-GGG GCC CCT CTC TCA GAG CTA GGA GAA TTC GAG GTC (reverse). For mutant Cdc25B416, it was 5’-GAC CTC AAG TAC ATC GCA CCA GAA ACG ATG GTG (forward) and 5’-GAC CTC AAG TAC ATC GCA CCA GAA ACG ATG GTG (reverse). For mutant Cdc25C416, it was 5’-GAC CTC AAG TAC ATC GCA CCA GAA ACG ATG GTG (forward) and 5’-GAC CTC AAG TAC ATC GCA CCA GAA ACG ATG GTG (reverse). For mutant Cdc25B470, it was 5’-ATG TCC TCT GAA GAG GGC CCA TTG CTA GAG AGC CGC (forward) and 5’-ATG TCC TCT GAA GAG GGC CCA TTG CTA GAG AGC CGC (reverse). Underlining indicates the restriction sites for BamHI. The mutated bases are underlined.

**Immunoblotting**—The cells were lysed in a buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 mM Na₃VO₄, 10 mM NaF, 80 mM β-glycerophosphate, 1 mM...
phenylmethylsulfonyl fluoride, 20 units/ml aprotinin, 5 mg/ml leupeptin and pepstatin. The lysates were clarified by centrifugation (10,000 × g for 10 min at 4 °C) and immunoblotted. The following antibodies were used: mouse anti-FLAG was purchased from Sigma. Rabbit anti-Cdc25B (H-85), mouse anti-Cdc25A (F-6), mouse anti-Cdc25C (H-6), rabbit anti-Cdk2 (M2), mouse anti-Cdk1 (C-19), rabbit anti-pT14pY15Cdk1 (cross-reacts with Cdk2), rabbit anti-cyclin B (H-433), and rabbit anti-cyclin A (H-432) were from Santa Cruz Biotechnology. Rabbit anti-Tyr(P)15 Cdk1 (cross-reacts with Cdk2) was from Cell Signaling Technology. Antibody to mouse anti-β-actin was from Oncogene Research Products. Peroxidase-conjugated goat antibodies against rabbit or mouse IGG (MP Biomedicals) were used as secondary antibodies. The signals were detected using ECL reagents (Amersham Biosciences) and autoradiography films (Labscientific, Inc.).

Colony Formation Assays—Each of the plasmids expressing Cdc25B, Cdc25C, Cdc25A, mutant Cdc25B, mutant Cdc25C, their derivatives, or empty vector was co-transfected with a plasmid conferring puromycin resistance into exponentially growing HCT116 cells using Lipofectamine™ reagent and Plus reagent (Invitrogen) following the manufacturer’s instructions. Forty-eight hours post-transfection, the cells were subjected to puromycin (2 μg/ml) selection for 12–14 days. The colonies were fixed and stained with Giemsa (Sigma-Aldrich).

Immunoprecipitations—HCT116 cells were transfected as described. The cells were lysed in the buffer described for immunoblotting. Mouse anti-FLAG® M2 affinity gel (Sigma) was used in immunoprecipitation assays, which were performed following manufacturer’s protocols. The immunoprecipitates were then immunoblotted as described.

Recombinant Adenoviruses—The FLAG-tagged open reading frames of Cdc25B, mutant Cdc25B, and Cdc25C were cut out of plasmids and subcloned into the pAdTrack-CMV shuttle vectors. These constructs were linearized by digestion with PmeI and were co-transformed together with the adenoviral backbone plasmid pAdEasy-1 into the homologous recombination-competent Escherichia coli strain BJ5183. The resultant recombinants were linearized with PacI and transfected into the adenovirus packaging cell line, 293T. High titer of recombinant adenoviruses was obtained by several rounds of infection. The cells were infected with multiplicity of infection of 8–12 for Ad-25B, 5–10 for Ad-25C, and 35–40 for Ad-mt25B.

RESULTS

Cdc25B but Not Cdc25C Inhibits Colony Formation—The long term consequences of overexpression of Cdc25B and Cdc25C on cell proliferation were investigated. The open reading frames of Cdc25B and Cdc25A were amplified from RNA extracted from WI-38 human fibroblasts using reverse transcription-PCR. The open reading frames of all three phosphatases were FLAG-tagged at their N termini and subcloned into the pcDNA3 vector (see “Experimental Procedures”). Each of these constructs or empty vector was co-transfected together with a plasmid conferring puromycin resistance into HCT116 cells. Immunoblotting using transiently transfected cell lysates and anti-FLAG antibody or specific antibody to each protein showed that these proteins were expressed at similar levels and exhibited the expected mobility (65 kd for Cdc25B, 55 kd for Cdc25C, and 60 kd for Cdc25A) (Fig. 1A). These constructs were then used in colony formation assays. Forty-eight hours post-transfection, HCT116 cells were subjected to puromycin selection for 12–14 days before fixing and staining the colonies with Giemsa. Representative plates are shown. C, bar graph shows the numbers of colonies formed in the presence of each construct as percentages of that of empty vector. The numbers of colonies in the presence of vector is set at 100%. Cdc25A and p53 were included as controls. The average values of two experiments performed in duplicate are shown.
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control for overexpression of an oncoprotein and appeared to impose a negative effect on cell proliferation (41%) but was not as effective as Cdc25B. Cdc25B significantly reduced the number of colonies (16%) compared with empty vector (set at 100%) (Fig. 1, B and C). By contrast, Cdc25C did not have any significant effect on the number, size, or shape of the colonies (90 and 110%, respectively) (Fig. 1, B and C). These results indicate that ectopic expression of Cdc25B but not Cdc25C reduces long term viability of the cells.

The Phosphatase Activity of Cdc25B Contributes to the Ability to Inhibit Cell Proliferation—It is well established that mutating the catalytic cysteine abolishes the phosphatase activities of the Cdc25s (6, 15, 34). To verify the role of the phosphatase activities of Cdc25B and Cdc25C in these assays, their catalytically inactive mutants Cdc25B (C488S) and Cdc25C (C377S) (referred to as mt25B and mt25C) were generated and were FLAG-tagged at their N termini. When compared with wild type Cdc25B (16%), the ability to suppress colony formation was impaired with the mutant Cdc25B (45%) (Fig. 1, A and C). This result argues that the phosphatase activity of Cdc25B accounts for a significant part of its antiproliferative ability. Nevertheless, the mutant Cdc25B retained some ability to suppress colony formation. This is most likely due to its ability to sequester its substrates and deprive the cell of their functions (6, 15, 34). By contrast, neither the inactive form of Cdc25C nor the wild type protein had any significant effect on the number, size, or shape of the colonies (90 and 110%, respectively) (Fig. 1, B and C). Thus, both the active and phosphatase-dead forms of Cdc25B and Cdc25C differ in their biological effects. Importantly, the phosphatase activity of Cdc25B dramatically enhances its ability to suppress cell growth.

The Catalytic Domain of Cdc25B Is Sufficient to Suppress Cell Viability—The N-terminal ends of Cdc25B and Cdc25C have been implicated in their regulation (14). To elucidate the role of the N terminus of Cdc25B in its antiproliferative activity, chimeras in which the N termini of Cdc25B and Cdc25C were exchanged were generated (25B/C and 25C/B) (Fig. 2). 25B/C consists of residues 1–390 of Cdc25B fused to residues 281–473 of Cdc25C (Fig. 2). The N terminus of 25C/B encompasses residues 1–280 of Cdc25C, and it was fused to residues 391–580 of Cdc25B (Fig. 2). The chimeras were generated by blunt end cloning to avoid possible influence of irrelevant sequences. As controls, constructs expressing only the catalytic domains of Cdc25B (ΔN25B) and Cdc25C (ΔN25C) and their corresponding catalytically inactive mutants were generated (Fig. 2). ΔN25B contains residues 391–580 of Cdc25B and ΔN25C residues 281–473 of Cdc25C. All of the constructs were FLAG-tagged at the N terminus. Each of these constructs or empty vectors was co-transfected with a puromycin-resistant plasmid into HCT116 cells. Twenty-four hours later, the cells were lysed, and expression levels of the proteins were analyzed by immunoblotting using anti-FLAG antibody or specific antibodies against the N or C terminus of Cdc25B or Cdc25C. β-Actin is loading control. A, 25B/C and 25C/B chimeras. B and C, ΔN25B and ΔN25C (8) and their corresponding catalytically inactive mutants ΔN25B/C and ΔN25C/B (C).

was 60% of that of empty vector (set at 100%) (Fig. 4, A and B). This was 4-fold greater than that of full-length Cdc25B (16%), suggesting that the N-terminal region of Cdc25B plays a role in its antiproliferative activity. However, the result with 25B/C was still 1.5-fold less than full-length Cdc25C (90%), arguing

FIGURE 3. Various Cdc25B and Cdc25C proteins are expressed at comparable levels. pcDNA3 vectors expressing the indicated FLAG-tagged proteins or empty vector were transfected into HCT116 cells. Twenty-four hours later, the cells were lysed, and expression levels of the proteins were analyzed by immunoblotting using anti-FLAG antibody or specific antibodies against the N or C terminus of Cdc25B or Cdc25C. β-Actin is loading control. A, 25B/C and 25C/B chimeras. B and C, ΔN25B and ΔN25C (8) and their corresponding catalytically inactive mutants ΔN25B/C and ΔN25C/B (C).
that the N-terminal part of Cdc25C may negatively regulate the activity. The 25C/B chimeras and ΔN25B on the other hand appeared to be more efficient than the full-length Cdc25B in inhibiting cell proliferation because the number of colonies formed following their overexpression were 5 and 0.5% compared with the empty vector (Fig. 4, A and B). These results indicate that the C terminus of Cdc25B is responsible for its ability to inhibit cell proliferation, and the N terminus of Cdc25B when fused to its C terminus negatively regulates this activity. The mtΔN25B reduced the number of colonies (50%), but not as efficiently as the wild type (0.5%), further confirming that the catalytic activity of Cdc25B plays a major role in its ability to suppress cell viability. The ΔN25C and its mutant behaved like the full-length Cdc25C and did not affect colony formation significantly (Fig. 4, A and B). These results also suggest that the N terminus of Cdc25B may also be involved in the suppression of colony formation that resulted from overexpression of 25B/C.

Given the high homology (61% identity) between the C-terminal domains of Cdc25B and Cdc25C, the effect of their overexpression on colony formation was surprising. The C termini of Cdc25B and Cdc25C, however, show much less homology at the extreme C-terminal end (24% identity) (the last 26 residues of Cdc25B and last 29 residues of Cdc25C). Biochemical studies have suggested that the two arginines, Arg556 and Arg562, within the last 17 residues (referred to as a “docking” region) of Cdc25B are important for its recognition and specific interaction with pT14pY15Cdk2/cyclin A (12, 13). To investigate the effect of this putative docking region on the ability of the C terminus of Cdc25B to inhibit cell proliferation, the C-terminal tails of ΔN25B and ΔN25C were exchanged. Constructs were generated by using fusion PCR so that there was no foreign sequence introduced as a linker. These constructs are referred to as ΔNB/C and ΔNC/B (Fig. 2). ΔNB/C consists of residues 391–554 of Cdc25B fused to residues 445–473 of Cdc25C (Fig. 2). ΔNC/B encompasses residues 281–444 of Cdc25C fused to residues 555–580 of Cdc25B (Fig. 2). They were validated by sequencing of constructs and immunoblotting of expressed proteins using either FLAG or specific antibodies against the C termini of Cdc25B or Cdc25C (Fig. 3C). These constructs were used in colony formation assays. Interestingly both ΔN25B/C and ΔN25C/B appeared to be able to suppress cell proliferation when overexpressed (Fig. 4, A and C). The number of colonies formed following overexpression of ΔN25C/B was 14% of that of empty vector. This is 28-fold greater than the number of colonies formed by ΔN25B (0.5%), suggesting a role for the last 26 amino acids of Cdc25B in its antiproliferative ability. The difference between the number of colonies formed by ΔN25C and ΔN25C/B was striking (91 and 22%, respectively) (Figs. 4, A and B, and 1, B and C). These experiments suggest that neither the expression of Cdc25C conferred antiproliferative activity to the catalytic domain of Cdc25C or the last 29 amino acids of Cdc25C negatively regulate its activity.

The N-terminal Portion of Cdc25B Is Dispensable for Its Affinity and Recognition of Its Known Substrates, Whereas Its Last 26 Residues Play a Role in Its Substrate Specificity—Ectopic expression of the C terminus of Cdc25B alone or fused to the N terminus of Cdc25C was more efficient in suppressing cell viability, whereas overexpression of its mutant was not (Fig. 4). This suggests that the C terminus of Cdc25B independently of its N-terminal end is capable of binding and dephosphorylating its substrates. Overexpression of Cdc25C or its derivatives, on the other hand, did not affect colony formation, which would be consistent with a lower affinity for its substrates. To examine this possibility, immunoprecipitation assays were performed. Each of the above constructs or their corresponding inactive mutants or empty vector were transfected into HCT116 cells. The expressed proteins were immunoprecipitated using anti-FLAG antibody. The immunoprecipitates were first immunoblotted for FLAG to verify expression and similar loading (Fig. 5). The presence of the known substrates for Cdc25B and Cdc25C in the immunoprecipitates was investigated. As shown in Fig. 5, mutant Cdc25B, mt25C/B and mtΔN25B formed stable complexes with equal amounts of pT14pY15Cdk1 and pT14pY15Cdk2 (top band of Cdk1 and bottom band of Cdk2) and pY15-Cdk1 (middle band), cyclin B, and cyclin A. Cdk1 is detected as three bands on a SDS-polyacrylamide gel (34). The middle band has been suggested to be phosphorylated only on tyrosine 15 (35). The bottom band is fully dephosphorylated on
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Serine 470 Is Essential for Interaction between Cdc25B and Its Substrates and Its Antiproliferative Activity—Cdc25B contains several serines that are followed by prolines, and as such they are candidates for phosphorylation by the Cdks (37). It was hypothesized that phosphorylation of at least one of these serines would enhance the interaction between Cdc25B and its substrates. Because the C terminus of Cdc25B behaved similarly to the full-length protein in colony formation and immunoprecipitation assays, two serines at positions 416 and 470 were mutated to alanines in both wild type and catalytically inactive Cdc25B. Wild type Cdc25B mutated at either position was referred to as Cdc25B416 and Cdc25B470, respectively. Catalytically inactive Cdc25B mutated at these residues will be referred to as mt25B416 and mt25B470. First, these mutants were used in immunoprecipitation assays. Interestingly, mt25B470 was incapable of forming complexes with either Cdk1 or Cdk2 and their cyclin partners (cyclins B and A) (Fig. 5). The affinity of mt25B416 for its substrates, on the other hand, showed lower affinities for these cell cycle regulators, as evidenced by the barely detectable amounts seen by immunoblotting (Fig. 5). As expected, none of the examined cell cycle regulators were found in immunoprecipitates from cells expressing the wild type forms of the above constructs (Fig. 5).

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Cyclin A

Cyclin B

FIGURE 5. The C-terminal 26 residues of Cdc25B but not its N-terminal region are important for recognition of its substrates. Constructs expressing the indicated proteins, their catalytically inactive mutants, or empty vector were transfected into HCT116 cells. Twenty-four hours later the cell lysates were prepared, and the proteins were immunoprecipitated using anti-FLAG antibody. The immunoprecipitates were immunoblotted to detect the FLAG epitope as well as with specific antibodies against the indicated cell cycle regulators.

FIGURE 6. Integrity of serine 470 is essential for interaction of Cdc25B with its substrates and its antiproliferative effect. A, the catalytically inactive mutants of either wild type Cdc25B, Cdc25B416, Cdc25B470, or empty vector were transfected into HCT116 cells. Twenty-four hours following transfection the cells were lysed, and the proteins were immunoprecipitated using anti-FLAG antibody. The immunoprecipitates were immunoblotted with the indicated antibodies. B, the indicated constructs were co-transfected with a puromycin-resistant plasmid into HCT116 cells. Twenty-four hours later, the cell lysates from these cells were subjected to immunoblot analyses to verify similar expression levels of the proteins using anti-FLAG antibody. β-Actin is a loading control. C, alternatively, the cells were subjected to puromycin selection for 2 weeks, and the colonies were stained with Giemsa. Representative plates are shown. D, bar graph shows the numbers of colonies formed in the presence of each construct as a percentage of that of empty vector. The numbers of colonies in the presence of vector is set at 100%. The average values of three experiments performed in duplicate are shown.

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growth arrest by Cdc25B correlates well with its activity as a tyrosine 15 phosphatase for Cdk1 and Cdk2. Taken together, these results strongly support the notion that Cdc25B inhibits colony formation by a mechanism involving inappropriate activation of its substrates.

DISCUSSION

In this study, biological evidence is provided that confirms biochemical studies comparing activities of Cdc25B and Cdc25C and their chimeras (12–15). It is demonstrated that overexpression of Cdc25B, but not Cdc25C, is detrimental to cell vitality. Furthermore, these data identify serine 470 of Cdc25B as an essential residue for interaction with its substrates, pT14pY15Cdk1/cyclin B and pT14pY15Cdk2/cyclin A. Finally, it is shown that Cdc25B mutated at this residue is also unable to inhibit cell proliferation. The antiproliferative effect of Cdc25B was not specific to HCT116 cells because similar results were obtained using U2OS (osteosarcoma) and HT1080 (fibrosarcoma) cells (data not shown). The level of Cdc25B expression has been suggested to be a critical regulator of its function (6, 9). Thus, alteration in the level of this phosphatase is expected to influence progression through the cell cycle.

The phosphatase activity of Cdc25B is clearly implicated in its ability to suppress cell proliferation. Although the catalytically inactive form retains some ability to inhibit cell growth, the fully active wild type Cdc25B suppresses colony formation to a substantially greater extent (16% versus 45%; Fig. 1, B and C). This is despite the greater ability of the mutant Cdc25B to interact with its substrates (Fig. 5). Thus, the molecular basis for the antiproliferative activity of wild type Cdc25B appears to be mechanistically distinct from that of the mutant form of the phosphatase and is likely to involve inappropriate catalytic activity toward its Cdk substrates. Although counterintuitive, the notion that proteins with oncogenic potential also exert negative effects on cellular growth is not unprecedented. Indeed, a paradigm has been established in which oncogenes such as Myc or Ras not only induce uncontrolled cell proliferation but also trigger so-called “oncogenic” checkpoints that need to be overcome for full transformation to occur (38). The studies presented here suggest that the Cdc25B oncogene should also be considered an example of this.

Previous studies have demonstrated that overexpression of Cdc25C has no effect on cell cycle progression in short term experiments (9, 19, 39). Colony formation assays in this study demonstrate that exposure to elevated levels of Cdc25C over an extended period of time can also be tolerated by cells. In support of this, stable cell lines derived from 293, U2OS, or HT1080 cells that overexpress Cdc25C were easily established. These lines were viable and did not show any alterations in their cell cycle profiles or morphologies even after numerous passages (Ref. 40 and data not shown). In vitro and cellular analyses have suggested that Cdc25C becomes active only after being modified by phosphorylation (9, 14, 39). Thus, the availability or activity of the kinase(s) that phosphorylate Cdc25C could be the limiting factors for its activity.

Removal of the N terminus of Cdc25B enhanced its ability to suppress cell viability (Fig. 4). This is in agreement with previous reports demonstrating that removal of the N terminus of Cdc25B augments its ability to induce mitotic catas-
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troph in cells and increases its activity toward its substrates in vitro (14, 22). This outcome may be due to inappropriate subcellular localization because the N terminus of Cdc25B is known to contain the nuclear import and export signals and binding sites for the 14-3-3 family members, which are known to regulate Cdc25B localization and activity (18, 29, 30). The N-terminal region is unlikely to affect the ability of the catalytic domain to bind to its substrates because similar amounts of pT14pY15Cdk1/cyclin B and pT14pY15Cdk2/cyclin A were found to bind to the mutant forms of full-length or the isolated catalytic domain of Cdc25B in immunoprecipitation assays (Fig. 5).

Similar to Cdc25B, the N-terminal end of Cdc25C contains nuclear import and export signals and binding sites for 14-3-3 proteins (19). In this study, removal of the N terminus of Cdc25C abolished its limited affinity for pT14pY15Cdk1/cyclin B and did not affect colony formation (Figs. 4 and 5). Phosphorylation of serine 216 on Cdc25C and subsequent binding to 14-3-3 family members are believed to negatively regulate its activity (19). Thus, removal of the N terminus of Cdc25C would be expected to stimulate its function. On the other hand, hyperphosphorylation of other sites in this region has been associated with its activation (11, 14).

The C-terminal domain of Cdc25B when expressed alone or fused to the N terminus of Cdc25C had a greater ability to suppress colony formation (Fig. 4), suggesting a negative role for the N terminus in regulating Cdc25B activity. This is in agreement with the in vitro observation showing that the catalytic activity of the bacterially expressed C-terminal domain of Cdc25B toward p-nitrophenyl phosphate, pT14pY15Cdk1/cyclin B, or pT14pY15Cdk2/cyclin A is greater than the full-length Cdc25B, and fusing the N terminus of Cdc25C to the C terminus end of Cdc25B did not affect this activity (14).

The difference between the consequences of overexpression of the C-terminal of Cdc25B and Cdc25C on colony formation was striking considering their high degree of homology. Exchanging the extreme tails from the C-terminal of Cdc25B and Cdc25C, however, significantly affected their ability for substrate recognition and conferred antiproliferative advantage to the catalytic domain of Cdc25C. Affinity of the C terminus domain of Cdc25B for pT14pY15Cdk1/cyclin B was also decreased. The ΔN25C/B chimera, on the other hand, showed an increase affinity for both pT14pY15Cdk1/cyclin B and pT14pY15Cdk2/cyclin A. Given the biochemical studies proposing the last 17 residues of Cdc25B as a modular “docking” site for pT14pY15Cdk2/cyclin A (12, 13), this is likely due to the ability of the last 26 residues of Cdc25B contributing to binding this complex rather than that the last 29 residues of Cdc25C inhibit this interaction. The data presented here suggest that this region is also important for the ability of the C terminus of Cdc25B to bind to pT14pY15Cdk1/cyclin B (Fig. 5). Nevertheless, the possibility that the last 29 residues of Cdc25C have a negative effect on its ability to bind to the examined cell cycle regulators cannot be excluded. Biochemical data, however, are not in favor of this possibility because removal of the last 20 residues of the C terminus of Cdc25C did not affect its activity toward pT14pY15Cdk2/cyclin A (12).

Ectopic expression of the catalytically inactive forms of Cdc25B or its derivatives inhibited colony formation, although not as efficiently as the wild type forms. Immunoprecipitation assays in this study confirms the previously proposed mechanism by which this mutant may prevent cell cycle progression (6, 15). This mutant was suggested to inhibit or delay entry into mitosis by forming a stable complex with pT14pY15Cdk1/cyclin B (6). As seen here, this mutant is also equally capable of binding and sequestering pT14pY15Cdk2/cyclin A in cells, suggesting that overexpression of this mutant may affect progression into or through the S phase as well. Thus, survival of 50% of cell population was surprising. One likely explanation is that levels of the inactive mutant were not high enough to compete with the endogenous wild type protein. In support of this is the observation that the inactive Cdc25B, when expressed at the same level as the wild type protein, did not affect cell proliferation. Indeed, it needs to be expressed at significantly higher levels than the wild type protein to achieve a cell cycle arrest with the majority of the cells accumulated at the G1/M transition (data not shown).

Overexpression of the catalytically inactive Cdc25C has been shown to lead to accumulation of cells in G1/M. In the colony formation assays performed in this study, however, number, size, or shape of colonies formed by cells transfected with this mutant was comparable with those of the control. One possibility is that the catalytically inactive Cdc25C is less efficient at competing with the endogenous protein as compared with Cdc25B and therefore has to be overexpressed at a considerable higher level. Another possibility is a lack of activating phosphorylations on the mutant Cdc25C. Phosphorylation of Cdc25C has been shown to be required for its activation (11, 14). Consistent with the results of the colony formation assays, mutant Cdc25C showed far less affinity for pT14pY15Cdk1/cyclin B relative to mutant Cdc25B in immunoprecipitation assays.

In summary, these results demonstrate key differences in the biological activities of Cdc25B and Cdc25C because of differential substrate affinity and recognition that could explain why Cdc25B, but not Cdc25C, acts as an oncogene. Mutation analysis demonstrates that both the phosphatase activity of Cdc25B as well as its ability to interact with its substrates contributes to the inhibition of cell proliferation. This supports the notion that the ability of Cdc25B to inhibit cell growth is dependent upon its catalytic activity, most likely involving hyperactivation of Cdk2 and inappropriate cell cycle progression. Importantly, this argues that similar to other transforming genes, the antiproliferative activity of Cdc25B needs to be overcome for it to act as an oncogene during tumorigenesis.

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