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Cdc25 Phosphatases and Cancer

Review

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The Cdc25 phosphatases function as key regulators of the cell cycle during normal eukaryotic cell division and as mediators of the checkpoint response in cells with DNA damage. The role of Cdc25s in cancer has become increasingly evident in recent years. More than 20 studies of patient samples from diverse cancers show significant overexpression of Cdc25 with frequent correlation to clinical outcome. Recent screening and design efforts have yielded novel classes of inhibitors that show specificity for the Cdc25s over other phosphatases and cause cell cycle arrest in vivo. Herein we provide a single source for those interested in the cellular functions of Cdc25 in cell cycle progression, its role in the progress of cancer and survival of cancer patients, and recent efforts in the design of specific inhibitors.

Introduction

According to the World Health Organization (2000), approximately 10 million new cases of cancer are diagnosed worldwide each year, causing 6%–12% of all human deaths. Liver, stomach, lung, and breast cancer are among the cancers with the highest morbidity. Although each type of cancer, even individual cases of cancer, can arise from diverse causes and can exhibit various traits, common features shared by all cancers are a disordered cell cycle and irregularities such as deletions, overexpression, or mutations in the molecules that control this cycle. The Cdc25 phosphatases are cell cycle control proteins whose overexpression is frequently associated with a wide variety of cancers. In this review of the Cdc25 phosphatases, we first consider the role of Cdc25 in cell cycle control and oncogenic transformation, and then discuss 20 different reports of Cdc25 overexpression in human cancers. We finish by reviewing recent progress toward inhibiting the Cdc25 phosphatases using small molecule inhibitors.

Cdc25 and Cell Cycle Control

The Cdc25 phosphatases are key for cell cycle control in eukaryotes under normal conditions and in response to DNA damage (reviewed in [1]). The physiological substrates of the Cdc25 phosphatases are the cyclin-dependent kinases (Cdk/cyclins), the central regulators of the eukaryotic cell cycle (Figure 1). The Cdk/cyclins are subject to numerous counteracting control mechanisms, including association with inhibitory proteins such as $p15^{Ink4b}$/$p16^{Ink4a}$ or $p21^{Waf1}$/$p27^{Kip1}$, activating phosphorylations on Thr160/161 of the T-loop by the Cdk-activating kinase, and inhibitory phosphorylation on Thr14 and Tyr15 by the Wee1 and Myt1 kinases [2]. The three human Cdc25s, Cdc25A, Cdc25B, and Cdc25C, are responsible for the dephosphorylation of pThr14 and pTyr15 and thereby trigger the final activation of the Cdk/cyclin complexes. Cdc25B and Cdc25C are regulators of G2/M through their activity on Cdk2/cyclin A, Cdk1/cyclinA, and Cdk1/cyclin B (Figure 1). Cdc25A has a more general role in controlling both the G1/S and G2/M transitions [3]. Positive and negative feedback activation loops between the Cdk/cyclins and the Cdc25s ensure abrupt and irreversible transitions during normal cell cycle progression.

The Cdc25 phosphatases also play a key role in integrating the specific signals of checkpoint control in response to damage by ionizing irradiation (IR), ultraviolet light (UV), replication inhibitors, and DNA damaging agents at each of the stages of the cell cycle (Figure 1) [4]. In response to IR, cells undergo rapid G1 arrest as a result of ubiquitin/proteasome-mediated degradation of Cdc25A and consequential maintenance of Cdk2/cyclin E in the phosphorylated and inactive state. This degradation of Cdc25A is triggered by Chk1 phosphorylation of Cdc25A as mediated through the ATM (ataxia-telangiectasia-mutated) pathway. Similarly, the response to UV irradiation and replication inhibitors also causes Cdc25A degradation, this time through Chk1 phosphorylation and the ATR (ATM- and Rad3-related) pathway. In the S-phase checkpoint response to IR, Cdc25A integrates the signals from the ATM-Chk2 and ATR-Chk1 pathways to yield a rapid and sustained cell cycle arrest, again leading to the preservation of Cdk2 in the inactive state. In the G2/M checkpoint, Cdc25A and Cdc25C become important downstream effectors of the Chk1 response. Phosphorylation of Cdc25A on Thr507 and Cdc25C on Ser216 leads to 14-3-3-mediated sequestration of the phosphatases away from their substrates and consequential maintenance of Cdk1/cyclin B in the phosphorylated and inhibited state. Cdc25B and the polo-like kinases PLK1 and PLK3 have also been implicated in the G2/M checkpoint response.

Cdc25 Structure and Mechanism

The human Cdc25s are between 423 and 566 amino acids long. The N-terminal regulatory domains have low sequence homology (20%–25% identity) and contain sites for phosphorylation, sequestration by 14-3-3, ubiquitination, and proline isomerization. Modifications at these sites are involved in both normal cell cycle control and in response to checkpoint signals. There exist at least three splice variants for Cdc25A, five for Cdc25B, and five for Cdc25C [5]. The question of splice variants is of specific importance for Cdc25B, as the most active

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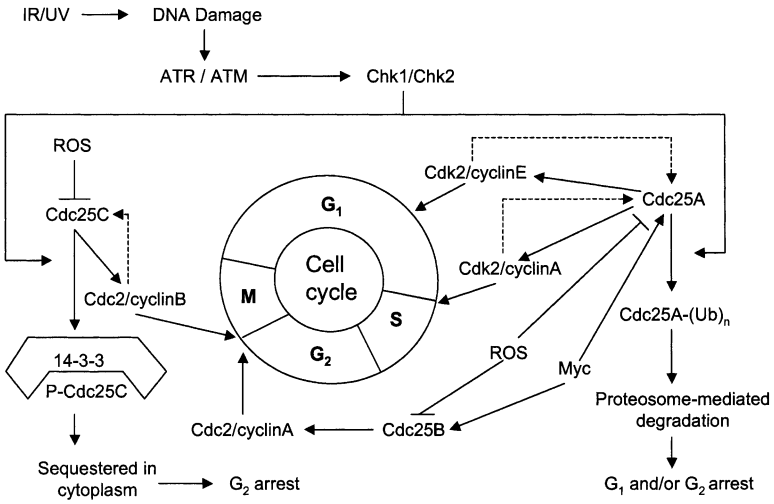

Figure 1. Cdc25s Are Involved in a Complex Web of Cellular Interactions Wherein They Function to Regulate Cell Cycle Progression under Normal Cell Growth and in Response to DNA or Oxidative Damage

of the splice forms (Cdc25B2) is the only variant detected in primary fibroblasts, whereas at least three different splice forms are found in immortalized fibroblasts [6]. As most studies do not differentiate between the different splice variants, little is known about the relative importance of the different forms. The catalytic domains are located in the more homologous C termini (~60% identity) and are able to dephosphorylate protein substrates as rapidly as full-length protein [7]. The catalytic domains contain the CX_5R motif common to all protein tyrosine phosphatases, where C is the catalytic cysteine and the amide backbones of the five X residues form a phosphate binding loop along with the arginine R (Figure 2). Until recently, the catalytic domains were not thought to undergo covalent modifications. However, in Cdc25A, Chk1 phosphorylation of Thr507 within the C-terminal substrate-docking tail is thought to govern 14-3-3 and cyclin B binding in the control of mitosis [8]. Also, oxidation of the active site cysteine by reactive oxygen species may be involved in the checkpoint response to oxidizing conditions in the cell [9–11].

The structures of the catalytic domains of Cdc25A [12] and Cdc25B [13] have been solved by X-ray crystallography. As suggested by the lack of sequence conservation outside of the CX_5R motif, the overall fold of the Cdc25s differs from other protein tyrosine phosphatases. The active site loop, however, is superimposable on the active sites of other diverse protein tyrosine phosphatases (Figure 2). A key feature of the Cdc25s is their lack of a deep active site pocket (Figure 3). There exists no obvious groove for binding protein or small molecule substrates, consistent with the lack of activity or specificity toward peptidic substrates [7]. It is presumed that a remote binding site mediates specific recognition of protein substrate.

The mechanism of the Cdc25 phosphatases has many similarities to the well-established mechanism of the protein tyrosine phosphatases, in particular the dual-specificity phosphatases (Figure 4) [14]. The active site cysteine exists as a thiolate with a pK_a of 5.9 [15, 16] and forms a transient covalent intermediate consisting of a phospho-cysteine. Para-nitrophenyl phosphate is a poor substrate ($k_{cat}/K_m = 15\ M^{-1}s^{-1}$) and probes the first half of the reaction, whereas the rate determining step is breakdown of the phospho-enzyme intermediate for the better substrate *O*-methyl fluorescein phosphate

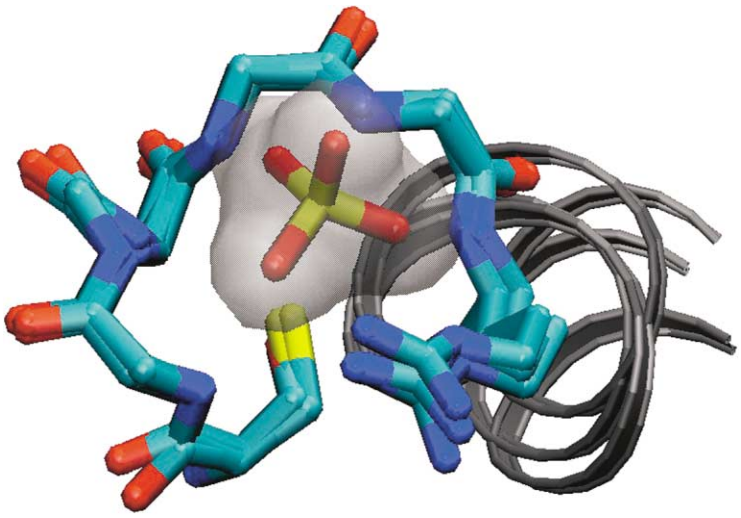

Figure 2. The Active Site Loop of Cdc25B Overlays with the Active Site Loops of the Human Vh1-Related Dual-Specificity Phosphatase and the *Yersinia* Protein Tyrosine Phosphatase, Despite Having Different Overall Protein Folds

The amide backbone and the arginine of the active site loop form hydrogen bonds with the bound sulfate that mimics phosphate binding. The beginning of a conserved α helix that provides a favorable dipole for maintenance of the cysteinyl anion is also shown. The figure was generated using VMD from Protein Data Bank ID codes 1qb0, 1vhr, and 1yts, and only the side chains of the active site cysteine and the arginine are shown for clarity.

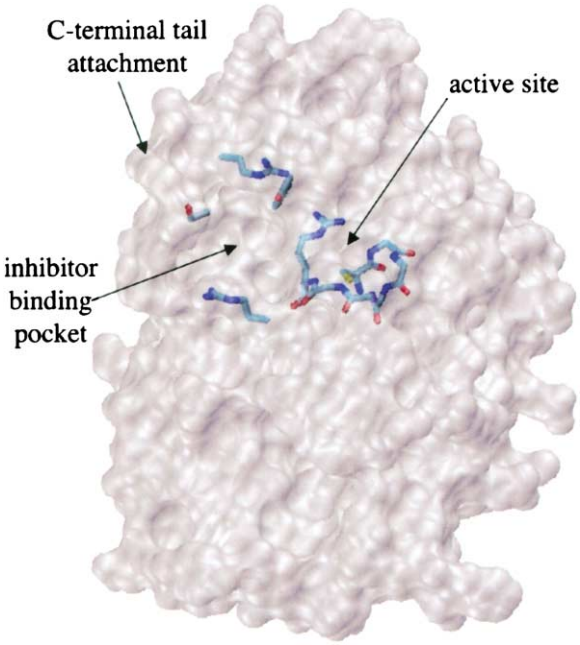

Figure 3. The Active Site of Cdc25B Is Adjacent to an Inhibitor Binding Pocket and the Attachment Site of the C-Terminal Tail

Shown is the active site surface of Cdc25B with key features in licorice, such as the active site loop, the residues that surround the inhibitor binding pocket adjacent to the active site (R482, R544, and T547), and the attachment point of the C-terminal tail, which is not visible in the crystal structure. The figure was generated using VMD from Protein Data Bank ID code 1qb0.

($k_{cat}/K_m = 10^4\ M^{-1}s^{-1}$). The bis-phosphorylated protein substrate Cdk2/CycA is by far the best substrate for Cdc25 ($k_{cat}/K_m = 10^6\ M^{-1}s^{-1}$). For the protein substrate, phospho-threonine is preferentially dephosphorylated, whereas phospho-tyrosine is preferred in the poorly utilized peptidic substrates ($k_{cat}/K_m = 1 - 10\ M^{-1}s^{-1}$) [7]. The origin of the catalytic acid that protonates the leaving group has not been unambiguously established. The glutamate adjacent to the catalytic cysteine (Glu432 in Cdc25A and Glu474 in Cdc25B) has been suggested to serve this role using small molecule substrates [17] but has been ruled out using the protein substrate [15, 16]. There is evidence that the catalytic acid resides on the protein substrate itself, either on an amino acid side chain or on the phosphate of the phospho-threonine. This proposed substrate-assisted catalysis would help explain the high specificity for, and reactivity with, the correct protein substrate.

Cdc25 and Oncogenic Transformation

From their initial discovery in humans, Cdc25s have been linked to oncogenic transformation [18]. Coexpression of oncogenic mutants of H-Ras with human Cdc25A or Cdc25B but not with Cdc25C in normal mouse embryo fibroblasts led to formation of transformed foci. Cells from individual foci grew readily in soft agar and induced tumor formation in nude mice. Expression of Cdc25A alone in retinoblastoma-deficient but not p53-deficient fibroblasts also lead to foci formation, growth in soft agar, and tumor formation in mice.

Subsequent studies in a number of model systems have since confirmed an oncogenic role for the Cdc25 phosphatases, although overexpression of Cdc25B in the mammary glands of mice has yielded conflicting data. In one study, enhanced proliferation of mammary epithelial cells resulted in the formation of precocious alveolar hyperplasia [19]. Using a similar overexpression strategy, another study saw no changes in mammary cells or elsewhere; however, the mice had an increased susceptibility to carcinogen-induced mammary tumors [20]. The oncogenic nature of the Cdc25 phosphatases has also been found in *C. elegans*, where a gain-of-function allele of the *cdc-25.1* gene causes excess proliferation of intestinal cells, and its reduction by RNAi leads to reduced proliferation of a variety of cell types [21]. Interestingly, the mitotic *cdc25C*$^{-/-}$ mouse is viable, develops normally, and does not display any obvious abnormalities [22]. The *cdc*25B$^{-/-}$ mouse is also viable and responds normally to DNA damage, although the females are sterile, as their oocytes are unable to undergo meiosis [23]. In both cases, it is speculated that the missing Cdc25 function may be complemented by one of the remaining Cdc25s.

Cdc25 Overexpression

Here, we summarize and comment on the techniques and results of the Cdc25 overexpression studies [18, 24–42]. Over half of the 15 different cancers studied (taken together) showed overexpression of both Cdc25A and Cdc25B isoforms (Table 1). The remainder showed overexpression of either Cdc25A or Cdc25B, and there was no significant overexpression of Cdc25C. We then discuss the mechanism and consequences of Cdc25 overexpression in human cancers.

Experimental Techniques

A total of 20 studies spanning from 1995 to 2004 that investigated the overexpression of Cdc25 phosphatases in human tumor samples were reviewed (Table 1). Cdc25A, Cdc25B, and Cdc25C overexpression was specifically addressed in 15, 19, and 10 of these studies,

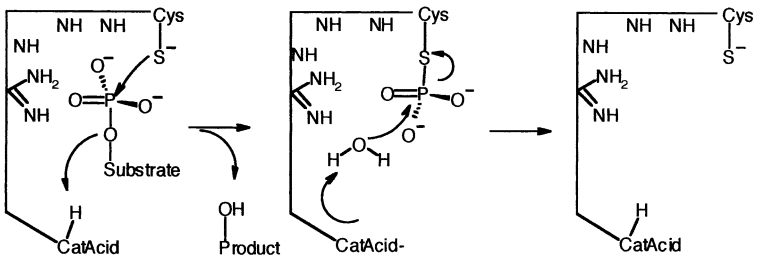

Figure 4. Two-Step Reaction Mechanism of the Cdc25 Phosphatases with a Covalent Phospho-Cysteine Intermediate

The identity of the catalytic acid has not been firmly established.

Table 1. Cdc25 Overexpression Is Found in Many Different Human Cancers

Citation	Type of Cancer	N Pts[a]	Cdc25s		Techniques[b]	Correlation between Overexpression and:	
			Studied	Overexpressed		Survival (A/B/C)	Other Factors
[24]	hepatocellular carcinoma	59P	A, B	A (78%)	IHC, WB, RT-PCR	yes/no/– (relapse risk 3-fold)	poor differentiation, fast growth
[25]	prostate cancer	30P	B	B (97%)	IHC	–/–/–	poor differentiation
[26]	esophageal squamous cell carinoma	61/16	B	B (79%)	IHC, cDNA, RT-PCR	–/–/–	no
[27]	esophageal squamous cell carcinoma	100	A, B	A, B (46%, 48%)	IHC, WB, RT-PCR	yes/no/– (76:33)[d]	tumor size
[28]	esophageal squamous cell carcinoma	47	A, B, C	A, B (40%, 17%)	IHC	–/–/–	deep infiltration, sensitivity to radiation
[29]	breast cancer	144P	A	A (47%)	IHC, Ptase, WB, RT-PCR	yes/–/– (24:5)[d]	no
[18]	breast cancer	124	A, B, C	B (32%)	AS riboprobes	–/yes/– (37:19)[f]	histological stage, microvessel density, nuclear atypia
[30]	colorectal carcinoma	34P	A, B, C	A, B (53%, 67%)	WB, NB, RT-PCR	no/yes/– (73:30)[e]	poor differentiation
[31]	colorectal carcinoma	181	A, B	A, B (47%, 43%)	IHC, WB, RT-PCR	no/yes/– (41:18)[d]	tumor size, distant metastasis
[32]	pancreatic ductal adenocarcinoma	48/24	A, B, C	B (72%)	IHC, cDNA, WB, RT-PCR, NB	–/–/-	metastatic
[33]	head and neck cancers	20	A, B, C	A, B (80%, 50%)	RT-PCR	–/–/-	–
[34]	gastric carcinomas	55	A, B, C	B (78%)	IHC, WB, NB	–/–/-	tumor stage, invasiveness, nodal metastasis, differentiation
[35]	nonsmall cell lung cancer	40P	A, B, C	A, B (60%, 45%)	RT-PCR	–/–/-	poor differentiation
[36]	nonsmall cell lung cancer	55	B	B (40%)	RT-PCR, IHC	-/yes/- (29:5)[c]	no
[37]	ovarian cancer	106	A, B, C	A, B (30%, 30%)	IHC, RT-PCR	yes/yes/- (50:30)[c]	–
[38]	thyroid neoplasms	172	A, B	A, B (65%, 61%)	IHC	no/no/-	high differentiation
[39]	non-Hodgkins lymphoma	63	A, B, C	B (56%)	NB, SB	–/–/-	cell proliferation, aggressive
[40]	non-Hodgkins lymphoma	89/9	A, B, C	A, B (35%, 39%)	RT-PCR, WB	–/–/-	aggressive tumor
[41]	neuroblastoma	20	B	B (85%)	RT-PCR, IHC	-/no/-	no
[42]	endometrial carcinoma	57/4	B	B (73%)	WB, RT-PCR	–/–/-	–

[a] N Pts, number of patients in study; P, paired: one tumor sample, one normal sample from surrounding tissue in same patient; x/x, tumor/normal tissue samples (not same patient).

[b] IHC, immunohistochemistry; WB, Western blot; RT-PCR, reverse transcriptase-polymerase chain reaction; cDNA, cDNA array analysis; Ptase, phosphatase activity assay; AS riboprobes, antisense riboprobes; NB, Northern blot; SB, Southern blot. Overexpression rates and mortality rates based on first mentioned technique.

[c] 3 year percentage mortality, high:low Cdc25 expression.

[d] 5 year percentage mortality, high:low Cdc25 expression.

[e] 7 year percentage mortality, high:low Cdc25 expression.

[f] 10 year percentage mortality, high:low Cdc25 expression.

respectively. The studies represent 14 types of human cancer and account for a majority of all cancer deaths. The total number of patients included in these studies was 1558 (range 20–181, mean 68). The diversity of cancers and patient populations make direct comparisons between studies essentially impossible. On the other hand, the consistent overexpression of Cdc25 testifies to the role of Cdc25 and its value as a marker, prognosticator, and important target for inhibition by small molecules.

In evaluating these Cdc25 overexpression studies, it is important to consider the experimental techniques employed. First, it is not expected that levels of mRNA, protein concentration, and protein activity will necessarily correlate. This is particularly true for the Cdc25 phosphatases in the context of cancerous cells, as the process of immortalization perturbs many of the mechanisms of post-translational modification known to affect Cdc25 phosphatases (see above). Second, from an experimental viewpoint, multiple techniques are valuable toward verifying results and eliminating bias. Commendably, we found that 14 of the 20 studies utilized multiple approaches to confirm overexpression of the Cdc25s.

The most common technique employed in the reviewed studies was immunohistochemistry (IHC). The strength of this technique is that it preserves the tissue architecture and thus allows identification of protein expression levels in cancerous cells compared to surrounding tissues, including intracellular localization. Strongly linking Cdc25 overexpression directly with cancer, both Cdc25A and Cdc25B staining in these 22 studies is found exclusively in cancerous tissues, with the exception of [32], where Cdc25B is also seen in the surrounding dedifferentiating cells and some fibroblasts. Cdc25A is found to be nuclear, except in [37], where it is mostly cytoplasmic, and in [27] and [38], where it is mixed. In contrast, Cdc25B is mostly cytoplasmic, with the exception of [17], where it is nuclear, and [26] and [32], where it is both. The problems with IHC arise from the variable percentage cut-offs used in determining overexpression, ranging from 10% [26, 34] to 75% [31, 37], with a more typical value of 50%. Also, there exist differences in antibody dilutions and sources (Santa Cruz and Cell Signaling were the most common suppliers), as well as tissue preparation and preservation.

The second most common technique employed is reverse transcription polymerase chain reaction (RT-PCR). Given the low levels of mRNA for the Cdc25 phosphatases, the increased sensitivity of RT-PCR over Northern blotting is essential to their detection (note the direct comparison in [30, 40]). Also, if properly designed, RT-PCR also allows for the detection of alternative splice variants, of which many have been found to exist, as noted above. Importantly, a high level of a constant splice variant can obscure changes in the low level of a different splice variant [40]. On the other hand, mRNA levels do not necessarily reflect protein levels for the Cdc25s and may not reflect phosphatase activity, as the splice variant Cdc25C2 lacks the catalytic domain.

Other techniques used include Western blotting (WB), anti-sense riboprobes, Northern blots (NB), phosphatase assays, and cDNA arrays. Of course, activity assays are of greatest relevance, but are difficult due to issues of substrate specificity (only phosphorylated Cdk/cyclin complexes should be used), low cellular levels of Cdc25, and the sensitivity of the phosphatase to mild oxidation during sample preparation.

Cdc25 Overexpression Results

From the initial observation of Cdc25B overexpression in breast cancer [18] to the more recent and very thorough study in [29] and the direct measurement of phosphatase activity in patient-derived cell lines [43], there has been a clear and consistent trend associating Cdc25 with breast cancer. These studies also consistently show that Cdc25 is overexpressed in only a subpopulation (32%–47%). Importantly, the overexpression of Cdc25A or Cdc25B in breast cancer correlates with clinical outcome, as is the case for ovarian [37] and colorectal cancer [31]. Cdc25A and Cdc25B are also both overexpressed in a subpopulation of non-Hodgkin's lymphoma [39, 40]. Although correlation with mortality was not reported, overexpression did correlate strongly with aggressive high-grade lymphomas. Esophageal [26–28], gastric [34], lung [35], thyroid [38], and head and neck cancers [33] also show overexpression of Cdc25A (40%–80%) and Cdc25B (17%–78%).

Cancers such as hepatocellular carcinoma [24] that specifically overexpress only Cdc25A appear to be rare. Pancreatic ductal carcinoma [32] and gastric carcinomas [34] are the only two types of cancer for which exclusive Cdc25B overexpression has been seen. The Cdc25C phosphatase appears to be a conundrum. Given its prominent role in promoting the G2/M transition [1] and its role in S phase in human cells [44], it was expected to play an important role in cancer progression. However, consistently from the first study in breast cancer [18], significant overexpression of Cdc25C has not been associated with any of the nine different cancers that have been tested.

In general, although both Cdc25A and Cdc25B are overexpressed in a majority of the cancers studied, a correlation between Cdc25A and Cdc25B overexpression has not been observed [27, 33], except in the study of non-Hodgkin's lymphoma [40]. In that study, 64% of the patients with high levels of Cdc25A also overexpressed Cdc25B. Higher levels of both were seen in 50% of aggressive tumors and in less than 4% of indolent tumors. Thus, although either Cdc25A or Cdc25B is sufficient to drive cell cycle progression in cancerous cells, the two can collaborate in more aggressive cancers. It should be cautioned, however, that overexpression of Cdc25B in many of these studies may be underestimated, and correlations between Cdc25A and Cdc25B may have been overlooked due to the nondiscrimination between splice variants of Cdc25B [30]. In the future, it will be necessary to examine the levels of the different splice variants and their actual cellular activity in greater detail.

Mechanism of Overexpression

The mechanism of Cdc25 overexpression is not clear despite numerous attempts to shed light on this important question. It is evident from studies in colorectal [30], gastric [34], non-Hodgkin's [39], nonsmall cell lung [35], and ovarian cancers [37] that gene amplification is not the cause of Cdc25 overexpression. The role of the

proto-oncogene and transcription factor c-Myc has been controversial. It was originally shown that both *cdc25A* and *cdc25B* were targets of c-Myc, as they both contain functional binding sites for Myc/Max [45]. Since then, many of the overexpression studies have looked for a correlation between c-Myc and Cdc25 expression. A positive correlation has been detected in non-Hodgkin's lymphoma, although other mechanisms of Cdc25B upregulation must exist, as the correlation was not 100% [39]. A positive correlation has also been reported for neuroblastoma [41] and for nonsmall cell lung in one study [36] yet not another [35]. There is also no correlation between Cdc25A and c-Myc in melanoma from patient-derived cell lines [46]. Given the presence of Myc target sites in both *cdc25A* and *cdc25B*, a correlation between phosphatase expression and Myc should also reveal a correlation between the two phosphatases. As noted above, this is rarely seen, although it may be underdetected due to the splice variant problem. An alternative mechanism of overexpression has been suggested wherein post-translational modification leads to an enhanced stability of Cdc25A [43]. Thus, the mechanism of Cdc25 overexpression, in most cases, is an important open problem, and its further elucidation may reveal novel anticancer targets.

Role of Cdc25 Overexpression in Cancer

As Cdc25 phosphatases promote cell cycle progression and are overexpressed in numerous rapidly dividing cancer cells, one might expect a correlation between Cdc25 overexpression and the rate of proliferation. In fact, no correlation has been seen in the majority of cases examined [28–31, 35, 38]. The only case with a reported correlation was non-Hodgkin's lymphoma [39], where 74% of cells with high levels of Cdc25B were found in S phase, compared to only 20% in those cells with low levels of Cdc25B. Thus, the role of Cdc25 overexpression is more complicated than that of a simple driver of cell proliferation. It is quite likely that Cdc25 overexpression in tumors is required to circumvent many of the checkpoints that would otherwise hinder cell proliferation. In support of this view, a lack of response to ionizing radiation was seen in breast cancer cell lines overexpressing Cdc25A [43], similar to the results for overexpression of Cdc25B in esophageal cancers [28]. Absence of a proper checkpoint response causes premature entry into the G2/M transition, leading to inappropriate distribution of chromosomes and aneuploidy. The lack of proper checkpoint control contributes to the malignant nature of the tumors, as noted in many of the overexpression studies.

Therapeutic Potentials

The therapeutic strategy prescribed by the strong link between Cdc25 and cancer has been to pursue the development of specific Cdc25 inhibitors. Although the shallow active site and the high reactivity of the catalytic cysteine of the Cdc25s suggests that this may be a difficult goal, a number of recent publications demonstrate that progress is being made. The detailed chemistry of many small molecule inhibitors of Cdc25 has recently been well reviewed [5, 47]. The inhibitors are derived from many diverse chemical classes, and some

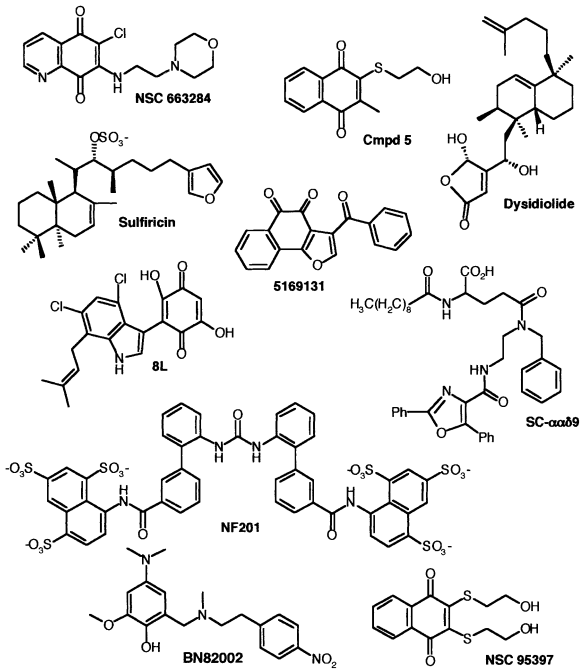

Figure 5. Representative Examples of a Number of the More Well-Studied or Recently Identified Inhibitors of the Cdc25 Phosphatases

of the more well-studied compounds include Vitamin K_3 and its derivatives, the dysidiolides, sulfiricins, quinolinediones, and naphthoquinone (Figure 5). Vitamin K_3 (menadione) and its thioalkyl derivatives (e.g., compound 5) have IC-50s of 1–15 μM and covalently modify Cdc25 phosphatases by arylation. In cell-based assays, these compounds lead to G1/S and G2/M arrest, consistent with inhibition of Cdc25s, although their potency may be diminished through reduction by quinone reductase [48]. The natural product dysidiolide and many subsequent derivatives have been probed as potential Cdc25 inhibitors (IC-50s of 0.8–16 μM), although the potency of the parent compound has been questioned. Sulfiricin, another natural product, has been investigated by extensive preparation and testing of derivatives (IC-50s of 2–9 μM), yet it shows no selectivity for Cdc25 over other tyrosine phosphatases.

A number of novel inhibitors have been discovered through a collaborative effort at the University of Pittburgh. SC-ααδ9 is a competitive inhibitor of the Cdc25s (IC-50 of 15 μM), blocks cells in G1 and G2/M, leads to increased phosphorylation of multiple Cdk/cyclin complexes, and is cytotoxic to breast carcinoma cells. The hydrophobicity of the side chain and the aromatic moiety on the oxazole ring appear to be the critical components for Cdc25 inhibition. The quinolinediones, originally identified from the NCI Diversity Set, have been extensively characterized. Compound NSC663284 has an IC-50 of 200 nM and a 20-fold specificity toward Cdc25B versus the Vh1-related dual specificity phosphatase (VHR). NSC663284, like compound 5, also covalently modifies the active site of Cdc25, surprisingly, through one of the serines, not the cysteine [49]. In cell-based assays, NSC663284 causes G2/M arrest, and, unlike the

Vitamin K derivatives, its potency is not affected by cellular quinone reductase concentrations [48]. The para-naphthoquinone NSC95397 is the most potent Cdc25 inhibitor to date (IC-50s of 22–125 nM). Again, this compound blocks the G2/M transition and shows growth inhibition against human carcinoma cell lines.

New inhibitor classes not previously reviewed include the indolyl-dihydroxy-quinones [50], the suramins [51], BN82002 [52], and the cyclopentaquinoline and napthofurandione inhibitors [53] (Figure 5). From the indolyl-dihydroxy-quinones, compound 8L is a competitive inhibitor, with a K_i of 0.43 μM against Cdc25B and appears to bind in the pocket adjacent to the active site (Figure 3). Inhibition by these compounds requires the C-terminal tail, shown previously to be involved in protein substrate recognition [54]. Compound 8L shows activity when challenged with the protein substrate, and treatment of HEK293 cells with 50 μM of compound 8L causes rapid apoptosis by an unknown mechanism. The suramin derivatives NF201, NF336, and NF339 have IC-50s from 2–4 μM and show 20- to 50-fold specificity over a series of other human phosphatases but have not been tested in cell-based assays. BN82002 has an IC-50 of 5.4 μM and has been shown to inhibit cell cycle progression in synchronized HeLa cells. Impressively demonstrating that Cdc25 is the likely intracellular target, BN82002 is able to reverse the premature chromosome condensation caused by transient overexpression of Cdc25B in HeLa cells. The newest and most interesting compounds discovered in Lazo's laboratory are the napthofurandiones (5169131) [53]. These compounds are competitive versus small molecule substrates, and docking studies suggest that they also bind in the pocket adjacent to the active site (Figure 3). In cell-based assays, 5169131 leads to both G1/S and G2/M arrest and causes a concomitant increase in the inhibitory phosphorylation of Cdk1.

There exists some discrepancy in the reported potencies of a number of Cdc25 inhibitors in vitro, which most likely arises from two causes. First, Cdc25 is particularly susceptible to nonspecific inhibitors that exhibit enzyme-concentration-dependent IC-50s (unpublished observations; see also [55]). Thus, the presence or absence of detergents, the clog p value of the compound, and the absolute concentration of enzyme can greatly alter the measured potency. Second, the highly reactive cysteine at the active site of the Cdc25s is particularly susceptible to covalent modification by many classes of compounds, and therefore inhibition constants will vary depending on assay conditions. For example, the Vitamin K derivatives [56], the quinolinediones [49], and BN82002 [52] all modify residues within the active site loop, although, surprisingly, not always the active site cysteine.

The real difficulty in Cdc25 inhibitor studies lies in demonstrating specific targeting of Cdc25 in cell-based experiments. The presence of three potentially complementing isoforms has precluded the clear delineation of a Cdc25 inhibition phenotype in higher eukaryotes using mouse knockout studies, siRNA, or antisense DNA. Based on the known biology described above, it is expected that a pan-Cdc25 inhibitor would block cell cycle progression. Thus, good cell-based assays will go beyond antiproliferation assays and evaluate the effect of compounds on synchronized cells, as done, for example, with the tsFT210 cell line [49] or synchronized HeLa cells [52]. Even better, a direct effect on the intracellular substrates of Cdc25 can be shown by increased inhibitory phosphorylation on the Cdk/cyclins (see for example [49, 52, 57]). However, as many different pathways feed into cell cycle control (Figure 1), non-Cdc25-specific cellular insults are also expected to cause G1/S or G2/M arrest. For example, cellular stress mediated by the p38 and Jnk kinases determines whether a cell progresses through the cell cycle, enters senescence, or undergoes apoptotic cell death, and thus inhibitors of dual-specificity phosphatases involved in MAP kinase control (e.g., MKP-3) can also yield cell cycle arrest phenotypes. Specifically, NSC663284, described as a Cdc25 inhibitor, was recently rediscovered in a screen for inhibition of ERK dephosphorylation [58]. Also, the promiscuity of the thioalkyl derivatives of Vitamin K is evident in the pull-down assays with biotinylated compound 5, which show a large number of other potential targets that remain to be characterized [56].

Based on Cdc25s effects on checkpoints in tumors, not the rate of cell proliferation, a better measure of Cdc25 inhibition in the context of human might be to test for compound-dependent restoration of the checkpoint response in cell lines overexpressing Cdc25 [43]. Alternatively, reversal of the mitotic effect caused by transient overexpression of Cdc25B in HeLa cells appears to be a novel cell-based assay with specificity for Cdc25 [52]. These alternative assays are difficult to implement in large-scale screening but are important in follow-up assays of interesting lead molecules, given the sensitivity of cell cycle control to multiple signaling pathways. Recently, a novel yeast-based screening method has been described that may be useful for discovering novel inhibitors, though not addressing any of the complications of human cells [59].

A completely alternative approach to Cdc25 overexpression, as suggested by some clinical correlations [28], is to aggressively treat Cdc25-overexpressing subpopulations with radiation therapy. Normal radiation-induced cell cycle arrest is abrogated in these tumors, and therefore radiation treatment results in apoptosis of the cancerous cells. From this point of view, an activator of Cdc25 activity could be a useful radiation- or chemo-sensitizer in tumors that do not overexpress Cdc25. The daunting task of developing activators of dual-specificity phosphatases has not yet been pursued. On the other hand, an increase in Cdc25 activity can also be attained by inhibition of a negative regulator of Cdc25 (Figure 1). To this end, inhibitors of Chk1 have shown good promise. For example, UCN-01 and the indolocarbazole SB-218078 are nanomolar inhibitors of Chk1 and enhance the cytotoxicity of DNA damage by abrogating the G2/M checkpoint response [60, 61]. Clinical trials combining UCN-01 with various chemo-sensitizers, such as prednisone, irinotecan, fluorouracil, and cis-platin, are underway.

Future Directions

Although much has been learned, the future of research in the Cdc25 field promises many exciting advances.

Analyses provided by cDNA arrays in both cell biology and patient studies should shed more light on the complex interactions that govern cell cycle control. Crystal structures of inhibitor-bound Cdc25s will allow major strides in the design of potent and specific inhibitors starting from some of the attractive leads discovered to date. Additionally, the elucidation of the detailed protein interactions that govern substrate recognition will pave the way for protein-protein interaction inhibitors that take advantage of Cdc25's specificity for the Cdk/cyclins.

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