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Forum Review

Redox Regulation of the Cdc25 Phosphatases

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ABSTRACT

The Cdc25 phosphatases are essential for cell-cycle control in eukaryotes under normal conditions and in response to DNA damage via checkpoint controls. Recent evidence indicates direct control of the Cdc25s, and therefore the cell cycle, in response to changes in cellular redox status. These redox changes may originate intracellularly from mitochondrial leakage or in response to specific external triggers leading to production of reactive oxygen species (ROS). This review shows that the known chemistry and biology of the Cdc25s favor a direct role for these phosphatases in temporarily blocking cell-cycle progression until favorable reducing conditions are restored. First, the Cdc25s contain a highly reactive cysteine at the active site that can react directly with ROS, leading to enzyme inactivation. Second, the ROS-inactivated form of Cdc25 is expected to prevent cell-cycle progression based on precedent from cellular responses to DNA damage. Third, ROS-mediated oxidation of the Cdc25s leads to an intramolecular disulfide that is readily reversible by the cellular reductant thioredoxin. Finally, *in vivo* data supporting a direct role for the Cdc25s in redox regulation are considered. *Antioxid. Redox Signal.* 7, 761–767.

INTRODUCTION

The Cdc25 phosphatases are essential for cell-cycle control in eukaryotes under normal conditions and in response to DNA damage. Recent evidence indicates direct control of the Cdc25s, and therefore the cell cycle, in response to changes in cellular redox status. These redox changes may originate intracellularly from mitochondrial leakage or in response to specific external triggers leading to production of reactive oxygen species (ROS). Thus, transient oxidation of the Cdc25s is proposed to halt cell-cycle progression temporarily until favorable reducing conditions are restored. If this is to be a reasonable proposal, the following criteria must be met: (a) The Cdc25s must contain a highly reactive functional group that can react rapidly and directly with ROS. (b) The consequences of Cdc25 modification by ROS must yield significant downstream effects. (c) There must exist a means of reversing ROS-mediated oxidation of the Cdc25s.

This review considers these three requirements in light of the known chemistry and biology of the Cdc25 phosphatases and finishes with a survey of *in vivo* data supporting such a direct role for the Cdc25s in redox regulation.

Cdc25 PHOSPHATASES CONTAIN A HIGHLY REACTIVE ACTIVE-SITE CYSTEINE

The Cdc25s belong to the dual-specificity phosphatase family, a subfamily of the protein tyrosine phosphatases (PTPs) (37). This family is defined by the active-site motif HCX_5R (Fig. 1). In this motif, H is a conserved histidine residue, C is the catalytic cysteine, the five X residues form a loop whose backbone amides hydrogen-bond to the phosphate of the substrate, and R is a highly conserved arginine that is required for binding of the phosphate and stabilization of the transition state. The catalytic cysteine (Cys^{431} in Cdc25A, Cys^{473} in Cdc25B, Cys^{377} in Cdc25C) forms a covalent phosphocysteine intermediate in a two-step reaction mechanism and exists as a thiolate anion in the free enzyme

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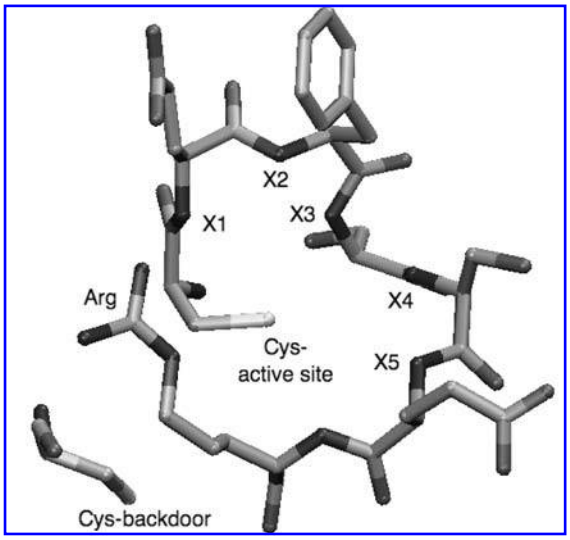

FIG. 1. Schematic of the active-site loop of Cdc25B generated from pdb file 1qb0 (61). The backdoor cysteine is positioned 5.4 Å from the active-site cysteine.

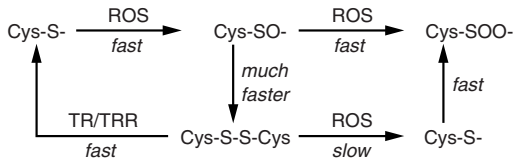

FIG. 2. The oxidation states of Cdc25B and their relative rates of formation. Adapted from Salmeen *et al.* (66).

(64). Although the Cdc25 phosphatases contain the signature active-site motif and follow the classical two-step mechanism characteristic of all PTPs (9, 24), they contain essentially no sequence or structural homology to other members of the PTP family (19, 63). The core structural fold resembles rhodanese much more than the PTPs (33). The shallow open active site not only lacks a peptide-binding groove, but is also missing the highly conserved aspartic acid that functions to protonate the leaving group in other PTPs (9).

It is the chemistry of the active-site thiolate that provides not only the catalytic prowess to the PTPs, but also the means for reversible regulation via direct oxidation by ROS. As in other characterized phosphatases, the pK_a of the active-site cysteine for Cdc25B is highly perturbed, specifically from the usual 8.3 to 5.8 (9, 64). This large shift in the solvent-accessible active site is brought about not only by the amide backbones and the arginine of the conserved HCX_5R motif, but also by the dipole of the α helix on top of which the active site is perched. Presumably, a similar pK_a exists for the catalytic cysteines of Cdc25A and Cdc25C, given the >60% pairwise identity in the catalytic domains of these homologues. Cysteines with such perturbed pK_a values are particularly susceptible to oxidation and concomitant inactivation by ROS. Hydrogen peroxide and superoxide react preferentially with the thiolate anion, not the protonated thiol (75). The product of the direct oxidation of the active-site cysteine is the sulfenic acid ($-SO^-$), itself a highly unstable anionic species that is often subject to further oxidation to the sulfinic ($-SO_2^-$) or even sulfonic acid ($-SO_3^-$) species. Historically, it has been difficult to isolate small organic molecules with sulfenic acids because of their high reactivity as either a nucleophile or an electrophile, depending on the reaction conditions (1). The sulfenic acids appear to be significantly more stable in the context of proteins than as small molecules in solution. Sulfenic acids have been detected at least transiently in a number of proteins, including Cdc25B (68), vaccinia H1-related phosphatase (14), and proteins with nonphosphatase catalytic functions (12). In protein tyrosine phosphatase 1B (PTP1b), the sulfenic acid oxidation state, along with higher oxidation states, has even been observed crystallographically (74).

For Cdc25B, the high reactivity of the active-site cysteine toward hydrogen peroxide has been experimentally confirmed *in vitro* (Fig. 2) (68). The second-order rate constant for oxidation of Cdc25B ($164 \pm 14\ M^{-1}s^{-1}$ at 20°C, pH 7.0) is ~400 times and ~15 times faster than the oxidation of glutathione and PTP1b, respectively. Oxidation occurs by hydrogen peroxide directly and leads to enzyme inactivation and the transient formation of the sulfenic acid species. The active-site cysteines in Cdc25A and Cdc25C (68) are essentially equally reactive, confirming that they also exist as thiolate anions. The high reactivity of the Cdc25s is consistent with their becoming oxidized under *in vivo* concentrations of ROS and explains Cdc25's dependence on reducing conditions for phosphatase activity (17). As expected, the sulfenic acid species in both Cdc25B and Cdc25C are unstable. In the presence of excess hydrogen peroxide, both isoforms oxidize further to generate the sulfinic acid at rates similar to the primary oxidation step (Fig. 2). Alternatively, at low concentrations of ROS, the sulfenic species of both Cdc25B and Cdc25C have been shown to form an intramolecular disulfide with the so-called backdoor cysteine (Figs. 1 and 2, and see below). Formation of this intramolecular disulfide significantly slows further oxidation to irreversibly modified forms (Fig. 2). For Cdc25C, the intramolecular disulfide species appears to have a significantly altered conformation compared with the unmodified protein as it interacts preferentially with 14–3-3 proteins and shows altered migration on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (67). As expected, all oxidative modifications of the active-site cysteines of the Cdc25s, including formation of the intramolecular disulfide, lead to a complete loss of catalytic activity.

INHIBITION OF Cdc25 PHOSPHATASES LEADS TO A BLOCK IN CELL-CYCLE PROGRESSION

The physiological substrates of the Cdc25 phosphatases are the cyclin-dependent kinases (Cdk/cyclins), the central regulators of the eukaryotic cell cycle (Fig. 3). Given their importance to cellular integrity, the Cdk/cyclins are subject to numerous counteracting control mechanisms. Regulatory

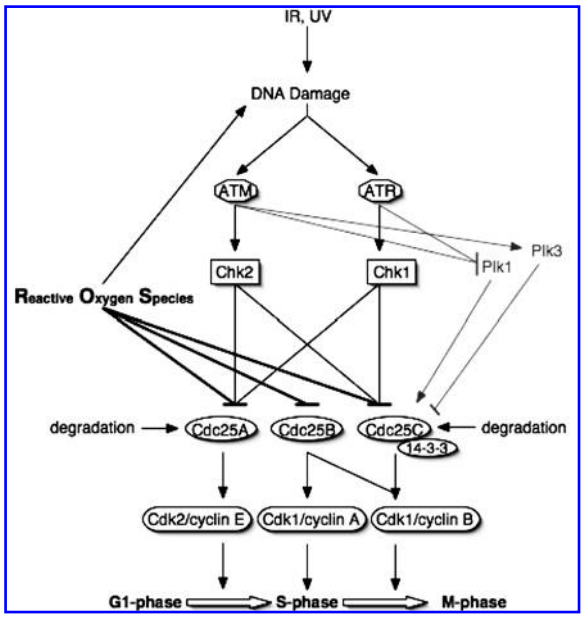

FIG. 3. The regulation and role of the Cdc25s in cell-cycle control. Adapted from Hofmann *et al.* (33).

mechanisms of the Cdk/cyclins include association with inhibitory proteins such as $p15^{Ink4b}/p16^{Ink4a}$ or $p21^{Waf1}/p27^{Kip1}$, activating phosphorylation on $Thr^{160/161}$ of the T-loop, and inhibitory phosphorylation on Thr^{14} and Tyr^{15} by the Wee1 and Myt1 kinases (52). The three human Cdc25s (Cdc25A, Cdc25B, and Cdc25C) are responsible for the dephosphorylation of $pThr^{14}$ and $pTyr^{15}$ and thereby trigger the final activation of the Cdk/cyclin complexes (56). Cdc25A controls the G_1/S transition by its activity on the Cdk2/cyclin E complex (38, 51), whereas Cdc25B and Cdc25C are regulators of G_2/M through their activity on Cdk2/cyclin A and Cdk1/cyclin B (Fig. 3) (22, 41). Positive and negative feedback activation loops between the Cdk/cyclins and the Cdc25s ensure abrupt and irreversible transitions during normal cell-cycle progression (16, 30–32).

The Cdc25 phosphatases also play a key role in integrating the specific signals of checkpoint control in response to damage by ionizing irradiation (IR) or ultraviolet light (UV) at each of the stages of the cell cycle (Fig. 3) (35). In response to IR, cells undergo rapid G_1 arrest as a result of ubiquitin/proteasome-mediated degradation of Cdc25A and consequential maintenance of Cdk2/cyclin E in the phosphorylated and inactive state (18, 49). This degradation of Cdc25A is triggered by checkpoint kinase 2 (Chk2) phosphorylation of Cdc25A at Ser^{123} as mediated through the ATM (ataxia-telangiectasia-mutated) pathway. Similarly, the response to UV irradiation 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 (18, 85). In the G_2/M checkpoint, Cdc25C becomes one of a number of key downstream effectors of the ATM-Chk2 and ATR-Chk1 pathways (46, 50, 84). Phosphorylation of Cdc25C on Ser^{215} leads to 14–3-3-mediated sequestration of Cdc25C in the cytoplasm and consequential maintenance of Cdk1/cyclin B in the phosphorylated and inhibited state (3, 21, 25, 57). ROS-mediated oxidation of Cdc25C to the disulfide form may also play a role in 14–3-3-mediated sequestration of Cdc25C in the cytoplasm (67). Cdc25A, Cdc25B, and the polo-like kinases PLK1 and PLK3 have also been implicated in the G_2/M checkpoint response (13, 83).

Confirming their importance in cell-cycle regulation, Cdc25A and Cdc25B, but not Cdc25C, have been strongly linked to many types of human cancers. Overexpression of Cdc25s has been seen in breast cancer (5, 23, 45), gastric carcinomas (40), colon cancer (15, 29), non–small cell lung carcinoma (79), ovarian cancer (4), aggressive non-Hodgkin's lymphomas (28), esophageal squamous carcinoma (34), thyroid neoplasms (36), prostate cancer (55), hepatocellular carcinomas (80), endometrial carcinoma (78), and pancreatic ductal adenocarcinoma (27). Additional support for the importance of the Cdc25s in cell-cycle regulation has come from forced overexpression of Cdc25B in mouse models (47, 48, 81). These have shown enhanced proliferation and induction of hyperplasia or increased sensitivity to mammary tumors by carcinogens. Also, numerous studies correlate inhibition of Cdc25 by small molecules with growth arrest or cell-cycle blocks (6, 42, 58, 59, 61, 69, 71–73, 77).

Thus, inactivation of the Cdc25 phosphatases by ROS can feed directly into these established and well studied pathways to halt cell-cycle progression temporarily as a short-term response to oxidative stress.

REACTIVATION OF Cdc25 PHOSPHATASES FROM A STABLE INTRAMOLECULAR DISULFIDE

In general, oxidation of cysteines past sulfenic acids to sulfinic or sulfonic acids is considered to be physiologically irreversible. That is, common cellular reductants such as thioredoxin/thioredoxin reductase (TR/TRR) and glutathione are capable of restoring a sulfenic acid to a thiolate, but are ineffective toward the higher oxidation states. Should higher oxidation states be generated, such modified proteins would presumably need to be degraded, perhaps a prelude to other more radical and long-lasting changes such as apoptosis. Thus, if regulation of Cdc25s in particular and PTPs in general is to be reversible, there must exist a mechanism to prevent excessive oxidation of the active-site thiolates and allow rapid intracellular rereduction. In an exception to the apparent irreversible nature of the higher oxidation states of cysteines, it has been found recently that the sulfinic acid in peroxiredoxins can be restored to the thiolate by an as-yet-unknown mechanism (76).

In the Cdc25s, the mechanism of reversibility toward oxidation is provided by the backdoor cysteines (Cys^{384} in Cdc25A, Cys^{426} in Cdc25B, Cys^{330} in Cdc25C) (Fig. 1). Under low concentrations of hydrogen peroxide, the sulfenic acid is rapidly trapped to generate the intramolecular disulfide between the active-site cysteine and the backdoor cysteine (Fig. 2). This

disulfide is readily reducible by the cellular reductant TR/TRR, but not glutathione (68). The presence of this backdoor cysteine is conserved in all known Cdc25 sequences, emphasizing its evolutionary significance. Intramolecular disulfide formation has also been observed with partial occupancy in the crystal structures of the catalytic domains of Cdc25A and Cdc25B (19, 63). Minimally, the backdoor cysteine exists to protect Cdc25 from spurious ROS damage that is an intrinsic danger to any phosphatase possessing a highly reactive cysteine. In the context of this review, however, the backdoor cysteine provides the means to regulate the cell cycle reversibly in accord with the redox status of the cell. The conservation of the backdoor cysteine in the Cdc25s is consistent with the evolutionary conservation seen for the pathways that monitor DNA damage in response to IR and UV.

Interestingly, intramolecular disulfide formation has been detected in several other phosphatases of the PTP family. In the low-molecular-weight phosphatases, disulfide formation between active-site Cys^{12} and conserved Cys^{17} allows for redox regulation of receptor stimulation of the platelet-derived growth factor (7, 10, 11). In the tumor suppressor PTEN (phosphatase and tensin homologue on chromosome 10), which is involved in cell migration, growth, and survival, active-site Cys^{124} forms a disulfide with conserved Cys^{71} in response to oxidation by hydrogen peroxide (43). In the Cdk-interacting protein phosphatase KAP, a disulfide of unknown significance has been crystallographically observed between active-site Cys^{140} and Cys^{79} (70). Most members of the PTP family, however, do not appear to have an available backdoor cysteine for disulfide formation. As recently reported, this has not precluded formation of a more stable, yet reversible, oxidation state for the active-site cysteine. In PTP1b, a novel sulfenylamide has been crystallographically observed between the sulfur and the nitrogen of the amide backbone of the residue following the active-site cysteine (66, 74). The kinetic mechanism and physiological relevance of this process remain to be explored. Particularly for the low-molecular-weight phosphatases, however, there exists increasing evidence that these secondary cysteines are present not merely to protect the sensitive active-site cysteine from spurious oxidation, but rather to provide a means of reversible regulation in response to ROS (20).

IN VIVO EVIDENCE THAT ROS CONTROL THE Cdc25 PHOSPHATASES

Clearly, cellular redox regulation is a very complicated process involving many different pathways ranging from calcium homeostasis to transcriptional control (39), and redox control of the cell cycle has also been demonstrated to exert effects through a number of different pathways involving p53, p21, and Chk1 (8, 65, 82). As enumerated above, the Cdc25 phosphatases themselves possess the three necessary elements to function directly in redox control of the cell cycle. They are rapidly and potently inactivated by ROS and therefore unable to promote cell-cycle progression under oxidative conditions until reactivation by thioredoxin takes place under reducing conditions. Do they actually serve this role *in vivo* and what evidence exists for such a novel role for the Cdc25 phosphatases? The first suggestion for direct involvement of the Cdc25 phosphatases in cell-cycle regulation in response to the redox status of the cell was surprisingly made more than 10 years ago (54). The *in vitro* study of embryonic development for purposes of reproductive research had long been hampered by the reduced efficiency of progression at the maternal–embryonic transition. It was shown by the Mori laboratory that ROS were directly involved in the two-cell block of the mouse embryo and that injection of superoxide dismutase or thioredoxin allowed for development past the two-cell stage *in vitro*. The mechanism for this thioredoxin-induced release was shown to act through the phosphorylation state of the Cdc2 kinase. Injection of thioredoxin led to dephosphorylation and activation of the Cdc2 kinase during M-phase of the second cleavage. The authors speculated that the Cdc25 phosphatases act as possible sources for this redox control of the cell cycle (54).

More recently, Finkel's laboratory at NIH has shown that heterologously expressed Cdc25C undergoes rapid nonproteasomal degradation in response to hydrogen peroxide treatment in HeLa cells (67). This response is due to a decreased half-life for Cdc25C and is dependent on intact active-site and backdoor cysteines. Cdc25C's response to ROS treatment does not depend on the Chk pathway, suggesting a direct interaction between ROS and Cdc25C (Fig. 3). Future *in vivo* studies are expected to elucidate these effects more extensively, also in regard to Cdc25A and Cdc25B regulation by ROS.

The observation that TR/TRR is the cellular reductant for restoring Cdc25 to the active state suggests that overproduction of Cdc25 alone may not be sufficient to drive cancerous growth. Interestingly, just as for the Cdc25s, overproduction of thioredoxin has also been linked to a variety of human tumors, including breast, thyroid, prostate, gastric, colorectal, and melanoma (2, 26, 44, 53, 62). As TR/TRR serves as a key cellular reductant for many cellular systems (60), a direct link is not necessarily indicated by these data. However, one should not forget the potential connection between these proteins suggested by the data from the Mori laboratory (54). As both Cdc25 and thioredoxin are found overexpressed in ~50% of patients with any particular tumor type, and overexpression for both is correlated with a worse clinical outcome, it would be interesting to see if there exists any correlation between Cdc25 overexpression and thioredoxin overexpression. Such a correlation would strongly support the relevance of ROS-mediated regulation of the Cdc25s.

In summary, Cdc25 phosphatase contains a built-in switch that is sensitive to the redox status of the cell. The active-site cysteine that facilitates its reactivity as a phosphatase is susceptible to rapid oxidization. The stable form of the oxidized protein exists as an intramolecular disulfide between the active-site and backdoor cysteines and has no activity, thus halting cell-cycle progression. This intramolecular disulfide can be reduced by thioredoxin, restoring phosphatase activity and allowing for resumption of the cell cycle. The role of redox regulation of the Cdc25s in cancer remains to be investigated.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ATM, ataxia-telangiectasia-mutated; ATR, ATM- and Rad3-related; Cdk, cyclin-dependent kinase; Chk, checkpoint kinase; IR, ionizing radiation; PLK, polo-like kinase; PTP, protein tyrosine phosphatase; PTP1b, protein tyrosine phosphatase 1B; ROS, reactive oxygen species; TR/TRR, thioredoxin/thioredoxin reductase; UV, ultraviolet light.

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