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Cdc25 Phosphatases: Structure, Specificity, and Mechanism†

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ABSTRACT: Cdc25 phosphatases, as activators of the Cdk/cyclins, play critical roles in the regulation of the eukaryotic cell cycle. Because of their overexpression and correlation with poor prognosis in many diverse cancers, Cdc25 phosphatases are attractive targets for anticancer drug development. Over the past few years, much knowledge of the basic enzymology of the Cdc25 phosphatases that may aid in the development of specific inhibitors has been gained. We review herein the structure, specificity, and mechanism of the Cdc25 phosphatases with a special focus on the activity of Cdc25 phosphatases with native protein substrates.

Cancer kills an increasing number of people every year, surpassing heart disease as the leading killer of Americans under 85 years of age. Although each type of cancer, even each individual case of cancer, can arise from diverse causes, all cancers share the common feature of a disordered cell cycle that is characterized by rapid and uncontrolled cell growth. Thus, targeting the molecules that regulate the cell cycle has become a major thrust in the development of anticancer therapeutics (*1*). Prominent among these targets are the cyclin-dependent kinases (Cdk/cyclins).[1] These protein kinases serve as the central regulators of the eukaryotic cell cycle and are responsible for driving each stage of cell division (Figure 1) (*2*). For example, one set of Cdk/cyclins phosphorylates proteins, leading to the initiation of DNA replication at the G1-to-S transition. At the G2-to-M transition, a different set of Cdk/cyclins phosphorylates and activates the proteins required for the structural reorganizations involved in chromosome separation and actual cell division.

Cdc25 phosphatases serve as key activators of the Cdk/cyclins (Figure 1), and thus, they too have become potential targets for the development of anticancer therapeutics (*3*). The three human Cdc25 phosphatases (Cdc25A, Cdc25B, and Cdc25C) are responsible for dephosphorylating Cdk/cyclins on pThr14 and/or pTyr15 residues. This dephosphorylation triggers the final activation of Cdk/cyclin activity during normal cell cycle progression (*4*, *5*). Cdc25A controls both the G1-to-S and G2-to-M transitions, whereas Cdc25B and Cdc25C are regulators of the G2-to-M transition. The Cdc25 phosphatases also play an important role in the checkpoint response that prevents Cdk/cyclin activation following DNA damage (Figure 1) (*6*, *7*). Confirming an important role for the Cdc25 phosphatases in cancer, Cdc25A and Cdc25B, but not Cdc25C, are overexpressed in many different primary human cancers (*3*). Emphasizing the potential application of inhibitors of the Cdc25s, the overexpression of Cdc25A and/or Cdc25B is frequently correlated with a poor clinical prognosis. Therefore, the Cdc25 phosphatases have been extensively targeted for anticancer drug development in both academia and industry. As part of these

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[1] Abbreviations: Cdk/cyclins, cyclin-dependent kinases; PTP, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase 1B; pNPP, *p*-nitrophenyl phosphate; mFP, *O*-methylfluorescein phosphate; Cdk2-pTpY−CycA, bis-phosphorylated Cdk2 (on Thr14 and Tyr15) complexed with cyclin A; mNBP, *m*-nitrobenzyl phosphate.

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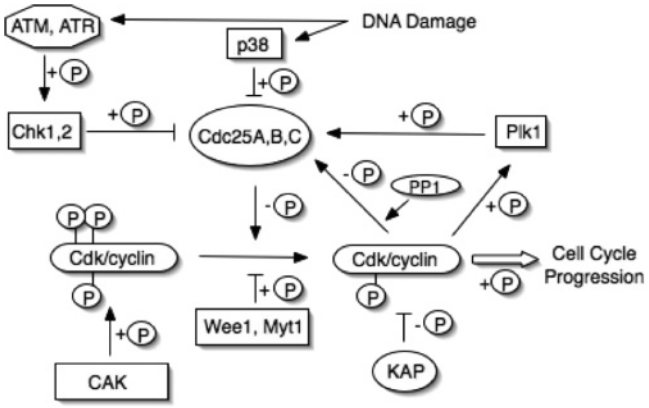


FIGURE 1: Regulation of Cdk/cyclin activity by protein kinases and phosphatases. The activity of the Cdk/cyclins on numerous targets is required for cell cycle progression, and these protein kinases are themselves regulated by numerous other kinases (+P) and phosphatases (−P) (*2*). Cdc25 phosphatases activate the Cdk/cyclins, thus counteracting the Wee1 and Myt1 kinases and promoting the cell cycle during normal cell growth. The activity of the Cdc25s is regulated by feedback activation through the Cdk/cyclins and the polo-like kinase Plk1 (*84*, *85*), as modulated by binding of 14-3-3 protein and dephosphorylation by protein phosphatase 1 (PP1) (*86*). Cdc25s also play a critical role in the checkpoint response (*6*). In response to DNA damage induced by ionizing radiation or ultraviolet light, activation of the ataxia-telangiectasia mutated (ATM) and/or ATM-related (ATR) kinases leads to phosphorylation and activation of the Chk1 and -2 kinases. Phosphorylation of Cdc25s by the Chk1 and -2 kinases leads to ubiquitin-mediated degradation (Cdc25A and -B) and/or 14-3-3-mediated cytoplasmic sequestration from the Cdk/cyclin substrates (Cdc25A−C). Additionally, there is evidence of phosphorylation of Cdc25B by the mitogen-activated protein kinase p38 in regulation of the G_2-to-M checkpoint in response to ultraviolet radiation (*87*).

efforts, the enzymology of the Cdc25s has been extensively studied. This work will review the current knowledge of the structure, specificity, and mechanism of the Cdc25 phosphatases, with a special emphasis on understanding the reactivity of Cdc25 phosphatases with native protein substrate. Because of the existence of recent reviews, we do not consider here the biology, regulation, or recent advances in drug discovery for the Cdc25s (*5*, *8*, *9*).

Structure

From one [in fungi (*10*)] to four [in *Caenorhabditis elegans* (*11*)] isoforms of the Cdc25 phosphatase exist in all eukaryotes. In humans, there are three isoforms, each with different possible splicing variants (*12*−*14*). The Cdc25 proteins are 300−600 residues in length and can be divided into two regions. The N-terminal regions are highly divergent in sequence, made even more so because of the alternative splicing therein. The N-termini contain numerous sites for phosphorylation and ubiquitination, which are involved in regulating phosphatase activity (*15*, *16*), protein levels (e.g., *17* and *18*), and/or association with other proteins (*19*−*21*). The N-termini also contain nuclear import−export signals that control the intracellular localization (nuclear vs cytoplasmic) of the Cdc25 phosphatases (*22*−*24*).

The more highly homologous C-terminal regions (∼60% pairwise identity over ∼200 amino acids) contain the catalytic functionality of the Cdc25s. The signature motif (HCX_5R) containing the catalytic cysteine that defines the active sites of all protein tyrosine phosphatases (PTPs) is found within this catalytic domain and represents the only significant region of homology between the Cdc25s and other PTPs. The structures of the catalytic domains of Cdc25A and Cdc25B have been determined (*25*, *26*). The overall core fold of the Cdc25s is unlike that of any other known PTP, consistent with the lack of overall sequence homology to other PTPs (Figure 2A). The small catalytic domains consist of a central five-stranded parallel β-sheet sandwiched by three α-helices from below and two α-helices from above and are most similar to rhodanese, a sulfur transfer protein found in bacteria and mitochondria (*27*). The 31 and 17 C-terminal residues of Cdc25A and Cdc25B, respectively, were not observed in these structures, with an additional α-helix seen near the active site of Cdc25B.

The active site loop of the Cdc25s contains the signature HCX_5R sequence and can be superimposed with essentially all other active site loops in PTPs with known structures (Figure 2B). Like in other PTPs, this signature motif is found perched atop a long α-helix, the helix dipole thereby favoring deprotonation of the active site cysteine within the motif (*28*). The five X residues contribute not their side chains but rather their backbone amides toward the functionality of the active site. In structures with bound sulfate, these amides, along with the arginine (R) of the motif, form multiple hydrogen bonds with the oxygen atoms of the sulfate, a mimic of a bound phosphorylated substrate (Figure 2C). The active site of Cdc25B appears to be preorganized for binding phosphorylated substrates. The structure of the apoenzyme from which sulfate was removed by soaking of the crystals in sulfate-free buffer is identical to the sulfate-bound enzyme (Figure 2C) (*29*). In fact, three water molecules are located in place of three of the oxygen atoms of the sulfate. The structure of the active site mutant of Cdc25B (C473S) is also essentially identical to that of the wild-type enzyme, having a more tightly bound sulfate that was recalcitrant to removal by multiple soakings in sulfate-free buffer (Figure 2C) (*30*). In contrast to other PTPs, the Cdc25s do not have a loop containing an aspartic acid (i.e., the catalytic acid) overhanging the active site.

One of the most striking structural features of the catalytic domains of the Cdc25s is the lack of any apparent substrate recognition site. As in other enzymes that recognize protein sequences, as for example the serine proteases (*31*), one might expect to find a specificity pocket for residues upstream or downstream of the site of dephosphorylation. In fact, the active site face of the Cdc25s is surprisingly flat, and even the active site pocket is extremely shallow (Figure 2D). In contrast to protein tyrosine phosphatase 1B (PTP1B), which has a sufficiently deep cavity to restrict substrate specificity to pTyr (and not pSer or pThr), the active site of the Cdc25 phosphatases is completely filled by the phosphoryl group of the substrate alone. The shallow active site is well-suited for allowing access to both pThr- and pTyr-containing substrates to the catalytic cysteine, in accord with the dual-specific nature of the Cdc25s.

Specificity

Association and Dissociation. Because Cdc25 phosphatases are protein phosphatases, elucidating the protein−protein interactions involved in substrate recognition is of utmost importance for understanding their overall reaction mechanism. On the basis of the flat structure of the active

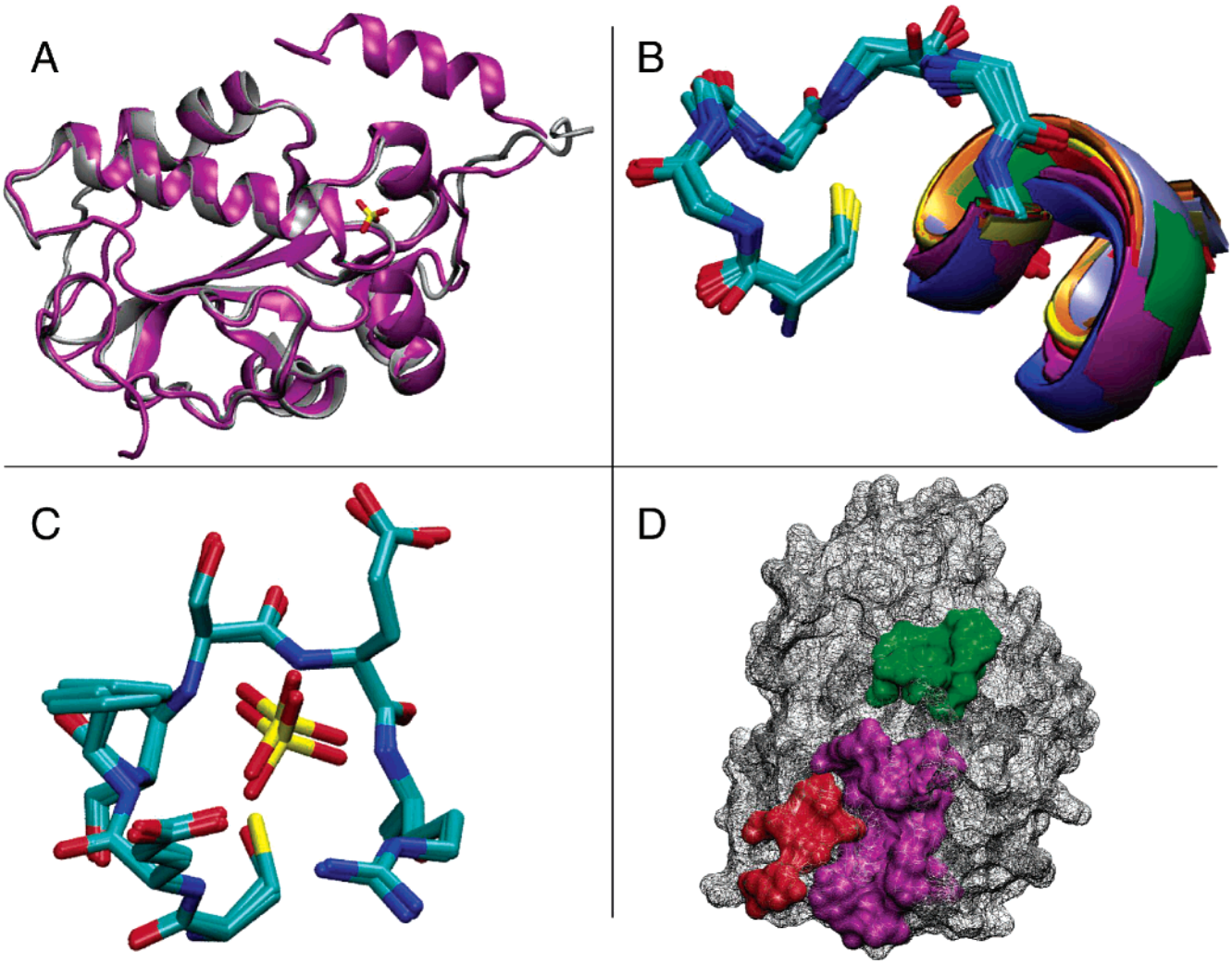
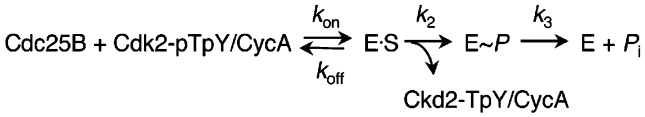


FIGURE 2: Structure of the catalytic domain of the Cdc25 phosphatases. (A) Overlay of Cdc25A (1c25, gray) and Cdc25B (1qb0, purple) structures shown as ribbon diagrams. The active site region can be identified by the bound sulfate in Cdc25B, a phosphate mimic, shown in licorice. (B) Overlay of the active site loops of Cdc25B (1qb0) with a diverse group of other PTPs (PDB entries in parentheses), including phosphatase and tensin homologue (PTEN, 1d5r), kinase-associated phosphatase (KAP, 1fpz), low-molecular weight (LMW) phosphatase (1phr), mammalian cell entry (Mce1) phosphatase (1i9s), proline-directed phosphatase Cdc14 (1ohc), PTP1b (1i57), *Yersinia* tyrosine phosphatase (YOP, 1yts), *Vaccinia* H1-related (VHR) phosphatase (1vhr), and Src homology domain phosphatases SHP1 (1gwz) and SHP2 (2shp). The active site cysteine residues are shown in licorice along with the backbone of the HCX_5R loop. The first five residues of the α-helix immediately following the active site loop are shown in cartoon with different colors for each phosphatase. (C) Overlay of the active site loop of Cdc25B (Cys473−Arg489) bound to sulfate (1qb0), as the apoenzyme (1ymk), and as the C473S mutant bound to sulfate (2a2k). (D) Surface view of Cdc25B as seen from the side that interacts with the Cdk2-pTpY−CycA substrate. The active site residues (Cys473−Arg489) are colored green. The hotspot residues (Arg488, Arg492, and Tyr497) are colored red. The residues of the potential inhibitor binding pocket adjacent to the hotspots are colored purple.

site surface and precedence from other PTPs, the primary sequence surrounding the site of dephosphorylation does not contribute significantly to the recognition of the protein substrate. A variety of peptides with different sequences and lengths exhibit poor and essentially identical activity with the Cdc25s (k_{cat}/K_m values of 1−10 M^{-1} s^{-1}) (*32*). In contrast, a native protein substrate, namely, bis-phosphorylated Cdk2 (on Thr14 and Tyr15) complexed with cyclin A (Cdk2-pTpY−CycA), is preferred over phosphopeptide substrates that contain the same primary sequence by at least 6 orders of magnitude (k_{cat}/K_m values of ∼1 × 10^6 M^{-1} s^{-1}). Specificity constants (k_{cat}/K_m) in this range have been observed for other PTPs (*33*) and appear to be typical for enzymes that utilize protein substrates, including the protein kinases. The high specificity of Cdc25B for Cdk2-pTpY−CycA emphasizes the physiological relevance of this enzyme−

Scheme 1: Kinetic Model for the Catalytic Reaction Performed by Cdc25B



substrate pair. Additionally, the preference for pTyr over pThr seen for phosphopeptide substrates (*32*), presumably based on their relative reactivities (*34*), is reversed for assays with the native protein substrate and is in accord with cell-based measurements (*35*). Cdc25s act on their protein substrates in a stepwise mechanism, wherein the enzyme dissociates after dephosphorylation of pThr and must re-associate for dephosphorylation of pTyr (*32*). Both full-length and catalytic domain constructs of Cdc25s have essentially

the same activity toward Cdk2-pTpY−CycA, suggesting that all important elements for substrate recognition are contained within the catalytic domain alone. Interestingly, both Cdc25A and Cdc25B exhibited essentially the same activity toward the physiological substrate Cdk2-pTpY−CycA. In contrast, Cdc25C, whose physiological substrate is thought to be the Cdc2-pTpY−CycB complex instead, was 100-fold less active (*36*). The difference in the reactivity of Cdc25C toward Cdk2-pTpY−CycA can be attributed to the least conserved part of the catalytic domain, namely, the C-terminal region that is not visible in the crystal structures. In fact, two arginines found in Cdc25A and Cdc25B but not in Cdc25C account for the majority of this effect.

More detailed studies have revealed the kinetic and thermodynamic basis for the dramatic preference for protein substrate (*37*, *38*). These studies rely on a three-step mechanism (Scheme 1) that describes the removal of pThr from Cdk2-pTpY−CycA. Formation of the enzyme−substrate (E•S) complex is governed by the rates of association (k_{on}) and dissociation (k_{off}). Transfer of a phosphate from pThr14 of Cdk2-pTpY−CycA to Cys473 of Cdc25B to form the phospho−enzyme intermediate (E∼P) with concomitant release of protein product (Cdk2-TpY−CycA) is governed by k_2 and is considered irreversible. Hydrolysis of the acid-labile phospho−enzyme intermediate is governed by k_3. As there is no observable burst of phosphate formation at high enzyme and Cdk2-pTpY−CycA substrate concentrations, neither hydrolysis of the phospho−enzyme intermediate nor release of the protein product appears to be rate-limiting. Thus, single-turnover kinetics have served as an ideal tool for elucidating the microscopic rate constants for the first half-reaction of the catalytic cycle performed by Cdc25B, namely, formation of the E•S complex followed by the chemical step of phosphate transfer leading to the phospho−enzyme intermediate.

The microscopic rate constant for association of protein substrate with Cdc25B phosphatase as determined by single-turnover kinetics is 1.3×10^6 M^{-1} s^{-1}, essentially equal to the specificity constant (k_{cat}/K_m) (*37*). A similar rate constant for association between the enzyme and the dephosphorylated protein product was observed using Biacore. Rate constants of this magnitude are often considered to be at or near the free-diffusion limit for functional protein−protein associations by theoretical calculations (*39*) or experimental observations (*40*). Faster rates of protein association are observed and are driven by long-range electrostatic steering (*41*). However, for Cdc25B and its protein substrate, the apparent rate of association is governed to a large part not by free diffusion but rather by specific short-range interactions. Thermodynamically, the enthalpic barrier ($\Delta H^{\ddagger}$ of 13.1 kcal/mol) dominates the transition state for substrate association (*38*). The small entropic contribution to the association of protein substrate of only 4.0 kcal/mol at 298 K is inconsistent with a purely diffusion-controlled process for such a significant burial of surface area and concomitant release of water molecules (see below). Consistent with the thermodynamics, there is only a small viscogen effect on the rate of association as deduced by k_{cat}/K_m measurements (*42*).

Once the E•S complex has formed, the rate of substrate dissociation (≤ 0.02 s^{-1}) is significantly slower than the rate of phosphate transfer to form the phospho−enzyme intermediate (1.1 s^{-1}; see below) (*37*). Cdk2-pTpY−CycA can thus be described as a "sticky" substrate wherein each binding event leads to catalysis. The estimated K_d derived from the single-turnover experiments (∼15 nM) does not reflect the experimentally determined K_m (400 nM) and is similar to the measured K_d for the substrate-trapping mutant in which the catalytic cysteine has been changed to a serine (C473S). Substrate-trapping mutants such as these are commonly used for studying the physiological substrates of PTPs in vitro and in vivo as they typically bind their protein substrates with high affinity (*43*). For example, substrate-trapping mutants have been used to demonstrate that Cdk1−CycB, Cdk2−CycA, and Cdk2−CycE complexes can be enriched from cell extracts using Cdc25A, whereas Cdc25B was found to interact only with Cdk2−cyclin A complexes (*44*). The substrate-trapping mutant of Cdc25B (C473S) not only has an affinity for the protein substrate similar to that of the wild-type E•S complex and a structure essentially identical to that of the wild-type protein (*30*) but also has rate constants for association and dissociation with Cdk2-pTpY−CycA similar to that of the wild-type enzyme (*37*).

Given that the rate of E•S dissociation is much slower than catalytic turnover, how is efficient expulsion of the protein product ensured? The C473D mutant provides potential insight (*37*). In contrast to the C473S mutant, the C473D variant has a much weaker affinity ($K_d = 2$ μM) and a rapid rate of dissociation (>2 s^{-1}) from the protein substrate (*37*), yet the C473D mutant has essentially the same rate constant for association as the C473S mutant as measured by Biacore (unpublished data). In the structure of C473D, the slightly longer aspartate residue compared to the wild-type cysteine or the substrate-trapping serine distorts the active site loop. This distortion presumably prevents the proper docking of the phosphorylated substrate in the active site. A similarly weak interaction was observed for the dephosphorylated protein product with the wild-type enzyme, despite a similar rate of association as measured by Biacore. Thus, formation of a high-affinity E•S complex is at least in part ensured by the extensive interaction of a phosphoryl group in the active site loop. When these interactions no longer occur, as for example following the transfer of a phosphate from substrate to enzyme, an increased rate of dissociation for the protein product is attained. Overall, Cdc25 appears to have evolved a highly suitable mechanism for simultaneously mediating substrate specificity and catalytic efficiency wherein rapid substrate binding is followed by rapid release of dephosphorylated protein product following the chemical step of phospho transfer.

Hotspot Mutants and a Docking Model of the E•S Complex. Because the association between the enzyme and protein substrate appears to be the primary mechanism for achieving specificity for the Cdc25B phosphatase, it was important to define the region on Cdc25B responsible for docking to Cdk2-pTpY−CycA. As expected from the flat and solvent-exposed active site region, Cdc25B utilizes a docking site remote from the catalytic cysteine. Mutants of the hotspot residues Arg488, Arg492, and Tyr497 located 20−30 Å from the active site specifically reduce the apparent rate of association (and correspondingly the specificity constant) by ∼200-fold (Figure 2D) (*30*, *45*). None of the other >30 residues on the same face of Cdc25B have a comparable effect. Importantly, controls using small molecule substrates ensure that these hotspot mutations do not nonspecifically

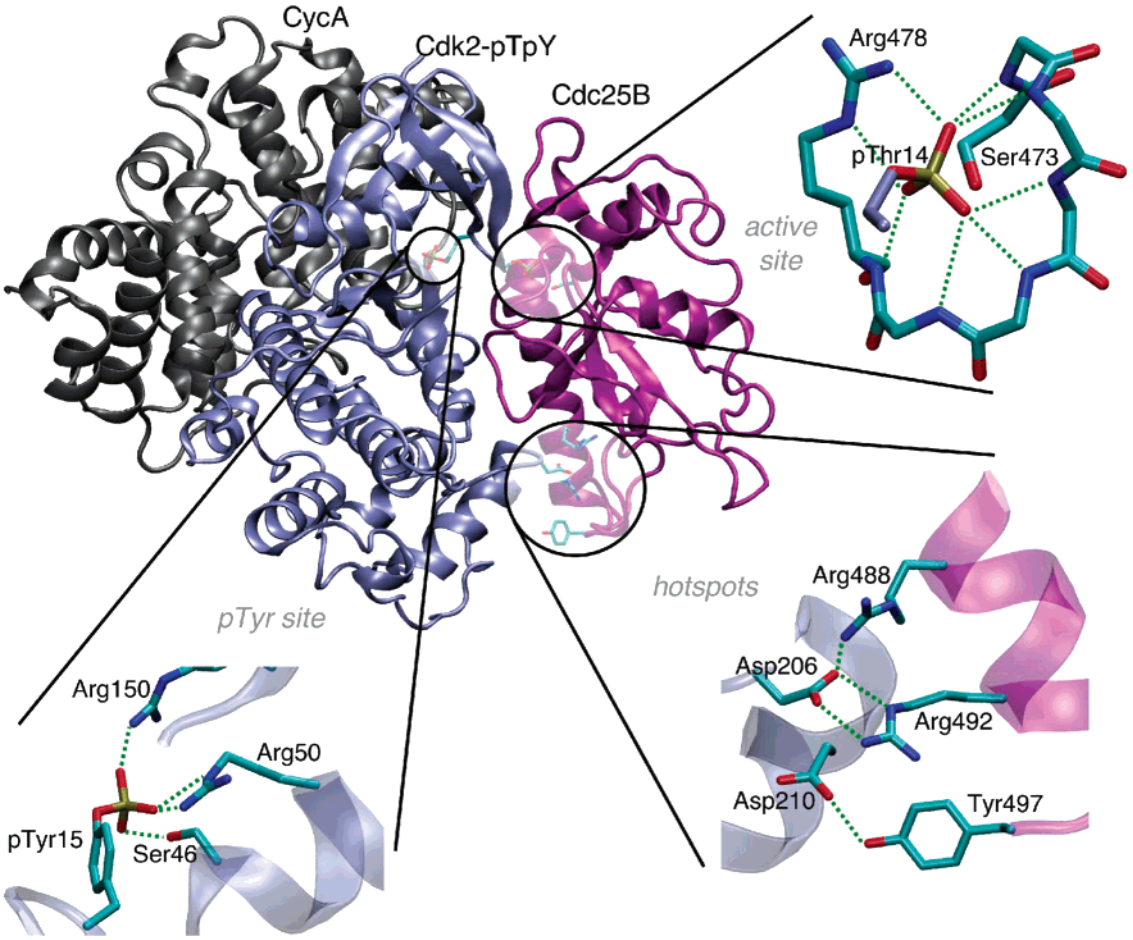


FIGURE 3: Docking model of Cdc25B with its substrate Cdk2-pTpY−CycA, highlighting the interactions at the active site of Cdc25B, at the remote docking site, and at the pTyr site. The substrate-trapping mutant of Cdc25B (C473S), bis-phosphorylated Cdk2 (Cdk2-pTpY), and cyclin A (CycA) are shown in cartoon representation colored purple, blue, and gray, respectively. The inset at the top right depicts the active site region, with the detail showing how the backbone amides of the active site loop along with Arg478 cradle the pThr14 of the protein substrate. The inset at the bottom right depicts the hotspot region, with the detail showing the ionic hydrogen bond interactions that govern the association between Cdc25B and its protein substrate. The inset at the bottom left depicts the pTyr15 of Cdk2, which interacts almost exclusively with the protein substrate as shown, not with the Cdc25B phosphatase.

affect phosphatase structure or reactivity. Under conditions that probe substrate binding [k_{cat}/K_m for the substrate *p*-nitrophenyl phosphate (pNPP)], formation of the phospho−enzyme intermediate (k_{cat} for pNPP), hydrolysis of the phospho−enzyme intermediate [k_{cat} for *O*-methylfluorescein phosphate (mFP)], or the integrity of the protein (burst equivalents with mFP), mutations of the hotspot residues show little if any change compared to the wild-type protein. Additionally, the structure of the R492L mutant shows a position of the Cβ, Cγ, and Cδ atoms of the substituted leucine identical to that for the wild-type arginine. Thus, the effects of the hotspot mutations are not global but encompass solely the loss of their contribution to the recognition of protein substrate.

Although the three hotspot mutants exhibit identically reduced rates of protein association compared to that of the wild-type enzyme, they have different effects on dissociation (*37*). In contrast to many cases with reduced affinity none of the rates of dissociation are increased compared to that of the wild-type enzyme. Instead, the changes in the upper limits for the rates of dissociation range from not detectable (R488L) to lower by 10-fold (Y497A) to 100-fold (R492L). Even for multiple substitutions at position Arg492, a variety of different apparent affinities that appear to be governed by variable rates of association and dissociation exist. Additionally, the hotspot mutants exhibit complex and varied enthalpy−entropy compensations compared to the wild-type protein (*38*). The subtleties of these differential rate constants and thermodynamic effects for the different mutations are difficult to interpret and reflect the complexity and cooperativity of molecular interactions (*46*).

Identification of the hotspots on Cdc25B served as a key step in generating a model for the docked complex of Cdc25B with Cdk2-pTpPy−CycA using a multistep computational protocol that relied on tools from computational geometry and molecular dynamics (Figure 3) (*30*). The docked model made several predictions that were experimentally tested. Most importantly, the hotspot residues at the remote docking site on Cdc25B interact via ionic hydrogen bonds with Asp206 and Asp210 of Cdk2. As for the original hotspot mutations on Cdc25B, mutation of Asp206 in the Cdk2-pTpY−CycA substrate led to a 200-fold reduction in the rate of association (and specificity constant) with wild-type Cdc25B. Numerous other mutations on the same surface of Cdk2 had no observable effect on activity as a substrate for Cdc25B. Control experiments demonstrated no change in the kinase activity of the D206A mutant using histone proteins as substrates. Further confirm-

ing the observed interfacial contacts between the hotspots on Cdc25B and Cdk2, significant coupling energies (3−4 kcal/mol) were observed by double-mutant cycle analysis (*30*). Furthermore, in an experiment inspired by genetic complementation, the two primary hotspot residues in this interaction could be successfully swapped. Relocating Arg492 from Cdc25B to the position of Asp206 on Cdk2 while simultaneously moving Asp206 from Cdk2 to the position of Arg492 on Cdc25B supports a functional interaction not too different from that of the wild-type proteins. Analysis using alternative substitutions and expansion to triple-mutant cycles has provided further insight how this cluster of hotspot residues contributes to substrate recognition (Figure 3) (*47*). Arg492 is at the center of the cooperative interactions at the remote docking site through its bidentate interaction with Asp206 of Cdk2. The primary role of the neighboring Tyr497 is to shield water from the bidentate interaction between Arg492 and Asp206. Arg488 participates in the electrostatic network with Asp206 and Asp210 of the protein substrate.

The docked model of Cdc25B with its protein substrate made a number of other correct predictions (*30*). pThr14 of the bis-phosphorylated substrate, not pTyr15, is seen docked into the active site, in accord with the preferred order of dephosphorylation observed experimentally (*32*, *35*). The orientation of the phosphoryl group mimicked exactly the structure of the bound sulfate in the crystal structures of the wild-type enzyme and the substrate-trapping mutant. In fact, pTyr15 of the protein substrate forms almost no interactions with the enzyme, in agreement with the essentially identical rates of dephosphorylation measured for the monophosphorylated substrate Cdk2-pT−CycA and the mutant Cdk2-pT-Y15F−CycA compared to that of Cdk2-pTpY−CycA. Finally, the overlap of the interaction site of Cdc25B with that of CksHs1 (*48*) explains the formerly cryptic observation that the yeast homologue of CksHs1 ($p13^{suc1}$) inhibits Cdc2 dephosphorylation of Tyr15 (*49*) and that overexpression of $p13^{suc1}$ leads to slowed growth and elongated cell growth in fission yeast (*50*).

In Vivo Validation of Hotspots. In general, it is difficult to intuit the importance of a docking site interaction in vivo given that in most cases one knows neither the binding affinity nor the intracellular concentrations of the corresponding proteins. However, such validation was possible for Cdc25B and its protein substrate given the rich history of cell cycle research using model organisms and the conservation of the hotspots on Cdc25B. In the *Xenopus* model for studying the cell cycle, intact immature oocytes can be dissected from female frogs and converted to fertilizable eggs by treatment with hormones such as progesterone (*51*). Oocyte maturation proceeds by a complex and poorly understood signaling pathway. The final intracellular step in this pathway is the dephosphorylation and activation of Cdk/cyclins by the Cdc25 phosphatase. The maturation event can be monitored by visual inspection of the oocytes (formation of a white spot on the vegetal pole of the oocyte). Conveniently, maturation can be triggered more directly by microinjection of the Cdc25 protein into *Xenopus* oocytes, even using such heterologous sources as humans (*52*, *53*). Phosphatase activity is required for maturation, as injection of the active site mutant has no effect. In addition to basic catalytic function, recognition of protein substrate is also required for maturation of *Xenopus* oocytes upon microinjection of Cdc25B (*30*, *45*). All three hotspot mutants have the same effect as the active site mutant, with no observable dephosphorylation of Cdk/cyclins and no oocyte maturation.

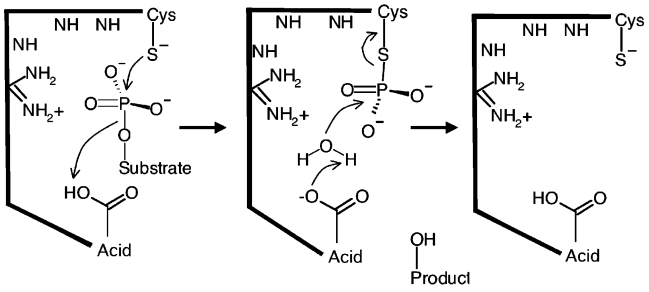


FIGURE 4: Cartoon of the reaction mechanism for PTPs, as described in more detail in the text.

The budding yeast *Saccharomyces cerevisiae* serves as another powerful model for studying cell cycle progression in vivo. Mih1p is the single homologue of Cdc25 in budding yeast (*54*). Although deletion of Mih1p by itself is not lethal, a sensitive functional readout of Mih1p activity exists in a yeast strain in which one of two negative regulators of Swe1p, the yeast homologue of the Wee1 kinase that adds inhibitory phosphates to the Cdk/cyclins, has been removed (*55*). This strain by microscopic inspection exhibits an elongated hyphal phenotype incapable of proper cell division unless it is complemented with a wild-type version of Mih1p. Introduction of Mih1p with an active site mutation or a mutation corresponding to any of the three hotspots in Cdc25B does not rescue the hyphal phenotype (*45*). Thus, even in the context of the full-length protein (not just the catalytic domain) under the control of its own promoter inside the cell (not just in vitro), recognition of substrate is as important to in vivo function as basic catalytic activity for the Cdc25 phosphatases.

Mechanism

Seminal mechanistic studies have established a general catalytic mechanism for PTPs containing the signature HCX_5R motif (Figure 4) (*56*−*60*). The active sites of the PTPs typically contain two key catalytic residues, an unprotonated thiolate and a protonated catalytic acid. The phosphoryl group of the substrate is cradled by the active site loop, forming hydrogen bonds to the backbone amides and the arginine of the HCX_5R motif. The thiolate anion of the catalytic cysteine lies directly below the phosphoryl group, which sets up an in-line attack of the thiolate on the P−O bond in the first chemical step of the reaction. Departure of the leaving group and formation of the phospho−cysteine intermediate are facilitated by protonation of the oxyanion by the catalytic acid, an aspartate located in a separate loop. In the second chemical step of the reaction, water serves as a nucleophile in the hydrolysis of the phospho−enzyme intermediate assisted by the aspartate, which now acts as a base to activate the water molecule. The details of the general reaction scheme for PTPs have been probed extensively using pH-dependent kinetics with a variety of small molecule substrates, mostly aryl phosphates such as pNPP. Additionally, kinetic isotope effects have been used to characterize the transition state and mechanism of

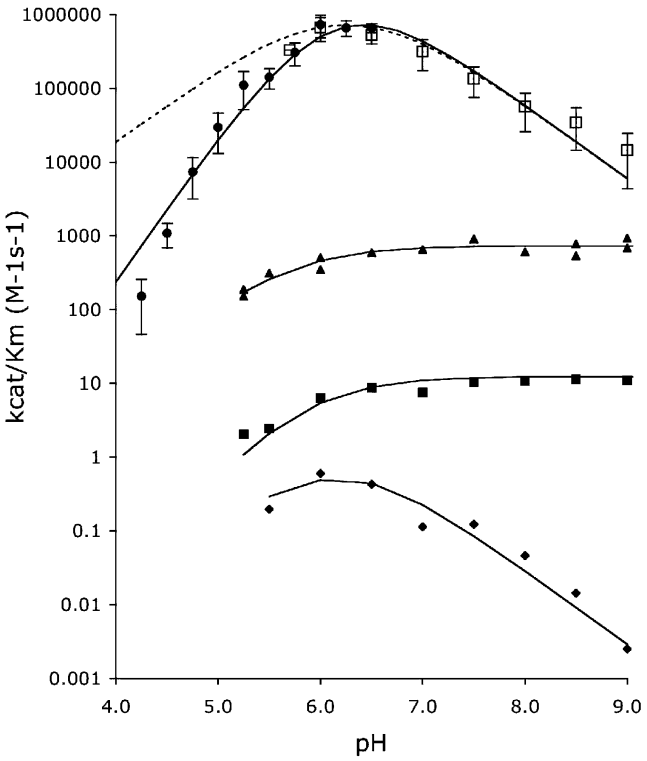


FIGURE 5: Cumulative pH-dependent data for Cdc25B with various substrates: α-naphthyl phosphate (◆), pNPP (■), mFP (▲), and Cdk2-pTpY−CycA (● and □). For Cdk2-pTpY−CycA, the data from pH 4.25 to 6.25 (●) are superimposed on the data from pH 5.7 to 9 (□) after correcting for the 50% reduced activity seen in 40% glycerol. The dotted line represents a hypothetical fit to a bell-shaped curve with only one acidic pK_a and one basic pK_a.

PTPs with the substrates pNPP (*61*) and *m*-nitrobenzyl phosphate (mNBP) (*62*). Although many aspects of this general mechanism for PTPs have been confirmed experimentally for the Cdc25 phosphatases using small molecule and protein substrates, the identity of the catalytic acid has remained puzzling.

A fact unique to the Cdc25s among the PTPs, no catalytic acid is required for substrates whose leaving groups have pK_a values of <8 (*63*, *64*). For both Cdc25A and Cdc25B, the pH dependence (k_{cat}/K_m and k_{cat}) between 6 and 9.5 is flat for the small molecule substrates pNPP and mFP (Figure 5). Isotope effect measurements have confirmed the lack of general acid catalysis for Cdc25A with pNPP as a substrate (*64*). As classically observed for substrates of serine proteases with good leaving groups (e.g., esters instead of amides), burst kinetics are observed for Cdc25 with the substrate mFP, but not pNPP, consistent with a two-step mechanism involving a phospho−cysteine intermediate (*36*, *65*) (Figure 4). Transfer of a phosphate from pNPP to generate the phospho−enzyme intermediate as determined using transition state theory ($\Delta G^{\ddagger} = 18$ kcal/mol at 298 K) is dominated by $\Delta H^{\ddagger}$ (20 kcal/mol), with a relatively small but favorable contribution from $T\Delta S^{\ddagger}$ (2 kcal/mol at 298 K) (*38*). This thermodynamic analysis of the reaction with pNPP is consistent with an active site preorganized for effective phosphoryl group transfer to Cys473 of Cdc25B with minimal conformational changes. This conclusion is consistent with existing structural data wherein the active site loop is fixed and identical in various forms of the enzyme (Figure 2C).

For the protein substrate Cdk2-pTpY−CycA and small molecule substrates with a leaving group pK_a of >8 (e.g., α-naphthyl phosphate and mNBP), the pH dependence (k_{cat}/K_m) for the Cdc25 phosphatases displays a bell-shaped curve typical of PTPs (Figure 5) (*64*, *66*). The slope of the acidic limb for protein substrate indicates two ionizations, as determined by stabilizing Cdc25B to pH conditions of <5.5 with glycerol concentrations of ≤50%. One pK_a of ∼5.9 reflects the catalytic cysteine (*63*), near the range seen for the catalytic cysteines of other PTPs (4.5−5.6) (*58*, *67*−*70*). The pK_a of the catalytic cysteine correlates well with the value of 5.9 determined by pH-dependent inactivation of activity by treatment with iodoacetic acid (*66*). The other pK_a (∼5.9) reflects the phosphoryl group of the substrate, as determined by comparison with thio-substituted protein substrate Cdk2-p$_S$Tp$_S$Y−CycA, in which the acidic limb of the bell-shaped profile reveals only one ionization (*42*). Thus, Cdc25s, like other PTPs, prefer a bisanionic over a monoanionic substrate. The slope of the basic limb indicates the ionization of the putative catalytic acid. Brønsted analysis and isotope effect measurements with mNBP support general acid catalysis by Cdc25A with small molecule substrates (*62*, *64*). Transfer of a phosphoryl group from Cdk2-pTpY−CycA to enzyme ($\Delta H^{\ddagger} = 11.6$ kcal/mol) is enthalpically more favorable than for the small molecule substrate pNPP, consistent with the involvement of a catalytic acid (*38*). Alternatively, the hydrophobic environment at the protein interface may favor the *m*-phosphate-like transition state typical in phosphoryl group transfer (*71*−*73*). Formation of the phospho−enzyme intermediate for protein substrate is entropically less favorable than for pNPP ($T\Delta S^{\ddagger} = -5.7$ kcal/mol at 298 K), consistent with rearrangements in the active site region that promote the subsequent rapid expulsion of the dephosphorylated protein product.

The identity of the putative catalytic acid indicated by the pH dependence and required by the chemistry has not been fully resolved for the Cdc25 phosphatases. As noted above, no sequence conservation for the Cdc25s with other PTPs exists, with the exception of the active site loop. Attempts to predict a fold for the Cdc25s prior to structure determinations erroneously identified Asp383 in Cdc25A as the catalytic acid on the basis of the severe reduction in activity of the D383N mutant (*74*). Because of the low activity of Cdc25s with non-aryl small molecule substrates, obtaining a convincing change in pH dependence for putative catalytic acid mutants has been difficult. That is, any mutations that result in reduced activity can typically not be thoroughly evaluated. The best data thus far have supported a catalytic role for Glu431 in Cdc25A for a substrate with a leaving group pK_a of 8.05, right at the limit of where the mechanism of catalysis changes from non-acid-catalyzed to acid-catalyzed (*64*). On the other hand, the corresponding mutation in Cdc25B (E474Q) results in an unchanged pH dependence with protein substrate, despite the significant reduction in activity (∼200-fold) (*63*). Mutation of other potential nearby catalytic acids (e.g., Glu478 or residues within the unstructured C-terminal tail) also yields an unchanged pH dependence. There are thus far no data supporting the hypothesis that protein substrate can provide the catalytic acid (*66*), despite the attractive location of Arg36 from Cdk2 near the active site in the docked model (*30*).

Open Questions

Despite the significant progress made toward understanding the detailed enzymology of the Cdc25 phosphatases, several unanswered questions that deserve further investigation remain. As noted above, the identity of the apparent catalytic acid in the reaction with the protein substrate has not been established unambiguously. Recall that the pH dependence ascribed to the catalytic acid is observed under k_{cat}/K_m conditions that reflect the association of the substrate with the enzyme. Thus, it is possible that some as yet unidentified residue on either Cdc25B or Cdk2-pTpY−CycA is required for successful protein docking. That is, the source of the observed pH dependence may not be a catalytic residue in the classical sense.

The field would also benefit from further in vitro studies of the specificity of Cdc25s using other Cdk/cyclin substrates. For example, the role of the conserved hotspot residues in the physiological pairings of Cdk1/cyclin B with Cdc25A and Cdc25C need to be elucidated. Are there also just these three hotspot residues, or are there additional specificity determinants? The difficulty in such experiments lies in the preparation of sufficient quantities of specifically phosphorylated protein substrates. Also, the binding site of the unstructured C-terminal tail on the Cdk/cyclin complex needs to be determined. It is tempting to speculate about the binding of the tail into the multipurpose recruitment site located on the cyclins, >40 Å from the site of dephosphorylation on the Cdks. This site has a known preference for binding protein substrates or inhibitors that contain an R*X*L motif (where R is arginine, *X* is any amino acid, and L is leucine), which is also found in the C-terminal tail of the Cdc25s (*75*−*80*). However, preliminary results with mutants that disrupt recognition in this pocket have no effect on dephosphorylation by Cdc25s (J. Rudolph, unpublished data), and thus, more work needs to be done. In a related question, the biochemical details of the feedback activation loop need to be elucidated given that the catalytic domains of Cdc25A and Cdc25B have the same activity toward Cdk2-pTpY−CycA as the full-length proteins (*32*) and that phosphorylation of full-length Cdc25A by Cdk/cyclins does not lead to an increase in phosphatase activity (J. Rudolph, unpublished data).

Finally, efforts to develop cancer therapeutics against the Cdc25 phosphatases must be reinitiated by taking into account the biochemical fundamentals of these elusive targets. Bis-phospho mimetics, proposed in the early stages as being potentially specific for Cdc25s because of the adjacent pThr14 and pTyr15 of the protein substrates, no longer seem reasonable in light of kinetic data and the docked model of the complex (Figure 3). Inhibitors directed at the featureless active site in general do not appear favorable, an observation made for many other protein phosphatases as well (*81*). The most favorable prospects for developing inhibitors against the Cdc25s lie in preventing enzyme−substrate association. Although inhibitors of protein−protein interactions are oft deemed inaccessible, Cdc25s appear to be a particularly tractable example. The dramatic effect of mutating the hotspot residues suggests that disruption of this single interaction is sufficient for inhibition. Perchance there exists a potential binding pocket for small molecules on Cdc25B directly adjacent to the hotspots (Figure 2D). Although none of the residues of Cdk2 protrude significantly into this pocket, the binding of a suitable ligand into the pocket could engage one or more of the hotspot residues at its lip. This pocket appears to be of adequate size (205 Å^3) by comparison to the binding pockets used by inhibitors of other protein−protein interactions (50−300 Å^3) such as FK506 (*82*) and the nutlins (*83*). In Cdc25B, the pocket is lined primarily by the side chains of 13 residues that are mostly conserved across the three human isoforms. Thus, core inhibitor structures that inhibit all three Cdc25s and thereby serve as general antiproliferation agents could be developed. More interesting for dissecting the biology of the Cdc25s and potentially developing more specific anticancer agents that target tumors with Cdc25 overexpression is the fact that there are selected differences in the pockets of the three Cdc25s that can be exploited in developing isoform-specific inhibitors. For example, Met505 sits at the base of the pocket in Cdc25B and is replaced with a leucine in both Cdc25A and Cdc25C. Alternatively, the hydrogen bond donor and acceptor Tyr382 in Cdc25B is a Phe in both Cdc25A and Cdc25C. Experimental and computational screening efforts to exploit this potential inhibitor binding pocket are currently underway in our laboratory and others.

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