The C-Terminal Tail of the Dual-Specificity Cdc25B Phosphatase Mediates Modular Substrate Recognition†

Manuel Wilborn,‡ Sarah Free,‡ Andrew Ban, and Johannes Rudolph*

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT: Cdc25 is a dual-specificity phosphatase that catalyzes the activation of the cyclin-dependent kinases (Cdk/cyclins), thus triggering initiation and progression of successive phases of the cell cycle. In our efforts to elucidate the interaction between Cdc25B and the natural substrate, bis-phosphorylated Cdk2/CycA (Cdk2-pTpY/CycA), we have previously found that the 17 residues of the C-terminal tail mediate a factor of 10 in substrate recognition. In the studies reported here, we localize the majority of this interaction using site-directed mutagenesis to two arginine residues (Arg556 and Arg562) located within this C-terminal region. We also show that the catalytic domain of Cdc25C, which differs most significantly from Cdc25B in this tail region, has a 100-fold lower activity toward Cdk2-pTpY/CycA. We further demonstrate that the proper presentation of the C-terminal tail of Cdc25B can be achieved in a "gain-of-function" chimeric protein consisting of the C-terminal tail of Cdc25B fused onto the catalytic core of Cdc25C. The >10-fold increase in activity seen only in the chimeric protein containing the two critical arginine residues demonstrates that the modular C-terminal tail of Cdc25B is the basis for most of the catalytic advantage of Cdc25B versus Cdc25C toward the Cdk2-pTpY/CycA substrate.

Kinases and phosphatases are responsible for much of the signal transduction and regulation that control cellular processes such as proliferation and differentiation. Each of the many kinds of kinases and phosphatases performs very simple reactions, namely, the transfer of a phosphate moiety to or from a serine, threonine, or tyrosine residue. Given the similarity of these reactions, the large number of these reactions that occur, and the necessity of avoiding undesired cross-talk, the exquisite control of substrate specificity is of prime importance. In the case of protein kinases and protein phosphatases, two primary mechanisms of substrate recognition have been discovered and extensively characterized. First, specificity can be mediated by the recognition of the primary sequence surrounding the site of phosphorylation. For example, the cyclin-dependent kinase (Cdk)[1] and mitogen-activated protein (MAP) kinase family members recognize a Ser/Thr-Pro motif, whereas protein kinases A and C (PKA and PKC, respectively) prefer an Arg/Lys/His motif (*1*). Modular domains that promote protein−protein interactions are the second mechanism for specific substrate recognition by protein kinases and protein phosphatases. The SHP-1 and SHP-2 phosphatases, for example, contain SH2 domains that target these phosphatases to their substrates, the receptor-associated protein tyrosine kinases (*2*).

However, there exist many interactions between protein kinases and protein phosphatases and their substrates that do not appear to be mediated by primary sequence motifs or modular domains. These types of interactions appear to be mediated by so-called "docking sites" and do not necessarily contain apparent conserved sequence motifs (*3*). These docking sites are especially hard to characterize given that the interactions between the enzymes and their protein substrates are transient during enzymatic catalysis, and thus, often no stable complexes can be isolated. We can speculate that these docking sites lead to the formation of a proper active site only upon formation of an interaction complex between the enzyme and its intact protein substrate. Thus, the enzymes in this class are expected to exhibit poor reactivity with peptidic substrates that do not provide the docking elements of the protein substrates. Cdc25, a phosphatase involved in cell cycle control, appears to fall into this category of protein phosphatases (*4*).

Cdc25 is the phosphatase that dephosphorylates and activates the central regulators of the eukaryotic cell cycle, the cyclin-dependent kinases (Cdks), thus triggering the initiation and progression of successive phases of the cell cycle (*5*). Three homologues of Cdc25 exist (Cdc25A, Cdc25B, and Cdc25C), each appearing to act on different Cdk/cyclin complexes and at different stages of the cell cycle. Cdc25C dephosphorylates Cdc2/CycB, thus triggering actual cell division at the G2/M transition. Cdc25A and Cdc25B are suggested to act on Cdk2/CycE, Cdk2/CycA, and/or

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* To whom correspondence should be addressed. Phone: (919) 668-6188. Fax: (919) 613-8642. E-mail: rudolph@biochem.duke.edu.

‡ These authors contributed equally to this work.

[1] Abbreviations: Cdk/cyclin, cyclin-dependent kinase; Cdk2/CycA, cyclin-dependent kinase 2/cyclin A; mFP, 3-*O*-methylfluorescein phosphate; pNPP, *p*-nitrophenyl phosphate; pT, phosphothreonine; pY, phosphotyrosine; Δ25B1, catalytic domain of Cdc25B (residues 377−548); Δ25B2, catalytic domain of Cdc25B (residues 377−566); Δ25B3, catalytic domain of Cdc25B (residues 377−562); Δ25C1, catalytic domain of Cdc25C (residues 280−453); Δ25C2, catalytic domain of Cdc25C (residues 280−473); DBL, R556L and R562L mutations in the context of the Δ25B2 construct; Δ25CB, C-terminal tail of Cdc25B (residues 549−566) fused onto the catalytic core of Cdc25C (residues 280−453); Δ25CB-DBL, R556L and R562L mutations in the context of the Δ25BC construct.

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Cdc2/CycB in G1/S through late S phases. Although Cdc25C appears to be the key mitotic regulator at the G2/M transition, it is not an essential gene in mice (*6*), and it is Cdc25A and Cdc25B that have been directly implicated in cancer and tumor development.

Cdc25 belongs to the class of dual-specificity phosphatases as it dephosphorylates both the phosphothreonine (Thr14) and phosphotyrosine (Tyr15) residues located in the Gly-rich loop of the Cdks (for a recent review on phosphatases, see ref *7*). As a member of the dual-specificity phosphatase family, Cdc25 proceeds via a two-step mechanism that involves formation of a phosphocysteine intermediate utilizing the active-site sequence motif HCX_5R, where H is a histidine residue, C is the catalytic cysteine, and the five X residues form a phosphate binding loop along with R, a highly conserved arginine. Recently, the structures of the catalytic domains of two of the three isoforms of Cdc25 have been determined (*8*, *9*). As predicted by homology modeling (*10*), Cdc25A and Cdc25B catalytic domains form a small α/β-domain with a central five-stranded parallel β-sheet sandwiched by three α-helices from below and two α-helices from above. Both of these structures have a disordered C-terminal region, consisting of 31 of 189 amino acids in the catalytic domain of Cdc25A and 18 of 211 amino acids in the catalytic domain of Cdc25B.

Cdc25A is a notoriously slow enzyme with most artificial substrates and phosphorylated peptides ($k_{cat}/K_m = 1-20$ M^{-1} s^{-1}) (*4*). However, it is a highly efficient phosphatase toward the natural bis-phosphorylated Cdk2/cyclinA substrate (Cdk2-pTpY/CycA) with a k_{cat}/K_m of $>1 \times 10^6$ M^{-1} s^{-1}. Given this dramatic difference in rates between phosphorylated peptides and the intact protein substrate, Cdc25A does not belong to the class of phosphatases that relies solely on recognition of the primary sequence at the site of dephosphorylation. The catalytic domain of Cdc25B has a similar low reactivity toward artificial substrates and high reactivity toward the natural substrate Cdk2-pTpY/CycA, whereas the catalytic domain of Cdc25C is significantly less active toward the Cdk2-pTpY/CycA substrate (*4*). We have recently shown that the 17-amino acid C-terminal tail mediates part of the higher reactivity of Cdc25B toward natural substrates (*11*). This tail, although structurally not defined in either the Cdc25A or Cdc25B crystal structure, nor having any known sequence motifs, provides 1 order of magnitude in rate enhancement toward Cdk2-pTpY/CycA and no significant change in activity toward artificial substrates. We have sought to understand the nature of this C-terminal-mediated substrate recognition using site-directed mutagenesis and chimeric Cdc25s using Cdc25B and Cdc25C.

EXPERIMENTAL PROCEDURES

Reagents. *p*-Nitrophenyl phosphate (pNPP) and 3-*O*-methylfluorescein phosphate (mFP) were purchased from Sigma. The pET3a vector and Pfu polymerase were purchased from Stratagene. The [γ-^{32}P]ATP, SP-Sepharose, and S-200 Superdex chromatography resins were obtained from Amersham-Pharmacia. Peptides were prepared by the Tufts University Sequencing Facility and purified by HPLC to >90% purity. All other reagents were of the highest grade commercially available.

Cloning of Cdc25B and Cdc25C. Cloning of the catalytic domain constructs Δ25B1 (residues 377−548) and Δ25B2 (residues 377−566) has been reported previously (*11*). Site-directed mutagenesis of the C-terminal region of Δ25B2 was performed using the Quick-Change method from Strategene with the primers listed in Table 1. The double mutant R556L/R562L was prepared from the R556L single mutant using the R562L primers listed in Table 1. The catalytic domain constructs Δ25B3 (residues 377−562 of Cdc25B), Δ25C1 (residues 280−453 of Cdc25C), and Δ25C2 (residues 280−473 of Cdc25C) were constructed as described for the catalytic domains of Cdc25B with the primers listed in Table 1 (*11*). The chimeric Δ25CB consisting of the C-terminal tail of Cdc25B (residues 549−566) fused onto the catalytic core of Cdc25C (residues 280−453) was constructed by fusion PCR. First, the Cdc25B and Cdc25C parts were amplified using the normal C- and N-terminal primers, respectively, and the Δ25CB primers listed in Table 1. Next, the amplified products were mixed together and re-amplified with the normal C- and N-terminal primers from Cdc25B and Cdc25C, respectively. The double arginine mutant in the context of Δ25CB-DBL was constructed by individual mutations, a process analogous to its construction in the context of Δ25B2. All constructs were sequenced in their entirety to ensure their integrity.

Table 1: Primer Pairs Used in the Preparation of the Site-Directed Mutants of Cdc25B, the Cloning of Δ25C1 and Δ25C2, the Catalytic Domain Constructs of Cdc25C and Δ25CB, and the Chimeric Cdc25B/Cdc25C Catalytic Domain

Mutation or clone	Primer sequences
Δ25B3	GACAGTGACCCCATGGAGCTGATTGG
	GGAGGGAAGCTTACTGGTCCTGCTACCGGC
W550F	GACTCGCAGCTtcGCTGGGGAGCGGAGCCGG
	CTCCCCAGCgaAGCTGCGAGTCTTGAGGCG
W550A	GACTCGCAGCgcGGCTGGGGAGCGGAGCCGG
	CTCCCCAGCCgcGCTGCGAGTCTTGAGGCG
A551E	CGCAGCTGGGagGGGGAGCGGAGCCGGCGG
	CCGCTCCCCctCCCAGCTGCGAGTCTTGAGG
S555A	GGGGAGCGGgcCCGGCGGGAGCTCTGTAGC
	CTCCCGCCGGgcCCGCTCCCCAGCCCAGC
R556L	GAGCGGAGCCtGCGGGAGCTCTGTAGCCGG
	GAGCTCCCGCaGGCTCCGCTCCCCAGCCC
S561A-	CGGGAGCTCTGTgcCCGGCTGCAGGACC
	CTGCAGCCGGgcACAGAGCTCCCGCCG
R562L	CGGGAGCTCTGTAGCCtGCTGCAGGACC
	GTCCTGCAGCaGGCTACAGAGCTCCCG
Δ25C1	GATTCTACCATGGGGCACCTGATTGG
	CCGCTCCCAAGCTTACACTTTGCTCTGGC
Δ25C2	GATTCTACCATGGGGCACCTGATTGG
	GGCTGGAAGCTTATCATGGGCTCATGTCC
Δ25CB	AGCCAGAGCAAAGTGTGGGCTGGGGAGCGG
	CCGCTCCCCAGCCCACACTTTGCTCTGGC

Expression, Purification, and Assays of Cdc25B and Cdc25C. Expression of all of the constructs was performed in *Escherichia coli* BL-21(DE3) cells as described previously, except that the double arginine mutant in the context of the chimera (Δ25CB-DBL) was grown at 18 °C. All steps in the purifications were performed at 4 °C as previously reported, except that an S-75 Sepharose column was used in the purification of the Cdc25C and chimeric constructs (*11*). All phosphatase reactions were performed in a three-component buffer system [50 mM Tris, 50 mM Bis-Tris, and 100 mM sodium acetate (pH 6.5)] containing 1 mM DTT at 25 °C. Reactions using *p*-nitrophenyl phosphate (pNPP) and 3-*O*-methylfluorescein phosphate (mFP) were followed by continuous UV−vis spectroscopy at 410 nm ($\epsilon = 5142$ M^{-1} cm^{-1}, at pH 6.5) and 477 nm ($\epsilon = 27\,200$ M^{-1} cm^{-1}), respectively. Assays using mFP at enzyme concentrations of 1−5 nM contained 0.01% Tween-20 to prevent the loss

of enzyme activity. All assays using pNPP and mFP consisted of complete K_m determinations using at least eight concentrations of substrate between $0.2K_m$ and $5K_m$ and were fitted as described previously (*11*). Reactions with the natural substrate Cdk2-pTpY/CycA were performed using 0.2−10 nM enzyme in the presence of 1 mg/mL bovine serum albumin as described previously (*11*). Assays using Cdk2-pTpY/CycA were limited to k_{cat}/K_m determinations at 100−300 nM substrate because of the limiting amounts and concentrations of the substrate. The time dependence (five points minimum), substrate concentration dependence (two differing concentrations), and enzyme concentration dependence (three to five differing concentrations) were tested and ensured for each assay. Burst experiments were performed using an Applied Photophysics stopped-flow instrument by monitoring the formation of product at 477 nm for the substrate mFP following a 1.3 ms mixing time in a 10 mm cuvette. Enzyme concentrations ranged from 1 to 5 μM, and substrate concentrations of mFP ranged from 31 to 500 μM. The primary burst data were analyzed as described previously (*11*), and the rate and amplitude of the burst at 250 μM mFP were used to determine the active site concentration for purposes of calculating turnover numbers.

CD Spectroscopy. Circular dichroism (CD) spectra were collected on an Aviv model 202 CD spectrophotometer with 10−20 μM peptide in 20 mM sodium phosphate buffer (pH 7.0) with 80 mM NaCl at 4 °C. Wavelength traces were scanned from 350 to 200 nm in a 1 mm cuvette at 1 nm increments with a bandwidth of 1 or 1.5 nm and averaged over a time of 5 s. The mean residue ellipticity was calculated from the absorbance at 222 nm in comparison to a peptide of the same length with 100% α-helicity.

RESULTS AND DISCUSSION

Experimental Rationale. The experiments and results described in this paper are part of our ongoing efforts to understand the basis for the efficient recognition by the Cdc25A and Cdc25B phosphatases of Cdk2-pTpY/CycA, a protein substrate whose k_{cat}/K_m indicates that it is dephosphorylated 5 orders of magnitude faster than the corresponding phosphopeptide (*4*). In our recent efforts to identify the catalytic acid of Cdc25B responsible for donating a proton to the leaving group (i.e., hydroxyl of Thr14 and/or Tyr15 of Cdk2), we observed that the catalytic domain lacking the last 17 residues (Δ25B1) was 1 order of magnitude slower at dephosphorylating Cdk2-pTpY/CycA than the full catalytic domain (Δ25B2). Yet the shortened Cdc25B (Δ25B1) was equally as active toward small molecule substrates such as Δ25B2. Although the 17 residues that comprise the C-terminal tail of Cdc25B appear to be unstructured in the crystal structure of the catalytic domain (*9*), this observation suggests that they are important specifically for the interaction between the protein substrate Cdk2-pTpY/CycA and Cdc25B, possibly by adopting a substrate-induced conformation. To localize and better understand the source of this effect, we decided to prepare a series of site-directed mutations in the C-terminal tail. To avoid having to make all 18 possible mutations of the C-terminal tail region, we started with a four-amino acid truncation to create Δ25B3. We then took into account the previous observation that both Cdc25A and Cdc25B are similarly efficient at dephosphorylating Cdk2-pTpY/CycA whereas Cdc25C is 2−3 orders of magnitude less efficient (*4*). The catalytic domains of the three human Cdc25s possess very high levels of sequence homology (Cdc25B and Cdc25C being 63% identical) with the greatest divergence within the C-terminal region (Cdc25B and Cdc25C only 33% identical) (Figure 1). We therefore speculated that at least part of the catalytic advantage of Cdc25A and Cdc25B versus Cdc25C arises from this difference. Thus, in our site-directed mutagenesis of the C-terminal tail, we chose to mutate only those residues that were identical in Cdc25A and Cdc25B, but different in Cdc25C (Figure 1). In a second approach to understanding the role of the C-terminal tail of Cdc25 in mediating substrate recognition, we took advantage of the dramatic difference in the activities between Cdc25B and Cdc25C toward Cdk2-pTpY/CycA. We were able to create a "gain-of-function" chimeric protein consisting of the C-terminal tail of Cdc25B fused to the catalytic core of Cdc25C, thus showing the modularity of the catalytic advantage of the C-terminal tail of Cdc25B as described in detail below.

Cdc25A	506	- T W A G E K S K R E M Y S R L K K L
Cdc25B	549	- S W A G E R S R R E L C S R **L Q D Q**
Cdc25C	453	- V Q E G E R Q L R E Q I A L L V K D M S P
Consensus		G E + R E L

FIGURE 1: Sequence alignments of the C-terminal regions of Cdc25A, Cdc25B, and Cdc25C. Residues of Cdc25B that have been deleted (to create Δ25B3) are denoted in bold, and those that have been mutated (in Δ25B2) are underlined.

Cdc25B Tail Mutants. We initially prepared one truncation mutant and seven site-directed mutants of the C-terminal tail in the context of the Δ25B2 catalytic domain. Given that the three Cdc25s show no significant degree of homology at the far C-terminal end, we started with a truncation mutant, Δ25B3, lacking the last four amino acids of Δ25B2. Each of the site-directed mutations we chose was intended to be conservative in terms of steric effects but dramatic in terms of electrostatics or hydrogen-bonding ability, reflective of the comparison between Cdc25B and Cdc25C (Figure 1). Thus, both Arg556 and Arg562 were mutated to Leu, whereas Ser555 and Ser561 were changed to Ala. Using the C-terminal tail of Cdc25C as a guide, we changed Ala551 to Glu. Trp550 was changed to both Phe and the less conservative Ala to probe a potential hydrophobic requirement. All mutant proteins were expressed in *E. coli* using the same protocol that was used for the wild-type protein and purified to homogeneity with similar yields. Both the W550A and W550F mutants exhibited faster migration on SDS−PAGE, which was not due to N- or C-terminal truncation as confirmed by LC−MS (data not shown). We later prepared the mutant containing the R556L/R562L double mutation (DBL), and it was also purified to homogeneity.

Initially, proteins with the C-terminal tail truncation or mutations were characterized for their activity toward the artificial substrates *p*-nitrophenyl phosphate (pNPP) and 3-*O*-methylfluorescein phosphate (mFP). As shown in Figure 2, the k_{cat} and k_{cat}/K_m values varied less than 1.5-fold for all of these mutants using either of these substrates. It should be noted that the substrate pNPP is a poor substrate and its kinetic parameters reflect the early steps of the kinetic reaction (binding and phosphate transfer to the catalytic cysteine). On the other hand, mFP is a much more potent substrate with the k_{cat} reflecting the rate-determining hy-

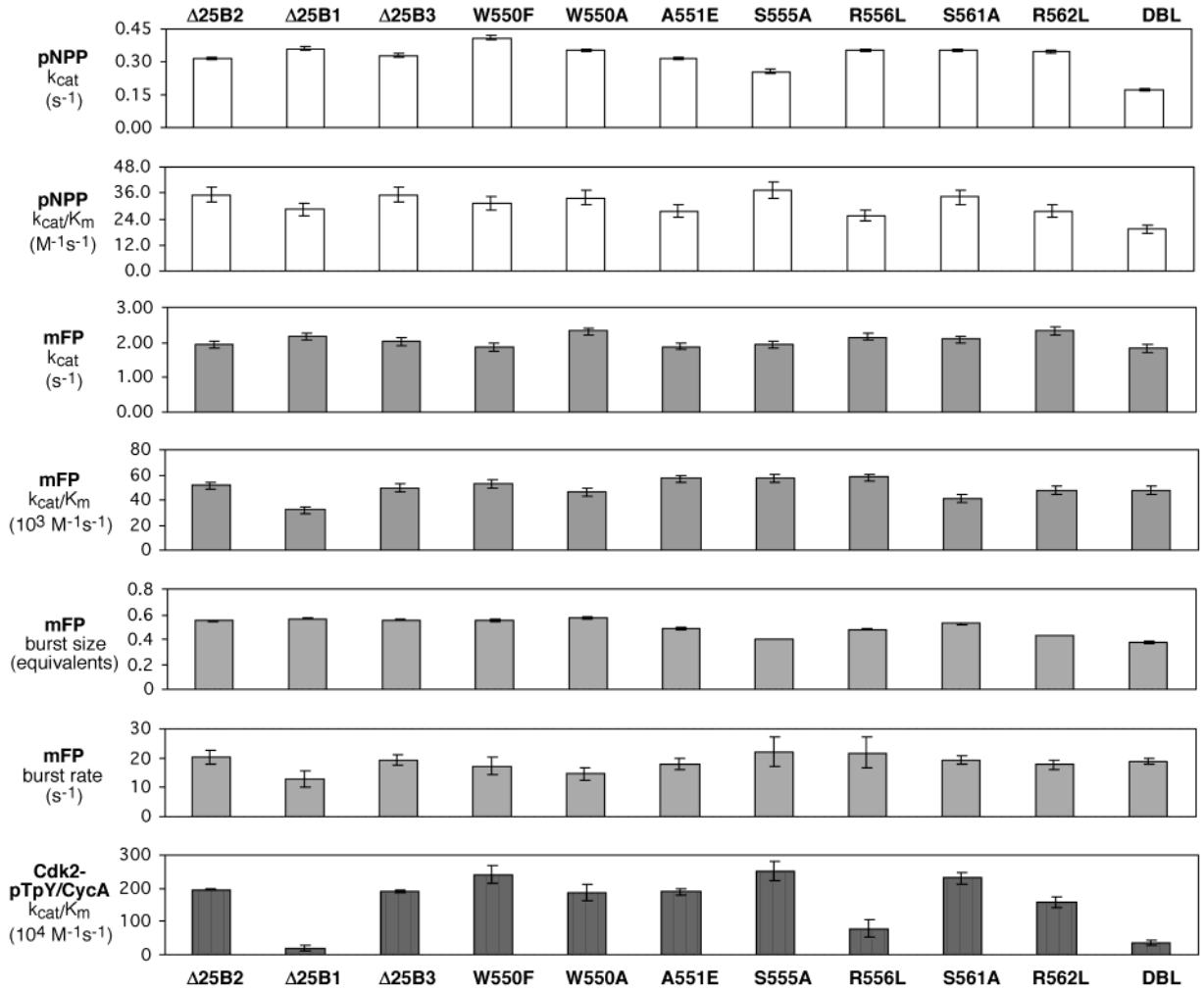

FIGURE 2: Summary of the kinetics of the catalytic domains of Cdc25B (Δ25B1 vs Δ25B2 vs Δ25B3) and the various site-directed mutants of Δ25B2 [W550F, W550A, A551E, S555A, R556L, S561A, R562L, and DBL (both R556L and R562L)]. The k_{cat} and k_{cat}/K_m values for the artificial substrates pNPP and mFP are shown in the top four rows. Rows 5 and 6 show the burst size and burst rate from the stopped-flow experiments with mFP, respectively. The last row shows the k_{cat}/K_m values of the various constructs and/or mutants for the Cdk2-pTpY/CycA substrate.

drolysis of the phosphocysteine intermediate (*11*). To further confirm the integrity of these mutants, we assayed each of them in a one-turnover burst experiment and found that the rate of burst formation and size of the burst at the fixed concentration of 250 μM mFP again varied less than 1.5-fold from the values found for the wild-type protein (Figure 2). Overall, the results of the kinetic assays using the artificial substrates pNPP and mFP were expected given that there had been no dramatic differences seen for complete removal of the C-terminal tail with the artificial substrates.

Next, the proteins were tested for their activity toward the Cdk2-pTpY/CycA protein substrate. As reported previously, the removal of the C-terminal tail in the Δ25B1 construct leads to a 10-fold drop in activity toward the natural substrate (Figure 2). Removal of only the last four amino acids (Δ25B3), on the other hand, did not affect the activity toward Cdk2-pTpY/CycA. Only two of the site-directed mutants gave significantly altered activities toward Cdk2-pTpY/CycA, namely, R556L and R562L. Although these changes did not appear to be large, they are highly reproducible from day to day and from one enzyme preparation to another. We therefore decided to pursue the simultaneous mutation of both R556 and R562 to leucines (DBL). Again, against the artificial substrates pNPP and mFP, the DBL mutant exhibited kinetic parameters that were reduced less than 2-fold compared to those of the wild-type protein. On the other hand, using the Cdk2-pTpY/CycA substrate, the effects of the individual mutations appeared to be additive and revealed an activity for DBL approaching that of Δ25B1, the construct lacking the entire C-terminal tail. Thus, it appears that these two arginine residues account for much of the catalytic advantage of the C-terminal tail in the reaction of Cdc25B with Cdk2-pTpY/CycA.

Inhibition and Activation by Peptides. We noticed that the C-terminal region of Cdc25B has a propensity toward α-helicity, as confirmed using the AGADIR program (*12*). It was therefore conceivable that free peptide containing this C-terminal region could adopt a conformation whereby it would act either as an activator of Δ25B1 or as an inhibitor of Δ25B2 using the substrate Cdk2-pTpY/CycA. In the case of Δ25B2, binding of such a peptide to Cdk2-pTpY/CycA could compete with binding of the actual C-terminal tail, thus inhibiting the reaction of Δ25B2 with Cdk2-pTpY/CycA. In the case of the Δ25B1, addition of high concentrations of the peptide could conceivably act synergistically with the truncated form of Cdc25 to promote catalysis using the protein substrate. We therefore prepared a peptide containing both of the critical Arg residues (C-term+) as well as a

Table 2: C-Terminal Peptides Tested as Activators of Δ25B1 or as Inhibitors of Δ25B2 in the Reaction with Cdk2-pTpY/CycA

peptide name	peptide sequence	α-helicity (%)
C-term+	E-R-S-R-R-E-L-C-S-R-L-Q	7.2
C-term−	E-R-S-L-R-E-L-C-S-L-L-Q	17.6
C-alpha	E-R-A-R-R-E-L-A-S-R-L-Q	24.5

control peptide with both of the Arg residues changed to Leu (C-term−) (Table 2). We also prepared a peptide containing selected mutations predicted to increase the α-helical character of this region (C-alpha) (*12*). The α-helicity of these peptides was confirmed using CD spectroscopy under aqueous buffer conditions and showed that a significant percentage of the peptides adopted an α-helical conformation (Table 2). However, when tested as activators of Δ25B1 or as inhibitors of Δ25B2 in reactions with Cdk2-pTpY/CycA, none of the peptides had a significant effect (<20% change in activity) up to a final concentration of 2.5 mM (data not shown). These results demonstrate that the catalytic advantage conferred by the C-terminal tail relies on proper presentation of this sequence, i.e., attachment to the C-terminus of Cdc25B. Whether the C-terminal region of Cdc25B is actually α-helical in solution remains unknown.

Cdc25C Constructs. To study the role of the C-terminal tail of Cdc25C and more quantitatively analyze the differences between the Cdc25B and Cdc25C catalytic domains using both artificial and natural substrates, we prepared the analogous catalytic domain constructs of Cdc25C, namely, Δ25C1 (residues 280−453) and Δ25C2 (residues 280−473). The proteins were purified to homogeneity using a protocol similar to that for the Cdc25B constructs except an S-75 column was used in the final step to better separate truncated forms of the proteins that would occur in some of the preparations. The catalytic domains of Cdc25C were stable and isolable in yields ranging from 1 to 5 mg/L of cell culture.

The Cdc25C constructs were initially characterized for their activity toward the artificial substrates pNPP and mFP. As shown in Figure 3, the k_{cat} and k_{cat}/K_m values were again less than 1.5-fold different for both artificial substrates. The kinetic parameters of Cdc25C were further analyzed using burst experiments with mFP performed and fitted as described previously (*11*). These data showed that Cdc25C, like Cdc25A and Cdc25B, exhibits burst kinetics in the reaction with mFP. The rates of burst formation are identical for the two catalytic domains of Cdc25C and thus independent of the presence of the C-terminal tail. In the comparison of the Cdc25C catalytic domains to Cdc25B, Cdc25C not only has a slower k_{cat} (2-fold), indicating slower hydrolysis of the phosphoenzyme intermediate, but also has a slower rate of burst formation (3-fold), indicating slower phosphate transfer to the enzyme (Figure 3). Thus, the ratio of k_{burst} to k_{cat} is 10 for Cdc25B and 7 for Cdc25C. Various catalytic domains of Cdc25A have ratios of the rates of burst formation to intermediate hydrolysis (k_{burst}/k_{cat}) essentially identical to those of Cdc25B and Cdc25C (unpublished data). The constant ratio of rates for the two steps of the catalytic cycle among the Cdc25s brings to mind the evolution of catalytic efficiency and balanced internal equilibria as studied in detail by Fersht using tyrosyl-tRNA synthetase (*13*). There

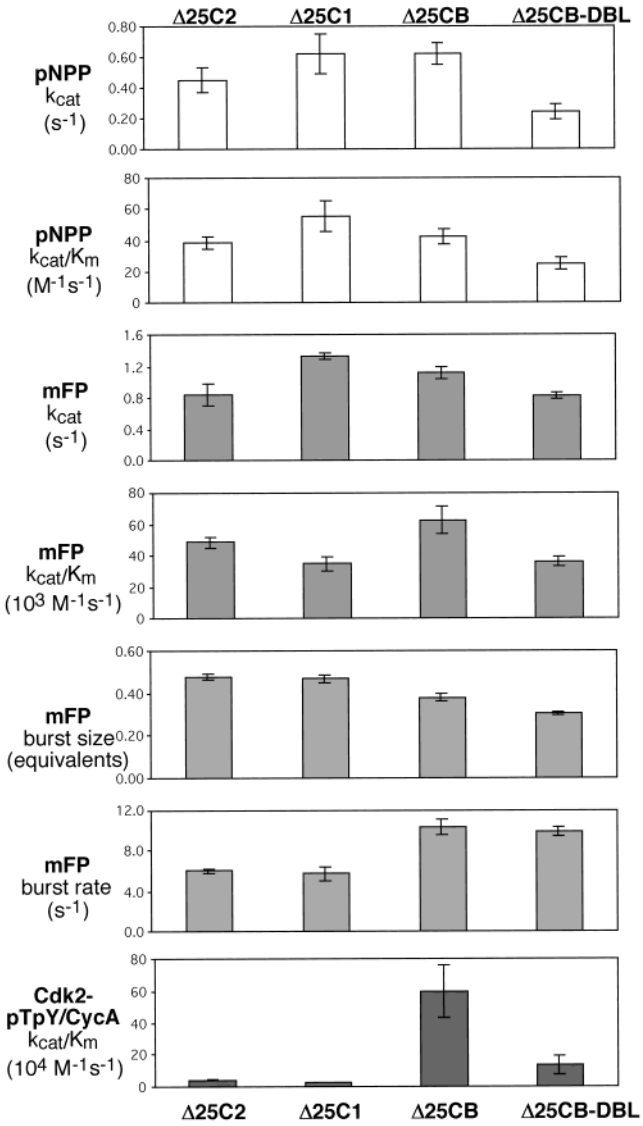

FIGURE 3: Summary of the kinetics of the catalytic domains of Cdc25C (Δ25C1 vs Δ25C2), the chimeric Cdc25 protein (Δ25CB), and its site-directed mutant of the two arginine residues (Δ25CB-DBL). The k_{cat} and k_{cat}/K_m values for the artificial substrates pNPP and mFP are shown in the top four rows. Rows 5 and 6 show the burst size and burst rate from the stopped-flow experiments with mFP, respectively. The last row shows the k_{cat}/K_m values of the various constructs and/or mutants for the Cdk2-pTpY/CycA substrate.

exists no need to increase the rate through the fast step of the reaction (e.g., transfer of phosphate from substrate to enzyme) further if a slower step (e.g., hydrolysis of the phosphoenzyme intermediate) is already rate-limiting. The essentially constant ratio of the rate of phosphate transfer to the enzyme and the breakdown of the phosphoenzyme intermediate among the Cdc25s may also be indicative of the functioning in vivo concentration of phosphorylated Cdk/cyclin complexes. That is, the substrate concentration would be expected to be less than 100 nM such that there exists a steady flux through the enzyme with no buildup of the phosphoenzyme intermediate. This expected concentration agrees well with the maximal in vivo concentrations of the Cdk/cyclin complexes of 2−10 nM (*14*). To summarize the comparison between Cdc25B and Cdc25C, despite the large amount of sequence identity shared between them, Cdc25B is a more active protein toward artificial substrates.

In measuring the activity of Δ25C1 and Δ25C2 using Cdk2-pTpY/CycA as the substrate, we found that the two catalytic domains of Cdc25C were very similar to one another (Figure 3). That is, the tail region of Cdc25C provides a less than 1.5-fold advantage in the activity toward Cdk2-pTpY/CycA, in contrast to what we found for Cdc25B. Also, the dramatically slower turnover by Cdc25C, compared to that of Cdc25B, with Cdk2-pTpY/CycA (100-fold) should be noted. Some of this difference (2−3-fold) can be attributed to the less optimal activity of Cdc25C compared to that of Cdc25B as detected in the mFP burst experiments. However, most of the difference between Cdc25B and CDc25C is observed only with the protein substrate. Given that the natural substrate for Cdc25C is expected to be Cdc2-pTpY/CycB, the much reduced activity of Cdc25C toward Cdk2-pTpY/CycA indicates the potential for the specificity of the Cdc25s to be regulated by specific recognition of the physiological protein substrate complex.

Chimeric Cdc25B/Cdc25C. The major disadvantage of most mutagenesis approaches to studying structure and function in enzymology is that many mutants have decreased activity compared to the wild-type enzyme, an observation that can have multiple noninformative origins such as protein misfolding or instability. Fortunately, our mutagenic approach to the tail regions of Cdc25B and Cdc25C contained an internal control in that the activities of all the mutants were essentially identical toward the artificial substrates pNPP and mFP. This indicated that the active sites were well-formed and that these mutant proteins were catalytically intact. All significant differences in activity we observed were restricted to the assay with Cdk2-pTpY/CycA, and thus, we were able to conclude that both Arg556 and Arg562 play crucial roles in the recognition of natural substrate by Cdc25B, but not in the recognition of artificial substrates.

An alternative approach to standard site-directed mutagenesis available when one has homologous proteins is the construction of chimeric proteins that exhibit a gain of function. These types of experiments can be very informative because an increase in activity is usually associated with a specific positive interaction. To obtain additional confirmation of the role of the C-terminus of Cdc25B in recognition of the protein substrate, we constructed a chimeric protein consisting of the catalytic core of Cdc25C and the C-terminus of Cdc25B to see if we could confer a catalytic advantage to Cdc25C in its interaction with Cdk2-pTpY/CycA. By carefully comparing the kinetics of all our Cdc25 catalytic domain constructs using mFP and Cdk2-pTpY/CycA, we were able to show that the C-terminus of Cdc25B mediates most of Cdc25B's catalytic advantage compared to Cdc25C in the reaction with Cdk2-pTpY/CycA.

Δ25CB (residues 549−566 of Cdc25B fused onto residues 280−453 of Cdc25C) was purified as were the Cdc25C catalytic domain constructs with a yield of 1−2 mg/L of cell culture. The protein was extremely labile and hard to purify intact as it was subjected to proteolytic clipping, to a length equivalent to the core Δ25C1 construct. This suggests that the C-terminal tail derived from Cdc25B has a fair amount of flexibility in at least the linker region. As expected, in assays using pNPP or mFP, the chimeric protein showed no significant changes in k_{cat} and k_{cat}/K_m values in comparison to Δ25C2 (Figure 3). These similarities in activity extend to the burst amplitudes and burst rates using mFP, providing additional evidence that the chimeric protein is similar to its Cdc25C-derived parent.

When assayed with the protein substrate Cdk2-pTpY/CycA, Δ25BC exhibited a dramatic (>10-fold) increase in activity compared to Δ25C2, which corresponds to a 20-fold greater activity than the Δ25C1 construct with no C-terminal tail. Although the C-terminus of Cdc25B has no effect as a free peptide in solution, it appears to be sufficiently modular that it can be attached to the core of Cdc25C and thus lead to a very significant increase in activity toward the Cdk2-pTpY/CycA substrate. Given that this increased activity was seen only with the protein substrate, it can specifically be attributed to better recognition of the Cdk2-pTpY/CycA complex by contacts between the C-terminal tail derived from Cdc25B and the protein substrate. This striking increase in activity for the chimeric protein leads to a k_{cat}/K_m that is ∼6-fold slower than for the Δ25B construct. Actually, as discussed above, given that the active site of Cdc25C is 2−3-fold less suited for phosphatase activity, it could be claimed that we have come to within 50% of the theoretical full activity of Cdc25C with the Cdk2-pTpY/CycA complex in the chimeric construct. Thus, most of the difference between Cdc25C and Cdc25B in recognition of Cdk2-pTpY/CycA lies in the region that is most disparate between these two proteins, namely, the C-terminal tail.

To confirm the significance and interpretation of these results, i.e., that these increases in activity as mediated by the C-terminal tail can be attributed to specific recognition of or activation by Cdk2-pTpY/CycA, we prepared and assayed the corresponding double arginine mutant of Δ25CB, namely, Δ25CB-DBL. This protein could only be isolated in small amounts as it tended to form inclusion bodies during expression, perhaps a sign of a less stable protein, and was also subject to significant proteolytic digestion during its preparation. Although the mutant chimeric protein appeared to have partially reduced activity using pNPP, in assays using mFP it showed no significant changes in k_{cat}, k_{cat}/K_m, burst amplitude, or burst rate in comparison to those of Δ25CB (Figure 3). In accord with our predictions, however, the reactivity of Δ25CB-DBL toward Cdk2-pTpY/CycA dropped back almost to the level seen for Cdc25C with its own native tail (Δ25C1). The residually higher activity seen in the DBL mutants in the contexts of both Δ25B2 (Figure 2) and Δ25CB (Figure 3) suggests that in addition to the two arginines, we are still missing one or more residues that are responsible for a minor component of the interaction. These will be extremely difficult to identify given their small overall effect within the reproducibility of the assays. However, using the nomenclature of Wells, we believe these two arginine residues can be termed the "hot spot" of the protein interaction surface (*15*).

In conclusion, we have characterized the effect of the C-terminal tail of Cdc25B in its reactivity with the natural substrate Cdk2-pTpY/CycA. We have shown that two Arg residues (R556 and R562) mediate the majority of this effect and that the effect is modular; i.e., it retains its positive interaction when transferred into the context of Cdc25C. As a matter of fact, the C-terminal tail of Cdc25B alone restores the majority of the activity that Cdc25C appears to lack toward the Cdk2-pTpY/CycA substrate. The effect is not preserved in the absence of a proper context as seen in the

lack of inhibition or activation seen upon addition of the C-terminal peptides derived from Cdc25B. We have thus identified a docking region on Cdc25B characterized by two positively charged residues.

In the MAP kinase/phosphatase field, recent progress has also led to the identification of docking domains consisting of short stretches of amino acids. For example, the extracellular signal-regulated kinase (ERK) binds to both the D domain and the FXFP motif located on its protein substrates (*16*, *17*). The D domain is characterized by a cluster of basic residues followed by an LXL sequence, whereas the FXFP motif relies primarily on the two hydrophobic phenylalanine residues. As these two sites function in multiple positions and arrangements within various protein substrates, they also appear to be modular. Although the interaction between ERK and its substrates has not been elucidated structurally, a groove has been identified on ERK that is required for interaction with the basic residues of the D domain (*18*). This groove contains two important aspartic acids and has been termed the CD (common docking) domain as it mediates interactions, in addition to substrates, with activators and other regulators of ERK. Given this precedent, we are now looking for an appropriate negatively charged region on Cdk2/CycA to interact with the two arginine residues of the C-terminal tail of Cdc25B. Interestingly, there is a patch of three negatively charged and highly conserved residues (D38, E40, and E42) seen in the crystal structure of Cdk2/CycA and located near the critical T14 and Y15 residues (12−20 Å) (*19*). However, given the apparently unstructured nature of the C-terminal tail of Cdc25B, it remains equally likely that the tail will wrap around the Cdk2-pTpY/CycA complex, reaching to a completely different region of the complex, perhaps even the cyclin partner of the kinase. Cdc25A and Cdc25B have been shown to interact with cyclin B in HeLa cells, as well as being activated several-fold in in vitro assays (*20*). Future experiments are aimed at determining the site of interaction on Cdk2-pTpY/CycA as a part of our ongoing efforts toward understanding the specific and efficient recognition by Cdc25B of its protein substrate.

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