The Cdc14 family of protein phosphatases is conserved within eukaryotes and antagonizes the action of cyclin-dependent kinases, thereby promoting mitotic exit and cytokinesis. We performed a detailed kinetic and mechanistic study of the Cdc14 phosphatases with both small molecule aryl phosphates and a physiological protein substrate hCdh1. We found that Cdc14 displays a strong preference for two-ringed aryl phosphates over smaller one-ringed or larger, multi-ringed substrates, a finding that may have important implications for inhibitor design. Results from both leaving group and pH dependence of the Cdc14-catalyzed reaction are consistent with a general acid-independent mechanism for substrates with leaving group \( pK_a \) < 7 and a general acid-dependent mechanism for substrates with leaving group \( pK_a \) > 7. The use of both low and high leaving group \( pK_a \) substrates, in combination with steady-state and pre-steady-state kinetic techniques enabled the isolation and analysis of both the phosphoenzyme (E-P) formation and hydrolysis step. We established the requirement of general acid catalysis for E-P formation in reactions with high leaving group \( pK_a \) substrates, and the presence of general base catalysis in E-P hydrolysis. Mutational study of invariant acidic residues in Cdc14 identified Asp^{253} as the general acid during E-P formation and the general base in E-P hydrolysis. We also identified several residues including Asp^{50}, Asp^{129}, Glu^{168}, Glu^{171}, and Asp^{177} in the Cdc14 active site cleft that are required for efficient dephosphorylation of hCdh1.

Cdc14 is a protein phosphatase conserved from yeast to man (1, 2). In the budding yeast, *Saccharomyces cerevisiae*, Cdc14 is essential for cell cycle progression from late anaphase into G1 of the subsequent cell cycle, a process referred to as exit from mitosis (3–5). Cdc14 triggers mitotic exit by antagonizing the cyclin-dependent kinase (CDK) activity in late mitosis. For example, Cdc14 dephosphorylates and stabilizes the mitotic CDK inhibitor Sic1, up-regulates Sic1 expression by dephosphorylating the transcription factor Swi5, and induces degradation of mitotic cyclins by dephosphorylating and activating the anaphase promoting complex (APC) regulatory protein Cdh1/Hct1 (6). Biochemical studies also suggest that human Cdc14 homologs can dephosphorylate products of CDKs, such as hCdh1 (7), cyclin E (8), and p53 (9).

Despite their structural similarities, the biological function of Cdc14 phosphatases may vary between species. Thus, in *S. cerevisiae*, Cdc14 is absolutely required for mitotic exit. In contrast, the Cdc14 homolog Clp1/Flp1 is not required for mitotic exit in *Schizosaccharomyces pombe*, but rather is mainly involved in regulating cytokinesis and affects the timing of entry into mitosis (10, 11). In addition, CcCdc14 phosphatase is required for cytokinesis in the *Caenorhabditis elegans* embryo (12). The functional roles for the two human Cdc14 homologs, hCdc14A and hCdc14B, have not been fully defined, though depletion of hCdc14A by RNAi causes a variety of mitotic defects, including cytokinesis failure (13). It is possible that Cdc14 phosphatases may antagonize CDK activity by dephosphorylation of different substrates and regulate distinct cell cycle transitions in different species, as CDK activity is important for many different cell cycle processes (14).

Although the role of the Cdc14 phosphatase in cell cycle has been studied extensively, relatively little is known about its catalytic mechanism. Given their critical roles in mitotic exit and cytokinesis, the Cdc14 phosphatases have been implicated as potential therapeutic targets for anticancer drug development. Thus it is important to define Cdc14 active site substrate specificity and identify residues that are essential for Cdc14 catalysis. Interestingly, the Cdc14 phosphatases contain the active site sequence CX_{\text{R}}R, which is a hallmark for the protein tyrosine phosphatase (PTP) superfamily. The PTPs are a diverse family of enzymes that includes tyrosine-specific, dual specificity, Cdc25, as well as low molecular weight phosphatases (15). Detailed enzymological studies suggest that the PTPs employ a conserved two-step mechanism (16), shown in Scheme 1, in which E is the enzyme, ArPO_{4}^{-}, the substrate, EArPO_{4}^{-}, the enzyme-substrate Michaelis complex, E-P, the phosphoenzyme intermediate, and ArOH, the leaving group phenol. In this mechanism, the side chain of the active site Cys residue serves as a nucleophile to accept the phosphoryl group.
Cdc14 Protein Phosphatases

from the substrate to form E-P. The active site Arg makes bidentate hydrogen bonds with the phosphoryl group in the substrate through its guanidinium group and plays an important role in both substrate binding and transition state stabilization during catalysis. To facilitate substrate turnover, PTPs also utilize an Asp residue, which acts as a general acid by protonating the ester oxygen of the leaving group, thus greatly enhancing the rate of E-P formation. In the second step, E-P hydrolysis occurs by the attack of a nucleophilic water molecule assisted by the same Asp, functioning as a general base, with subsequent release of the free enzyme and inorganic phosphate.

Aside from the conserved active site CX_R motif, there are little sequence similarities between Cdc14 and other members of the PTP superfamily. Consequently, it is not known whether other residues also participate in Cdc14 catalysis. This is in contrast to other PTPs for which a number of conserved, catalytically important residues have been identified and characterized (16). In the following, we describe a kinetic investigation of Cdc14 substrate specificity with both small molecule aryl phosphates and a physiological protein substrate hCdh1. We also describe results from site-directed mutagenesis experiments in combination with steady-state and pre-steady-state kinetic analyses that lead to the identification of several catalytically important residues including the general acid/base in Cdc14 catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials—p-Nitrophenyl phosphate (pNPP)** was purchased from Fluka. Phenyl phosphate, 4-methylumbelliferyl phosphate (MUP), β-naphthyl phosphate, and 3-O-methylfluorescein phosphate (OMFP) were obtained from Sigma. 6,8-DiFluoro-4-methylumbelliferyl phosphate (DiFMUP) was purchased from Molecular Probes. 2-Chloro-4-nitrophenyl phosphate, 4-cyanophenyl phosphate, 4-trifluoromethylphenyl phosphate, and 4-chlorophenyl phosphate were synthesized as described (17). 8-Fluoro-4-methylumbelliferyl phosphate (8-FMUP), 6-Fluoro-4-methylumbelliferyl phosphate (6-FMUP), and 5-Fluoro-4-methylumbelliferyl phosphate (5-FMUP) were prepared as described (18).

**Expression and Purification of Cdc14 and Mutant Proteins—**The cDNA for budding yeast Cdc14 was subcloned into vector pET-15b. The expression and purification of Cdc14 and mutant proteins were performed using a plate reader. For substrates 5-FMUP, 8-FMUP, 6-FMUP, MUP, β-naphthyl phosphate, 4-cyanophenyl phosphate, 4-trifluoromethylphenyl phosphate, 4-chlorophenyl phosphate, phenyl phosphate, the reaction mixtures were quenched with 10% trichloroacetic acid, and the amount of inorganic phosphate released was quantitated based on the method of Black and Jones (19). For the substrates 6,8-DiFMUP, 5-FMUP, 8-FMUP, 6-FMUP, MUP, continuous assays were performed. The reaction (1-ml total volume) was set up in a 1-ml quartz cuvette. The fluorescence of the product was monitored by a PerkinElmer Life Sciences 50B Luminescence spectrometer. When the substrate concentrations were well below the Km, the k_cat/Km values were obtained from a linear least square fit of the plot of initial rate versus substrate concentration using Equation 1:

\[
v = \frac{k_{cat}[E][S]}{K_m + [S]}
\]

where v represents the initial rate and [E] is the total enzyme concentration. In continuous assays, the k_cat/Km values were obtained by nonlinear least square fit to Equation 2:

\[
[P] = [S](1 - \exp(-k_{cat}[E][f]))
\]

where [P] represents the initial substrate concentration, whereas [S] is the initial substrate concentration. For the substrates 6,8-DiFMUP and DiFMUP were determined as a function of pH at 30 °C. To fit the k_cat/Km versus pH data, one of two equations (Equation 5 or 6) was used.

\[
h_{cat}K_m = \frac{(h_{cat}K_m)_{max}(1 + H/K_{ES})(1 + H/K_{ES} + K_{ES})}{H}
\]

where \(k_{cat}/K_m\)_{max} is the pH-independent value of \(k_{cat}/K_m\). H is the proton concentration, \(K_{ES}\) is the second ionization constant of the substrate, and \(K_{ES}\) and \(K_{ES}\) are the ionization constants of the enzyme (20).

To fit the h_cat versus pH data, one of two equations (Equation 5 or 6) was used.

\[
h_{cat} = \frac{(h_{cat})_{max}(1 + H/K_{ES} + K_{ES})}{H}
\]

where \((h_{cat})_{max}\) is the pH-independent value of \(h_{cat}\). H is the proton concentration, and \(K_{ES}\) and \(K_{ES}\) are the apparent ionization constants of the substrate–enzyme complex.

**Pre-Steady-state Kinetic Study—**Pre-steady-state kinetic measurements of the wild-type Cdc14 and its D253A mutant-catalyzed hydrolysis of pNPP and DiFMUP were conducted at pH 7.0 and 30 °C. Experiments were performed by mixing the enzyme and substrate in a temperature-controlled Applied Photophysics SX.18MV stopped-flow spectrophotometer. The excitation wavelength was 390 nm, and the emission wavelength was selected using a 420-nm cutoff filter. The reaction was monitored by the increase in fluorescence of the DiFMUP product. A standard curve was generated by measuring the fluorescence of solutions of DiFMUP that were reconstituted in the assay buffer. Data were collected and fit to Equation 7.

\[
y = At + B(1 - e^{-kt}) + C
\]

The burst amplitudes and rate constants were evaluated by the procedure described in Ref. 21. When [S] ≫ [E], values for \(K_E\) and \(h_k + h_s\) were obtained by plotting the first-order rate constant for the burst (h_cat, that is b in Equation 7) versus substrate concentration and fitting the data to Equation 8.

\[
h_{burst} = (h_k + h_s)[S]/(K_E + [S])
\]

The individual rate constants for the E-P formation (h_k) and hydrolysis (h_s) can be determined from the \(h_k + h_s\) value and Equation 9.

\[
A = h_k/h_s
\]
hCdh1 with His₆ tag fused to its N terminus was constructed using the Bac-to-Bac system (Invitrogen). SF9 cells were infected with the hCdh1 baculovirus at a multiplicity of infection (MOI) of 5 for 50 h. The SF9 cells were treated with 0.5 μg/mL of okadaic acid for 4 h prior to harvesting. The cells were lysed with a buffer containing 20 mM Tris (pH 7.7), 150 mM NaCl, and 0.1% Triton X-100. After centrifugation, the supernatant was incubated with Ni²⁺-NTA beads (Qiagen). On washing, hCdh1 was eluted with a step gradient of imidazole and dialyzed into the appropriate buffer for subsequent assays.

**Continuous Spectrophotometric Assay for Cdc14 and Its Mutants Using Phosphorylated hCdh1 as a Substrate**—Kinetic parameters for the dephosphorylation of the phosphorylated hCdh1 protein were determined using a continuous spectrophotometric assay. This assay incorporates a coupled enzyme system, which uses purine nucleoside phosphorylase and its chromogenic substrate 7-methyl-6-thioguanosine for the quantification of inorganic phosphate produced in the phosphatase reaction (23, 24). The change in absorbance at 360 nm was due to the conversion of 7-methyl-6-thioguanosine to 7-methyl-6-thioguanine in the presence of inorganic phosphate. Quantitation of the inorganic phosphate produced in the phosphatase reaction was determined using the extinction coefficient of 11,200 M⁻¹ cm⁻¹ at 360 nm and pH 7.0. Experiments of wild-type Cdc14 were carried out at 25 °C in a 200-μL reaction mixture containing 100 mM Tris at pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 0.1 mg/ml purine nucleoside phosphorylase (Sigma), and 50 μM 7-methyl-6-thioguanosine. The spectrophotometric measurements were conducted using a PerkinElmer Lambda 14 spectrophotometer equipped with a magnetic stirrer in the cuvette holder. The kₗ/Kₘ values for the Cdc14 catalyzed dephosphorylation of hCdh1 were obtained by nonlinear least square fit to Equation 1 at 360 nm and pH 7.0. Experiments with Cdc14 mutants were carried out at 25 °C in a 200-μL reaction mixture under the same conditions in 96-well plates. The spectrophotometric measurements were obtained using a Molecular Devices Spectro MAX 340 plate reader. The kₗ/Kₘ values were obtained from a linear least square fit of the initial rate versus substrate concentration to Equation 1. In these measurements, the phosphorylated hCdh1 concentration was less than 4 μM, and the concentrations of the Cdc14 and its mutants ranged from 6.4 to 64 nM.

**RESULTS AND DISCUSSION**

**Active Site Substrate Specificity**—The PTP active site binds and hydrolyzes phosphorylated amino acids such as Tyr(P). Consequently, although the physiological substrates of PTPs are phosphoproteins, members of the PTP superfamily are also capable of hydrolyzing small molecule aryl phosphates. An analysis of the active site substrate specificity of PTPs may furnish critical information with respect to the design of potent active site-directed inhibitors. For example, an investigation of PTP1B active site substrate specificity led to the identification of several potent small molecule aryl phosphate substrates (25, 26), which provided a foundation for the development of the most potent and selective PTP1B inhibitors to date (27, 28).

To begin to probe the active site properties of Cdc14, we explored the ability of Cdc14 to catalyze the hydrolysis of a panel of aromatic phosphates, including 1-, 2-, and multi-ringed substrates. The chemical structures for pNPP, DiFMUP, and OMFP are shown in Fig. 1. All of the experiments described in this study were performed with the full-length recombinant Cdc14 from the budding yeast with an N-terminal His₆ tag to enable facile purification. Steady-state parameters for the Cdc14-catalyzed hydrolysis of aryl phosphates were determined at pH 7.0 and 30 °C.

As shown in Table I, the kₗ values are similar for all aryl phosphate substrates while the Kₘ values differ in a dramatic fashion. Consequently, the kₗ/Kₘ value, which is a measure of substrate specificity, spans several orders of magnitude. A comparison of kₗ/Kₘ values reveals several important features of the active site substrate specificity for Cdc14. First, Cdc14 shows a remarkable (230-fold) preference for DiFMUP over OMFP, even though these two substrates have very similar leaving group pKₐ values. This is in stark contrast to the Cdc25A dual specificity phosphatase, which exhibits a strong (73-fold) preference for OMFP over DiFMUP (29). Cdc14 also shows a preference for 2-ringed substrates over 1-ringed substrates. The kₗ/Kₘ values for 6-FMUP and 5-FMUP are 11- and 9-fold higher than that of pNPP although these substrates share comparable leaving group pKₐ values. Similarly the kₗ/Kₘ value for β-naphthyl phosphate is 5.3-fold higher than that for 4-chlorophenyl phosphate even though these substrates also have the same leaving group pKₐ value. Unlike other dual specific phosphatases, such as Cdc25 phosphatases, mitogen-activated protein kinase phosphatases, and VHR-related (VHR), which display a striking preference (~2-3 orders of magnitude) for bulky multi-ringed substrates like OMFP over pNPP (29–34), the kₗ/Kₘ for the Cdc14-catalyzed hydrolysis of OMFP is only 8-fold higher than that of pNPP. Interestingly, the tyrosine-specific PTPs exhibit very limited preference for substrates with more than one aromatic ring (20, 25, 31). Collectively, the unique affinity of Cdc14 for 2-ringed aryl phosphate substrates suggests that the active site properties of the Cdc14 phosphatases are distinct from those of other dual specific and tyrosine-specific PTPs. This information regarding the range of functionality that can be readily accommodated by the active site of Cdc14 phosphatases may be useful when designing active site-directed inhibitors for these enzymes.

**Effects of Mutation on Invariant Acidic Residues in Cdc14**—With the exception of the active site CX₅XR motif, Cdc14 phosphatases display little sequence similarity with other members of the PTP superfamily but share a number of invariant residues with phosphatase and tensin homolog deleted on chromosome 10 (PTEN), phosphatase of regenerating liver (PRL), and kinase associated phosphatase (KAP) (Fig. 2). Although the importance of the cysteine and arginine residues in the CX₅XR motif to PTP activity is well established, it is not known whether other residues also participate in Cdc14 catalysis. This is in contrast to other PTPs for which a set of conserved, catalytically important residues has been defined, and functions have been determined for each of these residues (16). For example, a conserved acidic residue has been shown to play an important catalytic role as a general acid/base in almost all PTPs examined to date, including the Yersinia PTP (35), PTP1B (36, 37), the low molecular weight PTPs (38–41), VHR-related (VHR) (42, 43), and MKP3 (44). The general acid facilitates catalysis by protonating the leaving group oxygen, thereby neutralizing the negative charge that develops as the phosphate ester bond is broken. This stabilizes the transition state and enhances the rate of catalysis by >1000-fold in the
case of the *Yersinia* PTP (35). The identification of the general acid has enabled the design of more potent substrate trapping mutants of PTPs (45, 46), which should aid in the identification of physiological substrates of PTPs.

Interestingly, evidence suggests that, depending on the substrate, the requirement for general acid catalysis in PTPs may not be universal. For example, it appears that the RNA 5′-triphosphatase Mce1-catalyzed phosphoanhydride hydrolysis does not involve general acid catalysis, which is in accord with the finding that Mce1 lacks a functional group in a position suited for proton donation (47). In addition, no acidic residues in Cdc25 phosphatases or myotubularins were found in a position corresponding to the general acid-containing loop (the WPD loop) observed in most other members of the PTP superfamily. Recent mutational, kinetic and structural studies indicate that acidic residues located in the PTP signature motif may serve as general acids in the reactions catalyzed by the Cdc25 and myotubularin phosphatases (29, 48).

The requirement for general acid/base in Cdc14 catalysis has not been established. As shown in Table I, the $k_{\text{cat}}/K_m$ value for the Cdc14-catalyzed hydrolysis of pNPP is slightly less than that for DiFMU, but the $k_{\text{cat}}/K_m$ value for pNPP is −1,820-fold lower than that for DiFMU. The $k_{\text{cat}}/K_m$ parameter reflects the overall rate-limiting step of enzyme catalysis, and, in the case of PTPs, previous studies have shown this to be mainly associated with hydrolysis of the E-P intermediate ($k_i$ in Reaction Scheme 1) under acidic to neutral conditions (49). The fact that the Cdc14-catalyzed hydrolysis of pNPP and DiFMU, two substrates that differ markedly in their leaving group properties, does not involve general acid catalysis, which is in accord with the finding that Mce1 lacks a functional group in a position suited for proton donation (47). In addition, no acidic residues in Cdc25 phosphatases or myotubularins were found in a position corresponding to the general acid-containing loop (the WPD loop) observed in most other members of the PTP superfamily. Recent mutational, kinetic and structural studies indicate that acidic residues located in the PTP signature motif may serve as general acids in the reactions catalyzed by the Cdc25 and myotubularin phosphatases (29, 48).

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The kinetic parameter $k_{\text{cat}}/K_m$ monitors the PTP reaction beginning with binding of the substrate and up to and including the first irreversible step in the kinetic mechanism (Reaction Scheme 1), which is E-P formation accompanied by release of the leaving group. Kinetic isotope effect studies indicate that the chemical step (i.e., phosphoryl transfer from substrate to the enzyme nucleophile) is rate-limiting for the $k_{\text{cat}}/K_m$ term (36, 41, 43). In general, the PTP-catalyzed E-P formation exhibits little leaving group dependence due to protonation of the phenolic oxygen by the general acid. In contrast, general acid deficient mutant PTPs show large leaving group dependence on $k_{\text{cat}}/K_m$ (37, 40, 50). For Cdc14-catalyzed hydrolysis of pNPP and DiFMU, even when one excludes the contribution from preferential binding of 2-ringed substrates over 1-ringed substrates (10-fold), the $k_{\text{cat}}/K_m$ value for DiFMU is still 180-fold higher than that of pNPP. It is rare for a wild-type PTP to display such a large leaving group dependence on $k_{\text{cat}}/K_m$. Consequently, it is not clear whether the Cdc14-catalyzed reaction involves general acid catalysis.

In order to further define the catalytic mechanism of Cdc14 and to identify additional catalytically important residues, in particular the general acid/base, we performed site-directed mutagenesis in Cdc14 and assessed the mutational effects by kinetic analysis. Because previous studies have shown that the general acid/base functionality in PTP catalysis is associated with acidic residues, we primarily focused our attention on the nine invariant acidic residues in the catalytic domain of Cdc14, namely, Asp305, Asp309, Asp314, Glu366, Glu371, Glu373, Asp377, Asp383, and Glu386 (Fig. 2). We made the most conservative site-directed mutations possible (i.e., Glu to Gln and Asp to Asn) in order to remove the ability of these residues to function effectively in general acid-base catalysis while minimizing structural perturbations in the proteins. All of the mutations were verified by DNA sequencing. Recombinant proteins were expressed in *E. coli*, and purified to near homogeneity as judged by SDS-PAGE (data not shown).

While this article was in preparation, the x-ray crystal structure of the catalytic domain of human Cdc14B (residues Pro14–His386) was published (51). Unlike other members of the PTP superfamily, the structure of hCdc14B reveals a novel arrangement of two structurally equivalent domains arranged in tandem. The N-terminal domain (domain A, residues 44–198 in hCdc14B) may contribute to Cdc14 substrate specificity and shares no sequence similarity with other dual specific phosphatases. The C-terminal domain (domain B, residues 213–386 in hCdc14B) contains the conserved PTP motif. The active site of hCdc14B is located within a long groove between the interface of the A- and B-domain (Fig. 3). Residues of two surface loops of the A-domain, the extended WPD(A) (residues Thr177–Asp55 in Cdc14) and α5A/α6A (residues Leu177–Thr430 in Cdc14) loops, form one side of the groove. The WPD (residues Leu249–Asp258 in Cdc14) and Q-loops (residues Arg317–Gly323 in Cdc14) of the B-domain create the opposite face of the active site groove, whereas the interdomain linker α-helix (residues Leu164–Phe175 in Cdc14) is positioned at the entrance to one end of the channel. This structure provides a framework for the interpretation of our mutational data. All of the residues that were subjected to mutagenesis are highlighted in the hCdc14B structure (Fig. 3).

Table II summarizes the kinetic parameters of the wild-type Cdc14 Protein Phosphatases

**Table I.** Kinetic parameters of Cdc14-catalyzed hydrolysis of small molecule aryl phosphate substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$pK_a$</th>
<th>$k_{\text{cat}}$</th>
<th>$K_m$</th>
<th>$k_{\text{cat}}/K_m$</th>
<th>$k_{\text{cat}}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMFP</td>
<td>4.6</td>
<td>3.4 ± 0.3</td>
<td>1.0</td>
<td>0.1 ± 0.1</td>
<td>3,400 ± 340</td>
</tr>
<tr>
<td>DiFMUP</td>
<td>4.7</td>
<td>8.1 ± 0.2</td>
<td>0.0105 ± 0.0006</td>
<td>771,000 ± 63,000</td>
<td></td>
</tr>
<tr>
<td>8-FMUP</td>
<td>6.4</td>
<td>9.0 ± 1.0</td>
<td>0.7 ± 0.2</td>
<td>15,000 ± 1,500</td>
<td></td>
</tr>
<tr>
<td>6-FMUP</td>
<td>7.0</td>
<td>7.9 ± 0.7</td>
<td>1.6 ± 0.3</td>
<td>4,000 ± 500</td>
<td></td>
</tr>
<tr>
<td>5-FMUP</td>
<td>7.0</td>
<td>6.8 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>3,720 ± 350</td>
<td></td>
</tr>
<tr>
<td>MUP</td>
<td>7.8</td>
<td>7.0 ± 0.4</td>
<td>2.1 ± 0.2</td>
<td>3,305 ± 200</td>
<td></td>
</tr>
<tr>
<td>β-Naph</td>
<td>9.38</td>
<td>4.2 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>2,000 ± 150</td>
<td></td>
</tr>
<tr>
<td>2-CI-4-NO₂</td>
<td>5.45</td>
<td>3.09 ± 0.04</td>
<td>0.4 ± 0.01</td>
<td>7,725 ± 80</td>
<td></td>
</tr>
<tr>
<td>pNPP</td>
<td>7.14</td>
<td>4.5 ± 0.2</td>
<td>10.6 ± 0.9</td>
<td>424 ± 7</td>
<td></td>
</tr>
<tr>
<td>4-CN</td>
<td>7.95</td>
<td>3.35 ± 0.02</td>
<td>10.8 ± 0.9</td>
<td>311 ± 2</td>
<td></td>
</tr>
<tr>
<td>4-CF₃</td>
<td>8.68</td>
<td>ND*</td>
<td>ND</td>
<td>440 ± 5</td>
<td></td>
</tr>
<tr>
<td>4-Cl</td>
<td>9.38</td>
<td>4.2 ± 0.2</td>
<td>11.1 ± 1.1</td>
<td>376 ± 4</td>
<td></td>
</tr>
<tr>
<td>PhP</td>
<td>9.99</td>
<td>2.55 ± 0.20</td>
<td>11.1 ± 0.8</td>
<td>225 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.

**y**

The assays were performed at pH 7.0, 30 °C as described under "Experimental Procedures." Listed are the leaving group $pK_a$ values for the substrates. The substrates include: 1) multi-ringed aryl phosphate OMFP, 2) 2-ringed aryl phosphates, DiFMUP, 8-FMUP, 6-FMUP, 5-FMUP, MUP, and β-Naph (β-naphthyl phosphate), and 3) 1-ringed aryl phosphates, 2-CI-4-NO₂ (2-chloro-4-nitrophenyl phosphate), pNPP, 4-CN (4-cyanophenyl phosphate), 4-CF₃ (4-trifluoromethylphenyl phosphate), 4-Cl (4-chlorophenyl phosphate), and PhP (phenyl phosphate).
and the mutant Cdc4s at pH 7.0 and 30 °C, using both pNPP and DiFMUP as substrates. It is apparent that mutants D143N, E168Q, E174Q, and E308Q have kcat and Km values very similar to those of the wild-type Cdc14. These results indicate that Asp143, Glu168, Glu174, and Glu308 are not essential for the Cdc14-catalyzed hydrolysis of small molecule aryl phosphates. In contrast, greater than 30-fold decrease in kcat was observed for D50N and D129N, while the kcat/Km values for these two mutants were only decreased 1.2–2.8-fold with pNPP as a substrate. Since Asp50 (in the WPD(A) loop) and Asp129 (in the \(\text{H92}^{5A}/\text{H92}^{6A}\) loop) are located within the active site cavity (Fig. 3), it is possible that these two residues may play more important roles in the hydrolysis of the E-P intermediate. Only moderate reduction in kcat and no change or a slight increase in kcat/Km was observed for E171Q and D177N, suggesting that Glu171 and Asp177 are not essential for the Cdc14-catalyzed pNPP hydrolysis.

Asp55 is invariant among all Cdc14 phosphatases, and it is also conserved in more related phosphatases including PTEN, PRL, and KAP (Fig. 2). The corresponding Asp92 in PTEN (52) and Asp110 in KAP (53) have been found in a position equivalent to those of the general acid/base residues observed in the WPD loop of other PTPs. In addition, the structure of the hCdc14B/C314S mutant with a Ser(P) peptide reveals that Asp253 in the WPD loop is placed to donate a proton to the O\(\gamma\) atom of the Ser(P) substrate (Ref. 51 and Fig. 3). Based on the structural data, it seems that Asp253 could function as a general acid/base in the Cdc14-catalyzed reaction. Previous studies have shown that mutations of the general acid in PTPs generally result in two to three orders of magnitude decrease in both...
loop (residues His282 protonated at residue 55). The invariant residues (labeled using Cdc14 numbering) mutagenized in this study are shown in yellow. Key active site loops, WPD(A) (residues Thr37–Asn38, Asp253–Asp258), WPD loop (residues Leu246–Asp258), the Q-loop (residues Arg187–Gly192), and the PTP-loop (residues His292–Arg298), are also labeled.

$k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ as shown in Table II, the $k_{\text{cat}}$ values for the Cdc14-catalyzed hydrolysis of pNPP and DiFMUP decrease 640 to 1200-fold when Asp253 is replaced by an Ala. Less dramatic reductions in $k_{\text{cat}}$ (50–100-fold) are observed for the D253A mutant. Surprisingly, the $k_{\text{cat}}/K_m$ values for D253A and D253N drop only 7- and 26-fold respectively, with pNPP as a substrate, while the $k_{\text{cat}}/K_m$ values are virtually the same as those of the wild-type Cdc14 using DiFMUP as a substrate. In order to determine whether Asp253 indeed functions as a general acid/base in Cdc14 catalysis, we carried out additional experiments with both the wild-type and Asp253 mutant Cdc14 enzymes.

Characterization of Asp253 as a General Acid/Base in Cdc14 Catalysis—PTPs with functional general acids show bell-shaped pH profiles where the maximum catalytic rate usually occurs around pH 5–6 with aryl phosphates as substrates. PTPs with general acids also show relatively flat leaving group dependencies, i.e. the rate of catalysis is affected minimally by the $pK_a$ of the leaving group. Removal of the general acid through site-directed mutagenesis results in a flat pH profile with a significant drop in the rate of catalysis, and a significant leaving group dependence where the rate of catalysis drops as the leaving group $pK_a$ rises.

Leaving Group Dependence—The leaving group dependence for the hydrolysis of aryl phosphates catalyzed by Cdc14 was investigated at pH 7.0 and 30 °C using a series of substrates with leaving group $pK_a$ values ranging from 4.7 to 10. Since we have shown that the size of the aryl substrate can have substantial effects on the rate of Cdc14 catalysis (Table I), we analyzed the leaving group effect with the one-ringed and two-ringed substrates separately, in order to exclude steric effects. Fig. 4 shows the Brønsted plots, which relate the logarithm of $k_{\text{cat}}/K_m$ to the $pK_a$ values of the leaving group. The slope of the Brønsted plot corresponds to the $\beta_{pK_a}$ value, which reflects the amount of negative charge developed on the phenolic oxygen at the transition state.

The most dramatic feature of the Brønsted plot with two-ringed substrates for Cdc14 is a break at the $pK_a$ value of 7. A similar break may also exist in the Brønsted plot with one-ringed substrates. In the low $pK_a$ region, from 4.7 to 7, the slope for the two-ringed substrates is substantially negative, giving a $\beta_{pK_a}$ value of $-1.01 \pm 0.01$. This value is similar to that of the uncatalyzed aryl phosphate dianion reaction ($\beta_{pK_a} = -1.23$, Ref. 54), which indicates a full negative charge on the leaving group oxygen at the transition state and suggests that no general acid catalysis occurs for substrates with leaving group $pK_a$ values between 4.7 and 7. Interestingly, the leaving group dependence for two-ringed substrates with leaving group $pK_a$ values from 7–9.4 is flat, giving a $\beta_{pK_a}$ value of $-0.12 \pm 0.02$. In addition, the leaving group dependence for one-ringed substrates with leaving group $pK_a$ values from 7.14 to 9.99 is also flat, yielding a $\beta_{pK_a}$ value of $-0.66 \pm 0.05$. These values are similar to those determined for PTPs with functional general acid catalysis (20, 40, 55) and the uncatalyzed monoanion reaction ($\beta_{pK_a} = -0.27$, Ref. 54), in which the leaving group is protonated in the transition state.

The results described above suggest that the Cdc14-catalyzed reaction does not involve a general acid for substrates with low leaving group $pK_a$ values (4.7–7), whereas the hydrolysis of substrates with high leaving group $pK_a$ values (>7) appears to require a general acid. To determine if Asp253 fulfills the function of a general acid, we also quantified the leaving group effect for the Cdc14/D253A-catalyzed reaction. Unfortunately, the activities of Cdc14/D253A-catalyzed hydrolysis of one-ringed substrates with leaving group $pK_a$ values >8 are too low to be measurable. However, as shown in Fig. 4, we were able to determine the kinetic parameters for the Cdc14/D253A-catalyzed hydrolysis of 2-ringed substrates with leaving group $pK_a$ values 4.7–7.8, which yielded a $\beta_{pK_a}$ value of $-1.19 \pm 0.07$. This value indicates that no general acid catalysis occurs for substrates with leaving group $pK_a$ values between 4.7 and 7.8. That the $k_{\text{cat}}/K_m$ values for the D253A mutant catalyzed hydrolysis of substrates with leaving group $pK_a$ between 4.7 and 7 are similar to those of the wild-type enzyme is consistent with the notion that the dephosphorylation of substrates with leaving group $pK_a$ below 7 does not require general acid catalysis. Importantly, there was clearly no break at $pK_a = 7$ in the leaving group profile for the D253A mutant as the reaction rates for substrates with leaving group $pK_a$ values higher than 7 continued to drop. The fact that a $\beta_{pK_a}$ value of $-1.19$ was obtained for the Cdc14/D253A-catalyzed reaction that extends beyond for substrates with leaving group $pK_a$ above 7 and that a further decrease in $k_{\text{cat}}/K_m$ for substrates with leaving group $pK_a$ values >7 suggest that Asp253 may indeed act as a general acid in Cdc14-catalyzed hydrolysis of high leaving group $pK_a$ (7.0 ~ 9.99) substrates.

pH Dependence—To further substantiate that Asp253 is the general acid/base in Cdc14, we determined the pH-rate profiles for the hydrolysis of pNPP and DiFMUP by both the wild-type and the D253A mutant Cdc14 phosphatases (Fig. 5). Since the leaving group $pK_a$ for DiFMUP (4.7) is <7, we predicted that the wild-type Cdc14-catalyzed DiFMUP hydrolysis would not exhibit general acid catalysis for the E-P formation step ($k_{\text{cat}}/K_m$) but may require general base catalysis for the E-P hydrolysis step ($k_{\text{cat}}$). As predicted, DiFMUP shows a flat pH-$k_{\text{cat}}/K_m$ profile between pH 5.5 and 8.0 (Fig. 5A), consistent with a lack of general acid and with the leaving group dependence described above. Since no general acid catalysis is involved, removal of the carboxyl group at position 253 has no effect on $k_{\text{cat}}/K_m$, and the D253A mutant also displays a flat pH dependence for DiFMUP with $k_{\text{cat}}/K_m$ values virtually identical to those of the wild-type enzyme (Fig. 5A).

The pH-$k_{\text{cat}}$ profile (Fig. 5B) indicates the presence in the enzyme-substrate complex an ionizable residue that must be deprotonated with a $pK_{\text{cat}}$ value of 6.4 ± 0.1 for maximal turnover ($k_{\text{cat}}$)max = 10.5 ± 0.5 s$^{-1}$). Because of the ease of expulsion of the leaving group, the rate-limiting step for the Cdc14-catalyzed DiFMUP reaction is most likely E-P hydrolysis ($k_3$ in Scheme 1, see below). Consequently, the apparent
The lines were generated by linear least squares fit to the log($k_{cat}/K_m$) versus leaving group $pK_a$ values 4.7–7.8 gave a $pK_a$ value of $-1.19 \pm 0.07$, while the substrates with leaving group $pK_a$ values $>8$ had $k_{cat}/K_m$ values that were too low to be measurable.

ionization constant ($pK_{ES1} = 6.4$) corresponds to the general base functionality in E-P. Indeed, the $k_{cat}$ values for the Cdc4/D253A-catalyzed hydrolysis of DiFMUP (−0.007 s$^{-1}$) are more than 3 orders of magnitude slower than ($k_{cat})_{max}$ of the wild-type enzyme and are insensitive to pH values (Fig. 5B). Since removal of Asp$^{253}$ in Cdc4 does not affect E-P formation, the large reduction on $k_{cat}$ for D253A appears primarily due to an impairment of E-P hydrolysis. Thus the rate-limiting step for the D253A-catalyzed DiFMUP reaction is also likely E-P hydrolysis (also see below). Consistent with Asp$^{253}$ serving as a general base in the Cdc4-catalyzed reaction, mutating residue 253 from Asp into Ala eliminates the acid limb of the pH-$k_{cat}$ profile for DiFMUP.

Since the leaving group $pK_a$ for pNPP is 7.14, we predicted that the wild-type Cdc4-catalyzed pNPP hydrolysis would exhibit characteristics of general acid/base catalysis. As expected, the wild-type Cdc4-catalyzed pNPP hydrolysis displays bell-shaped pH dependences for both $k_{cat}$ and $k_{cat}/K_m$, as observed with other PTPs using pNPP as a substrate (20, 35, 42). As shown in Fig. 5C, pNPP shows a pH optimum at 5.4 and steadily decreasing $k_{cat}/K_m$ values at higher pH values, quite unlike the pH dependence of DiFMUP. This behavior is consistent with a Cdc4 residue that must be protonated for optimal catalytic efficiency, possibly a general acid. Because of the instability of Cdc4 at low pH values, the $k_{cat}/K_m$ values below pH 5.0 could not be accurately measured. Consequently, the data were fitted to Equation 4 with the second ionization constant of pNPP ($pK_{E2}$) fixed at the experimentally determined value of 5.1 (29). From this analysis, the $pK_{ES1}$ (ionization constant of the active site Cys residue) was determined to be 5.5 ± 0.5, the $pK_{E2}$ (ionization constant of the general acid) 5.1 ± 0.4, and the ($k_{cat}/K_m)_{max}$ (2.3 ± 1.6) $\times 10^4$ M$^{-1}$s$^{-1}$. These ionization constants are similar to those obtained for other PTPs. Consistent with the involvement of a general acid in the Cdc4-catalyzed hydrolysis of substrates with leaving group $pK_a$ values $>7$, the $k_{cat}/K_m$ values for Cdc4/D253A are dramatically reduced, especially at the optimal pH, and replacement of Asp$^{253}$ with an Ala abolishes the basic limb of the pH profile for pNPP (Fig. 5C). The $pK_{ES1}$ value for Cdc4/D253A could not be measured because of its low activity and instability at pH below 6. Therefore, we conclude that D253 is responsible for the basic limb of the pH-$k_{cat}/K_m$ profile seen for wild-type Cdc4 with pNPP and that Asp$^{253}$ must be protonated for optimal catalysis of this substrate.

As shown in Fig. 5D, the pH versus $k_{cat}$ profile for the Cdc4-catalyzed pNPP reaction shows a pH optimum at 6.8, and the ($k_{cat}/K_m)_{max}$, $pK_{ES1}$, and $pK_{E2}$ values are determined to be 12.9 ± 1.4 s$^{-1}$, 6.7 ± 0.1, and 6.9 ± 0.1, respectively. It is important to recognize that $pK_{ES1}$ and $pK_{E2}$ are apparent values and may not represent microscopic ionizations of particular groups (56). It is also important to recognize that there are uncertainties in $pK_a$ values calculated from kinetic data when the differences in $pK_a$ are less than 0.6 units (57). Nevertheless, the acidic limb of the pH-$k_{cat}$ profile for pNPP is similar to that of DiFMUP, suggesting that the rate-limiting step likely corresponds to E-P hydrolysis in this pH range and that $pK_{ES1}$ likely reflects the ionization of the general base function of Asp$^{253}$. In accordance with this, removal of Asp$^{253}$ reduces the $k_{cat}$ by 3 orders of magnitude and eliminates the acidic limb of the pH-$k_{cat}$ profile (Fig. 5D). Interestingly, unlike the DiFMUP reaction, whose $k_{cat}$ values level off at a maximum plateau at pH > 7 (Fig. 5B), the pH-$k_{cat}$ profile for pNPP shows a steady decrease in $k_{cat}$ as the pH is raised (Fig. 5D). This may be caused by the fact that under more basic conditions, Asp$^{253}$ is deprotonated, so that E-P formation step eventually becomes the rate-limiting step for substrates that require general acid catalysis. Consequently, the apparent $pK_{ES1}$ (6.9) derived from the basic limb of the pH-$k_{cat}$ profile for pNPP may not represent an intrinsic ionization constant but reflect a change in the rate-limiting step, from E-P hydrolysis at lower pH values to E-P formation at higher pH values. Since Asp$^{253}$ is also in-

TABLE II
Kinetic parameters of wild-type and mutant Cdc4 phosphatases with both pNPP and DiFMUP as substrates

<table>
<thead>
<tr>
<th>Cdc4</th>
<th>pNPP</th>
<th>DiFMUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
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<tr>
<td></td>
<td>s$^{-1}$</td>
<td>mM</td>
</tr>
<tr>
<td></td>
<td>s$^{-1}$</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>WT</td>
<td>4.5 ± 0.2</td>
<td>10.6 ± 0.9</td>
</tr>
<tr>
<td>D50N</td>
<td>0.14 ± 0.03</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>D128N</td>
<td>0.13 ± 0.01</td>
<td>0.87 ± 0.11</td>
</tr>
<tr>
<td>D143N</td>
<td>3.5 ± 0.4</td>
<td>9.6 ± 1.0</td>
</tr>
<tr>
<td>E168Q</td>
<td>4.7 ± 0.4</td>
<td>12.9 ± 1.5</td>
</tr>
<tr>
<td>E171Q</td>
<td>0.94 ± 0.01</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>E174Q</td>
<td>4.3 ± 0.4</td>
<td>11.2 ± 1.3</td>
</tr>
<tr>
<td>D177N</td>
<td>0.86 ± 0.01</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>D253A</td>
<td>0.007 ± 0.001</td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>D253N</td>
<td>0.09 ± 0.01</td>
<td>5.75 ± 1.31</td>
</tr>
<tr>
<td>E308Q</td>
<td>4.0 ± 0.3</td>
<td>11.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>8.1 ± 0.2</td>
<td>10.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.13 ± 0.01</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>5.4 ± 0.1</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>8.7 ± 0.01</td>
<td>16.6 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>ND</td>
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<td></td>
<td>8.2 ± 0.4</td>
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<td></td>
<td>8.2 ± 0.4</td>
<td>9.6 ± 1.1</td>
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<tr>
<td></td>
<td>0.007 ± 0.001</td>
<td>0.0079 ± 0.0014</td>
</tr>
<tr>
<td></td>
<td>0.08 ± 0.01</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>5.7 ± 0.4</td>
<td>8.7 ± 3.1</td>
</tr>
</tbody>
</table>
volved in E-P formation, its removal also abolishes the basic limb of the pH-\(k_{\text{cat}}\) profile for pNPP (Fig. 5D).

We also analyzed the pH dependence of the D253N-catalyzed hydrolysis of DiFMUP and pNPP (data not shown). Similar to D253A, the D253N mutant exhibits flat pH-rate profiles for both \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\). The \(k_{\text{cat}}/K_m\) values for the D253N-catalyzed reaction are only slightly lower than those of the D253A mutant. Interestingly, the \(k_{\text{cat}}\) for the D253N mutant is approximately 10-fold higher than D253A (Table II), suggesting that the carboxyamide group in D253N is more efficient than the methyl group in D253A in promoting E-P hydrolysis.

Collectively, the results from both leaving group and pH dependence of Cdc14-catalyzed reaction are consistent with a general acid independent mechanism for substrates with leaving group pNPP reaction are consistent with a general acid independent mechanism for substrates with leaving group pNPP reaction.

The \(k_{\text{cat}}/K_m\) for the D253N mutant is approximately 10-fold higher than D253A (Table II), suggesting that the carboxyamide group in D253N is more efficient than the methyl group in D253A in promoting E-P hydrolysis. Collectively, the results from both leaving group and pH dependence of Cdc14-catalyzed reaction are consistent with a general acid independent mechanism for substrates with leaving group pNPP reaction are consistent with a general acid independent mechanism for substrates with leaving group pNPP reaction.

Pre-Steady-state Burst Kinetics—We next proceeded to carry out pre-steady-state burst kinetic experiments in order to determine the rate of E-P formation and breakdown for the Cdc14-catalyzed reaction and to further define the role of Asp253 in catalysis. Unfortunately, burst kinetics was not feasible for the pNPP reaction because of the large \(K_m\) value, which makes it difficult to saturate the enzyme. However, we successfully performed a burst kinetic analysis of the Cdc14-catalyzed hydrolysis of DiFMUP at pH 7.0 and 30 °C. If E-P formation is sufficiently rapid relative to its breakdown, we would expect to see a burst of product (DiFMU) upon rapid mixing of the enzyme and substrate. As shown in Fig. 6A, the time course for the Cdc14-catalyzed hydrolysis of DiFMUP is biphasic, characterized by a rapid, exponential burst phase, which is then followed by a slower linear phase. By varying the initial concentration of the enzyme and substrate we found that the amplitude of the burst is proportional to the enzyme concentration. The presence of a pre-steady-state burst upon the hydrolysis of DiFMUP by Cdc14 is consistent with rate-determining E-P hydrolysis for this substrate. This is in full agreement with the conclusion derived from the steady-state kinetic evidence discussed above. The rate constants of the individual chemical steps \(k_2\) and \(k_3\) as well as the dissociation constant \(K_d\) can be obtained by plots of \(k_{\text{burst}}\) (rate constant for the burst phase) versus DiFMUP concentrations (Fig. 6B) as described under “Experimental Procedures” (21). The \(k_2 \cdot k_3/K_d\) values are correspondingly 151 ± 29 s\(^{-1}\), 20 ± 5 s\(^{-1}\), and 209 ± 66 μM. Thus, for the hydrolysis of DiFMUP, \(k_2 \cdot k_3/K_d\) is 7.6-fold greater than \(k_1\). Since \(k_{\text{cat}} = k_2 \cdot k_3/K_d + k_3\), and \(K_d = k_2 \cdot k_3/K_d + k_3\), \(k_{\text{cat}}/K_d\) can be transformed to \(k_2/K_d\). The \(k_2/K_d\) value (720,000 M\(^{-1}\) s\(^{-1}\) determined by the stop-flow method is similar to the \(k_{\text{cat}}/K_m\) value (770,000 M\(^{-1}\) s\(^{-1}\)) obtained by steady-state experiments.

We also measured the Cdc14/D253A-catalyzed DiFMUP hydrolysis by the stopped-flow technique. As shown in Fig. 6A, a burst was also observed in the D253A-catalyzed reaction, indicating E-P hydrolysis as the rate-limiting step. From the substrate concentration dependence of \(k_{\text{burst}}\) (Fig. 6B), the \(k_2\), \(k_3\), and \(K_d\) values for the D253A reaction were determined to be 85 ± 10 s\(^{-1}\), 0.01 ± 0.001 s\(^{-1}\), and 145 ± 33 μM. The \(k_2/K_d\) value (586,000 M\(^{-1}\) s\(^{-1}\) obtained from the stopped-flow experiments is similar to the \(k_{\text{cat}}/K_m\) value (880,000 M\(^{-1}\) s\(^{-1}\)) measured by steady-state experiments. Both the \(k_2\) and \(K_d\) values for D253A are close to those of the wild-type enzyme, indicating that Asp253 does not significantly contribute to E-P formation for D253A.

Since \(k_3\), the rate constant for E-P hydrolysis, must be identical for dephosphorylation of either substrates, we can approximately solve for the kinetic parameter \(k_3\) in the Cdc14-catalyzed pNPP reaction based on \(k_{\text{cat}} = k_2 \cdot k_3/K_d + k_3\). The calculated \(k_3\) for pNPP is 5.8 s\(^{-1}\). Therefore, the rate of E-P formation is 26-fold faster with DiFMUP as a substrate than with pNPP. Since \(k_2\) and \(k_3\) for the pNPP reaction are comparable, both E-P formation and breakdown contributes to the
rate-limiting step in pNPP hydrolysis. This also support the conclusion derived from the pH-dependence analysis of the pNPP reaction that the $k_{cat}$ is rate-limited by both $k_2$ and $k_3$ at pH 7. Since $k_{cat}/K_m = k_2/K_m$, $K_m$ for pNPP can be calculated to be 13.8 mM using the estimated $k_2$ value and the experimentally determined $k_{cat}/K_m$ for pNPP (420 M$^{-1}$ s$^{-1}$). Thus, the dissociation constant ($K_d$) of Cdc14 for DiFMUP (209 mM) is 66-fold lower than that for pNPP, providing further evidence that Cdc14 possesses intrinsically higher affinity for 2-ringed aryl phosphates. This property may relate to the existence of a hydrophobic pocket adjacent to the active site that recognizes the $p + 1$ Pro residue in Cdc14 substrates (51). The enhanced substrate binding may be partially responsible for the faster rate of E-P formation with DiFMUP than with pNPP.

Activity of Cdc14 Toward Its Physiological Substrate hCdh1—The crystal structure of hCdc14B shows that acidic residues Glu$^{168}$, Glu$^{171}$, and Asp$^{177}$ in the interdomain linker $\alpha$-helix (residues Leu$^{164}$–Phe$^{177}$ in Cdc14) cluster at the entrance of the catalytic site, suggesting that these residues may confer specificity for basic residues in the substrates (51). However, substitution of these acidic residues with an Ala reduces the $k_{cat}/K_m$ for hydrolysis of a peptide substrate, Ala-Ser(P)-Pro-Arg, by only 2-fold (51). It is not known whether these acidic residues play a role in recognizing protein substrates.

To gain further insight into the molecular basis of Cdc14 substrate specificity, we chose to analyze the Cdc14-catalyzed dephosphorylation of a physiological protein substrate Cdh1.

Cdh1 is a substrate-specific co-activator of the APC (58, 59), which functions as an E3 ubiquitin ligase to degrade the mitotic cyclin Clb2 and other substrates during the G1 phase of the cell cycle. Cdh1 is present throughout the cell cycle, but its binding to APC is blocked by Cdk phosphorylation (22, 60). Cdc14 activates APC by dephosphorylating Cdh1, leading to destruction of Cdc28-Clb2 and mitotic exit (4). In addition, human Cdc14A can also dephosphorylate human Cdh1 (hCdh1) to promote the activation of APC$^{\text{Cdh1}}$ in vitro (7).

To follow the Cdc14-catalyzed hCdh1 dephosphorylation, we employed a continuous spectrophotometric enzyme-coupled assay in which the coupling enzyme, purine nucleoside phosphorylase, uses the inorganic phosphate, generated by the action of Cdc14, to convert 7-methyl-6-thioguanosine to 7-methyl-6-thioguanine and ribose-1-phosphate, resulting in an increase in absorbance at 360 nm (23, 24). The reaction was carried out at pH 7 and 25 °C. Fig. 7 shows a typical progress curve of the Cdc14-catalyzed hCdh1 dephosphorylation. To ensure first-order conditions, the reaction was conducted at several hCdh1 concentrations ranging from 0.5 to 4 μM. Identical $k_{cat}/K_m$ values were obtained by fitting the progress curves to Equation 2, indicating that the substrate concentrations were $\ll K_m$. The $k_{cat}/K_m$ values for hCdh1 dephosphorylation by the full-length Cdc14 and the catalytic domain (residues 1–374) were determined to be (1.8 ± 0.04) × 10$^5$ and (1.3 ± 0.01) × 10$^5$ M$^{-1}$ s$^{-1}$, respectively. This is consistent with previous observations that C-terminal domain of Cdc14 (residues 375–551) is not required for its phosphatase activity (1). Interestingly, under the same conditions the human Cdc14A catalytic domain (residues 1–379) was able to dephosphorylate hCdh1 with a $k_{cat}/K_m$ value of (4.0 ± 0.04) × 10$^5$ M$^{-1}$ s$^{-1}$, indicating that hCdh1 is an efficient substrate for both the yeast and human enzymes.

We subsequently evaluated the ability of the Cdc14 mutants to carry out hCdh1 dephosphorylation. As expected, a more than 100-fold decrease in $k_{cat}/K_m$ was observed for D253A. In contrast, the $k_{cat}/K_m$ values for D143N, E174Q, and E308Q were almost identical to that of the wild-type Cdc14. Thus, Asp$^{143}$, Glu$^{174}$, and Glu$^{308}$ are not essential for hCdh1 dephosphorylation. Compared with wild-type Cdc14, the $k_{cat}/K_m$ values of E168Q, E171Q, and D177N were reduced by 2-fold. These results suggest that the acidic cluster in the interdomain linker, Glu$^{168}$, Glu$^{171}$, and Asp$^{177}$, may contribute to Cdc14
protein substrate recognition. Interestingly, more dramatic decreases in the rate of hCdh1 dephosphorylation were observed for D50N and D129N. The $k_{\text{cat}}/K_m$ ratio of the D50N and D129N-catalyzed hCdh1 dephosphorylation was reduced by 5- and 10-fold, respectively, in comparison with that of the wild-type Cdc14. Thus, Asp50 and Asp129 may play a more important role in Cdc14 substrate recognition.

The residues that we identified as important for hCdh1 dephosphorylation are located in structural elements that surround the Cdc14 catalytic site (Fig. 3). Thus, Asp50 and Asp129 reside in the WPD(A) (residues Thr37–Asp55) and α5α6α7 (residues Leu117–Thr140) surface loops respectively. Asp50 is located at the WPD loop (residues Leu249–Asp256). And Glu168, Glu171, and Asp177 in the interdomain linker α-helix (residues Leu164–Phe175) are positioned at the entrance to one end of the active site groove. Although none of these residues (with the exception of Asp257) are found in direct contact with the phosphopeptide Ala–Ser(P)–Pro in the crystal structure of the hCdc14 Protein Phosphatases.
Kinetic and Mechanistic Studies of a Cell Cycle Protein Phosphatase Cdc14
Wei-Qing Wang, Joshua Bembenek, Kyle R. Gee, Hongtao Yu, Harry Charbonneau and
Zhong-Yin Zhang

doi: 10.1074/jbc.M402217200 originally published online May 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402217200

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