Cdc14 Phosphatases Preferentially Dephosphorylate a Subset of Cyclin-dependent kinase (Cdk) Sites Containing Phosphoserine*

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Running Title: Substrate specificity of Cdc14 phosphatases

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Background: Cdc14 phosphatases help control mitosis by dephosphorylating sites (Ser/Thr-Pro) targeted by cyclin-dependent kinases (Cdks).

Result: Cdc14 family phosphatases strongly prefer phosphoserine over phosphothreonine at Cdk sites.

Conclusion: By discriminating among Cdk sites, Cdc14 may participate in setting the order and timing of Cdk substrate dephosphorylation.

Significance: Mechanisms governing the timing of Cdk site dephosphorylation are crucial for proper coordination of late mitotic events.

Mitotic cell division is controlled by cyclin-dependent kinases (Cdks), which phosphorylate hundreds of protein substrates responsible for executing the division program. Cdk inactivation and reversal of Cdk-catalyzed phosphorylation are universal requirements for completing and exiting mitosis and resetting the cell cycle machinery. Mechanisms that define the timing and order of Cdk substrate dephosphorylation remain poorly understood. Cdc14 phosphatases have been implicated in Cdk inactivation and are thought to be generally specific for Cdk-type phosphorylation sites. We show that budding yeast Cdc14 possesses a strong and unusual preference for phosphoserine over phosphothreonine at Pro-directed sites in vitro. Using serine to threonine substitutions in the Cdk consensus sites of the Cdc14 substrate Acm1, we demonstrate that phosphoserine specificity exists in vivo. Furthermore, it appears to be a conserved property of all Cdc14 family phosphatases. An invariant active site residue was identified that sterically restricts phosphothreonine binding and is largely responsible for phosphoserine selectivity. Optimal Cdc14 substrates also possessed a basic residue at the +3 position relative to the phosphoserine, whereas substrates lacking this basic residue were not effectively hydrolyzed. The intrinsic selectivity of Cdc14 may help establish the order of Cdk substrate dephosphorylation during mitotic exit and contribute to roles in other cellular processes.

During cell division, mitosis is triggered when cyclin-dependent kinases (Cdks)8 in association with mitotic cyclins phosphorylate hundreds of proteins at Ser-Pro and Thr-Pro sequences to promote chromosome condensation, nuclear envelope breakdown, centrosome separation and assembly of a bipolar microtubule spindle. After chromosome segregation initiates at anaphase, mitotic cyclin proteolysis leads to Cdk inactivation
and disassembly of the molecular machinery of mitosis, a process called mitotic exit. Cyclin destruction and Cdk inactivation is necessary but not sufficient for mitotic exit (1). Dephosphorylation of Cdk substrates by opposing protein phosphatases is also required (1). In the budding yeast, *Saccharomyces cerevisiae*, the Cdc14 phosphatase is essential for Cdk inactivation (2, 3) and is thought to be the primary phosphatase responsible for reversing Cdk phosphorylation events to drive mitotic exit (2). Cdc14 activity towards a limited number of substrates *in vitro* confirms its ability to counteract Cdns (4, 5).

During interphase and early mitosis Cdc14 is sequestered in an inactive state in the nucleolus (2, 6). At the onset of anaphase, active Cdc14 is released to the nucleoplasm where it dephosphorylates a distinct subset of nuclear Cdk substrates to stabilize the mitotic spindle and establish the spindle midzone, promote the timely separation of rDNA and telomeres, and correctly position the nucleus (7, 8). These activities ensure proper spindle elongation and chromosome segregation. In late anaphase, Cdc14 accumulates in the nucleus and cytoplasm. Dephosphorylation of Cdc14 targets in the cytoplasm triggers mitotic cyclin destruction and Cdk inhibition, events required for exit from mitosis (2, 3). Cdc14 also localizes to the bud neck where cell abscission occurs and is thought to be involved in promoting onset of cytokinesis by unknown mechanisms (9).

Cdc14 phosphatases are conserved in eukaryotes with the exception of higher plants. Most vertebrates express two paralogs typically designated Cdc14A and B. Unlike the budding yeast enzyme, Cdc14 orthologs of most other species are not required for mitotic exit (10). In vertebrates, there are conflicting reports on the functions of the Cdc14 isoforms but there is evidence for involvement in regulating mitotic entry, centromere duplication, DNA repair, and cytokinesis (10).

Cdc14 phosphatases are members of the protein tyrosine phosphatase (PTP) superfamily (11). Based on structural and partial sequence similarities and *in vitro* activity with artificial substrates, Cdc14 phosphatases are classified as dual specificity phosphatases, a subgroup of PTPs capable of dephosphorylating Ser/Thr as well as Tyr residues (12, 13). However, few sites targeted by Cdc14 *in vivo* have been directly identified and the specificity of Cdc14 phosphatases has not been defined by biochemical analyses. In this study, we examined the substrate selectivity of Cdc14 phosphatases and found they possess a conserved ability to discriminate between phosphoserine (pSer) and phosphothreonine (pThr) at Cdk sites. The strong preference of Cdc14 for Cdk sites containing pSer suggests previously unrecognized complexity in the regulation of Cdk targets and highlights a mechanism that could allow Cdk substrates and individual Cdk sites to be differentially regulated.

**EXPERIMENTAL PROCEDURES**

*Peptide synthesis and purification*—Peptide synthesis and purification was performed using solid-phase Fmoc chemistry on a Prelude Parallel Peptide Synthesizer (Protein Technologies, Tucson, AZ, USA) essentially as described (14) with the following changes. Coupling times for phosphorylated amino acids were increased to 3 h. Completed peptides were cleaved with 5 ml 95% TFA, 2.5% water, and 2.5% triisopropylsilane, precipitated, and washed 3 times with 35 ml diethyl ether prior to purification by reverse phase HPLC.

Purified peptides were dissolved in 50 mM Tris-HCl (pH 8.0) and concentrations determined as described previously (15).

*Purification of Cdc14 phosphatases and Cdc14 substrates*—His6-tagged budding yeast Cdc14 (wild-type and the A285G mutant) or His6-tagged fission yeast Clp1 were expressed in *Escherichia coli* and affinity purified using a Ni2+-chelate column. The catalytic domains of the hCdc14A (residues 1-379) and hCdc14B (residues 1-418) isoforms were expressed as N-terminally tagged glutathione S-transferase (GST) fusion proteins in *E. coli* and affinity purified using glutathione agarose. Full-length, recombinant forms of the budding yeast proteins Acm1, Cdc6, and Fin1 were expressed as GST fusion proteins in *E. coli* and affinity purified using glutathione agarose beads and phosphorylated by Cdk1 for use as substrates for Cdc14. See Supplemental Information for detailed methods used for protein purification and phosphorylation of protein substrates.
Phosphatase assays—Dephosphorylation of phosphopeptides was measured by detection of released inorganic phosphate with either a 2.5% (w/v) ammonium molybate:0.15% (w/v) malachite green solution dissolved in 1N HCl as described (15) or BIOMOL Green™ reagent (Enzo Life Sciences). Assays were performed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 0.1% β-mercaptoethanol at 30 °C for varying times. Reactions were stopped with either an equal volume of 1.2 N HCl (for malachite green assays) or the BIOMOL Green reagent as directed by the supplier and absorbance measured at 620 nm. Standard curves were generated using sodium phosphate under identical buffer conditions. Km and kcat values were obtained by measuring initial rates at varying substrate concentrations and fitting the Michaelis-Menten equation to these data using nonlinear regression with either KaleidaGraph (Synergy Software) or GraphPad Prism (GraphPad Software) programs. For phosphopeptides exhibiting substrate inhibition, data were fit with a modified Michaelis-Menten equation containing a substrate inhibition (K_i) term.

Dephosphorylation of recombinant protein substrates—Cdk1-phosphorylated GST-Fin1 (3 µM), GST-Acm1 (5 µM) or GST-Cdc6 (1 µM) were treated with budding yeast His6-Cdc14 (200 nM) and incubated at 30 °C for the indicated times. For Acm1 and Cdc6, aliquots were removed, added to equal volumes of 2x SDS-PAGE loading dye, heated at 95 °C to stop the reaction, and separated by SDS-PAGE. Gels were stained with Coomassie blue and excised bands subjected to in-gel digestion with either Lys-C (for Acm1) or trypsin (for Cdc6), and prepared for mass spectrometry (MS) analysis as described (16). For Fin1, reactions were stopped by addition of an equal volume of acetonitrile. Samples were diluted to 20% acetonitrile with fresh 50 mM ammonium bicarbonate, digested with 50 ng Lys-C (Sigma Aldrich) overnight at 37 °C, and analyzed by MS.

LC/MS analysis and label-free quantification—Peptides derived from recombinant proteins were analyzed by liquid chromatography coupled to electrospray ionization MS on an LTQ-Orbitrap Velos instrument (Thermo Scientific). Phosphopeptides were identified and phosphorylation sites confirmed based on product ion spectra. Extracted ion chromatograms for all detectable parent phosphopeptides and several non-phosphorylated peptides were generated using XCalibur and integrated using GraphPad Prism. To quantify dephosphorylation at each Cdk site, phosphopeptide signals were first normalized using the non-phosphorylated standard peptides and then converted to percentages of the zero timepoint signals. For the Cdc6 peptides containing two Cdk sites, the Orbitrap was operated in targeted tandem MS mode and extracted fragment ion chromatograms specific for each phosphorylation site were generated. Quantification was the same as for the other sites. For additional details, see supplemental Methods.

Co-immunopurification (co-IP) and immunoblotting—The acm1-S4T allele was generated using the QuikChange Multi site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. ACM1 and acm1S4T were integrated under control of the natural ACM1 promoter at the TRP1 locus in a W303 acm1Δ background using the pRS404 vector. 3HA-CDC14 and the 3HA-cdc14-C283S substrate trap mutant were integrated under control of the GAL1 promoter at the LEU2 locus using the pRS305 vector. To analyze the effect of Cdc14 on Acm1 phosphorylation and stability, cultures were grown to mid log phase (OD600 = ~0.4) in YP-raffinose medium and 2% galactose was added for two h to induce 3HA-Cdc14 expression. Soluble protein extracts were generated by glass bead lysis in IP buffer (50 mM sodium phosphate (pH 7.5), 100 mM NaCl, 10% glycerol, 0.1% Triton X-100, and 5 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin, 100 µM leupeptin, 20 mM sodium fluoride, and 20 mM β-glycerophosphate. Extracts were then separated by SDS-PAGE on a 10% gel and analyzed by immunoblotting. Acm1 and Acm1-S4T were detected with an affinity-purified polyclonal antibody directed against recombinant GST-Acm1 (Pacific Immunology, 0.04 µg/ml working concentration). 3HA-Cdc14 was detected with anti-HA 12CA5 (Roche, 0.4 µg/ml). Rabbit anti-G6PD (0.2 µg/ml) was from Sigma-Aldrich. Cultures for co-IP analysis were grown and processed similarly except that the 3HA-Cdc14-
C283S substrate trap mutant was expressed by galactose induction. Soluble whole cell extracts in IP buffer (4 mg) were incubated with 12.5 µl EZView anti-HA affinity resin (Sigma Aldrich) for 2 h at 4 °C with gentle mixing, washed 4x with 1 ml IP buffer, and bound proteins eluted by boiling in SDS gel loading buffer without reducing agent. Recovered proteins were supplemented with 25 mM dithiothreitol, reheated, and analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

We showed that the budding yeast Cdh1 inhibitor Acm1 is a substrate of Cdc14 (16). In subsequent studies, a phosphopeptide containing the conserved Thr161 Cdk phosphorylation site of Acm1 could not be dephosphorylated in vitro by Cdc14, even at high enzyme concentrations. This finding suggested that Cdc14 might act only on a subset of Cdk sites. To test this possibility, we synthesized over 20 phosphopeptides matching sequences from budding yeast Cdk substrates and examined their ability to be dephosphorylated by Cdc14 (Fig. 1, and Table 1 and supplemental Table S1). Without exception, Cdc14 exhibited little or no detectable activity towards peptides containing a pThr. In contrast, Cdc14 exhibited a broad range of rates and catalytic efficiencies towards pSer-containing peptides. Acm1pS31, the most effective pSer-containing peptide for budding yeast Cdc14, had a catalytic efficiency (kcat/Km) more than three orders of magnitude greater than the best peptide substrate bearing pThr (Table 1).

To more directly determine if Cdc14 distinguishes between pSer and pThr, we replaced pSer with pThr in Acm1pS31. The catalytic efficiency for Acm1pT31 was over 3,000-fold lower than Acm1pS31 (Fig. 1C; Table 1). Conversely, when pThr in the Cdc6pT7 peptide was replaced with pSer, catalytic efficiency increased more than 1,000-fold (Fig. 1D; Table 1). Additional phosphopeptide substrates (supplemental Table S2) confirmed the +1 proline specificity of Cdc14 and its ability to dephosphorylate tyrosine (13), albeit weakly and largely independent of a +1 Pro.

The selectivity of Cdc14 for pSer at Cdk sites was also observed with full-length protein substrates. We purified recombinant forms of the physiologic Cdk substrates Acm1, Fin1, and Cdc6, and phosphorylated them in vitro with purified Cdk1 (Clb2-Cdc28). MS analyses of all three proteins confirmed the sites of phosphorylation and showed all three proteins contained both pSer and pThr residues at roughly equivalent average stoichiometry (supplemental Table S3 and Dataset 1). Following treatment with Cdc14, we used proteolytic peptide MS signals to independently quantify relative rates of dephosphorylation at each detectable Cdk phosphorylation site in the three protein substrates. In Acm1, Cdk sites containing pSer were efficiently dephosphorylated by Cdc14, whereas pThr161 was not significantly affected (Fig. 2A). Similarly, in Fin1 and Cdc6 (Fig. 2, B and C and Dataset 2) all pSer sites were almost completely dephosphorylated but no significant hydrolysis of pThr was observed. Ser selectivity was manifested clearly on three peptides containing both a pSer and a pThr Cdk site. In all three cases, signal for the pSer form decreased during the reaction, whereas the pThr form accumulated due to pSer dephosphorylation from the doubly phosphorylated substrate population (Fig. 2, B and C and Dataset 2).

We next considered the source of the wide variation in catalytic efficiencies among pSer peptide substrates. Activity of Cdc14 towards pSer substrates correlated strongly with the number of basic residues C terminal to pSer (supplemental Table S1). The best Cdc14 substrates contained three basic residues occupying the +3 to +5 positions relative to pSer, whereas peptides with no basic residues in this region were virtually unreactive. We synthesized peptide variants of an efficient (Acm1pS31) and poor (Cdh1pS169) pSer substrate that had 0, 1, 2, or 3 basic residues occupying the +3 to +5 positions relative to pSer (supplemental Table S1). The best Cdc14 substrates contained three basic residues occupying the +3 to +5 positions relative to pSer, whereas peptides with no basic residues in this region were virtually unreactive. We synthesized peptide variants of an efficient (Acm1pS31) and poor (Cdh1pS169) pSer substrate that had 0, 1, 2, or 3 basic residues occupying the +3 to +5 positions (Fig. 3 and supplemental Table S2). For both, the dephosphorylation rate was very low in the absence of basic residues. The rate increased dramatically with a single basic residue at the +3 position and was further increased by additional basic residues at +4 and +5 (Fig. 3). Preliminary analyses of a larger peptide library revealed that +3 was the only position in the +2 to +6 region where a single basic amino acid conferred high catalytic efficiency to a pSer-containing substrate.
peptide (data not shown).

To test the physiological importance of Cdc14 pSer selectivity, we overexpressed Cdc14 from the \textit{GAL1} promoter and monitored the phosphorylation status and stability of wild-type Acm1 and an Acm1 mutant in which its four Ser-containing consensus Cdk phosphorylation sites were changed to Thr (Acm1-S4T, Fig. 4A). Our previous work demonstrated that Cdk phosphorylation stabilizes Acm1 and reduces its SDS-PAGE mobility, whereas Cdc14-catalyzed dephosphorylation triggers Acm1 proteolysis (16). Consistent with our prior work, the slow mobility form of Acm1 was lost upon Cdc14 overexpression and the overall level of Acm1 was strongly reduced (Fig 4B). In contrast, the level and SDS-PAGE mobility of Acm1-S4T were unaffected by Cdc14 overexpression.

We also examined the ability of an active site Cdc14 mutant (Cdc14-C283S) that binds substrates tightly (16) but is catalytically inactive to associate with Acm1 and Acm1-S4T by co-IP analysis. Consistent with our previous work (16), wild-type Acm1 was efficiently recovered in an IP of 3HA-Cdc14-C283S (Fig. 4C). Acm1-S4T was completely absent from the 3HA-Cdc14-C283S IP samples indicating that Cdc14 has low affinity for Acm1 if its Ser Cdk sites are converted to Thr Cdk sites. The overall phosphorylation status of Acm1 and Acm1-S4T appeared similar based on their identical SDS-PAGE mobility pattern. Together, the resistance of Acm1-S4T to dephosphorylation by wild-type Cdc14 and lack of binding to the Cdc14-C283S substrate trap mutant clearly demonstrate that Cdc14 distinguishes between pSer and pThr Cdk sites in vivo.

To determine if specificity for pSer was evolutionarily conserved, we purified several Cdc14 orthologs and tested their activities on a subset of the synthetic phosphopeptide substrates. The catalytic domain of human Cdc14A (residues 1-379) exhibited efficient dephosphorylation of pSer peptides and poor activity towards pThr peptides (Fig. 5A). The difference in catalytic efficiency between the best pSer and pThr substrates was 625-fold (supplemental Table S1). The catalytic domain of human Cdc14B (residues 1-418) and fission yeast Clp1 showed a similar preference for pSer peptide substrates (Fig. 5, B and C). The strong preference for a +3 basic residue was also conserved in hCdc14A and hCdc14B, although the human enzymes appeared less sensitive to additional basic residues (supplemental Fig. S1, A-E). We conclude that intrinsic selectivity for pSer-Pro-X-Lys/Arg sites is a conserved property of the Cdc14 phosphatase family.

The structure of hCdc14B bound to a short pSer peptide substrate (17) provided insight into the molecular basis for pSer selectivity. Without altering the conformation of hCdc14B or the backbone of the bound peptide, the pSer residue of the peptide was replaced with pThr in the model (Fig. 6). This change revealed a steric clash between methyl side chains on pThr of the peptide substrate and Ala316 of hCdc14B, which is invariant among Cdc14 phosphatases (supplemental Fig. S2). The structure of the KAP phosphatase (18) is similar to that of the catalytic subdomain of hCdc14B (17). This similarity is particularly strong within a 54 amino acid region encompassing the active site where residues 292-345 of hCdc14B could be superimposed on the corresponding segment of the KAP phosphatase, residues 115-172, with a root-mean-square deviation of 1.1 Å for aligned Cα atoms. KAP dephosphorylates a pThr in the activation segment of Cdks (19) and interestingly, has Gly at the position corresponding to Ala316 in hCdc14B (supplemental Fig. S2). Consistent with these observations, further analysis of the structural model of hCdc14B suggested that replacement of Ala316 by Gly might alleviate the steric interference with the pThr side chain and permit efficient pThr binding and hydrolysis (Fig. 6). To test this, we replaced the homologous Ala285 in budding yeast Cdc14 with Gly, generating a Cdc14 A285G mutant that exhibited a net 100-fold decrease in selectivity for pSer compared to pThr (Fig. 6, B and C). This result suggests that pSer selectivity in Cdc14 enzymes arises largely from steric occlusion of pThr from the active site, but indicates additional features of the enzyme must also confer pSer specificity since the catalytic efficiency of Cdc14 A285G towards pThr-containing peptides is significantly lower than that for peptides containing pSer.
DISCUSSION

Cdc14 substrate specificity has previously been assumed to include either pSer or pThr residues located within Ser/Thr-Pro consensus sequences recognized by Cdks. Our work provides crucial refinements in our understanding of sites targeted by the Cdc14 phosphatases. We show that efficient dephosphorylation by Cdc14 phosphatases not only requires Pro at the +1 position, but also requires Ser as the phosphoamino acid and a basic amino acid at +3. At least with the budding yeast enzyme, additional adjacent basic residues further enhance activity. Importantly, mutational analysis of Cdk phosphorylation sites in Acm1 clearly demonstrated that the phosphoserine selectivity detected in vitro was observed in cells and is physiologically relevant.

An invariant active site Ala residue and adjacent acidic groove of Cdc14 phosphatases are major determinants of substrate specificity and function. The methyl group of an Ala residue located at the edge of the active site cleft restricts access to pThr residues bestowing the ability to discriminate between pSer and pThr. Gray et al. (17) described a negatively charged groove extending from the active site of hCdc14B that contains three acidic residues conserved in Cdc14 phosphatases from diverse species (supplemental Fig. S2). The negative effect of mutating residues E168, E171, and D177 on the activity of budding yeast Cdc14 towards human Cdh1 (20) and our results revealing the importance of basic residues at +3 to +5 positions in substrates confirm the importance of this acidic channel in substrate recognition.

The unexpected specificity of Cdc14 phosphatases for pSer-Pro-X-Lys/Arg sequences has broad implications for the mechanisms by which Cdk site phosphorylation is reversed during cell cycle progression and for understanding functions of Cdc14 enzymes in other cellular processes. Selectivity for pSer within Cdk sites is biologically relevant because a substantial fraction of physiologic Cdk phosphorylation sites contain pThr. Cdk substrates do not appear to distinguish between Ser or Thr (21, 22) and large-scale phosphoproteomic data suggests that 22 percent of in vivo budding yeast Cdk sites contain pThr (23). In HeLa cells, 27 percent of Cdk-like sites upregulated in mitosis contain pThr, whereas 21 percent of total proline-directed phosphosites are pThr (24).

Our results imply that Cdc14 phosphatases are restricted in their capacity to oppose Cdks and may therefore play specialized roles in reversal of Cdk phosphorylation. Cdk substrates are dephosphorylated at different times during mitosis (25) to ensure events like chromosome segregation, exit from mitosis, and cytokinesis are properly orchestrated, and the intrinsic specificity of Cdc14 could contribute to this timing. For example, budding yeast Cdc14 is transiently activated in early anaphase to target a small set of substrates important for anaphase spindle function (7, 8). As predicted by our data, the known early anaphase Cdc14 substrates have significantly higher Ser-Pro to Thr-Pro ratios and frequencies of Ser-Pro sites with a +3 basic residue than the average yeast protein and known Cdk substrates (Table S4). The different specificities of S phase and mitotic Cdks combined with the differential timing of cyclin degradation during mitosis has also been proposed to contribute to the temporal dephosphorylation of Cdk substrates in yeast (26), a mechanism that is not mutually exclusive with the Cdc14 specificity reported here.

Cdk sites containing pThr could allow proteins to avoid premature dephosphorylation by Cdc14 in early anaphase. One example in budding yeast is the Cdc14 inhibitor, Net1 or Cfi1, which sequesters Cdc14 in an inactive state in the nucleolus for most of the cell cycle (2, 6). Cdk1-mediated phosphorylation of an N-terminal region of Net1 at the onset of anaphase is required for the initial transient release of Cdc14 from the nucleolus (27). Four of the six Cdk sites in this region are Thr and the two Ser sites lack +3 basic residues (27), suggesting they are all poor Cdc14 substrates that could have evolved to prevent Cdc14 from inhibiting its own release. Before the onset of anaphase, there may be an active pool of Cdc14 in the nucleolus (28, 29), thus pSer selectivity may be particularly important in preserving the interaction of Cdc14 with Net1 in interphase and early mitosis and sustaining normal Cdc14 regulation.

In budding yeast, Cdc14 is thought to be
Responsible for dephosphorylating most mitotic Cdk substrates during mitotic exit. Our results imply that at least one other protein phosphatase is likely required to oppose Cdns. The Ser/Thr protein phosphatases PP2A and PP1 are involved in mitotic regulation and are good candidates for targeting pThr-Pro sites (6). The involvement of multiple phosphatases permits diversity in the mechanisms governing reversal of mitotic phosphorylation and may allow more refined temporal and spatial control of mitotic exit.

More specialized roles for Cdc14 in opposing Cdns is also consistent with the fact that Cdc14 is not required for mitotic exit in most eukaryotes (10). Currently, the functions of vertebrate Cdc14 enzymes during cell division are not clear (10). Although the functions and substrates of Cdc14 orthologs, and the specific kinases they oppose, may have diverged substantially during evolution, our findings clearly show their enzymatic specificity has been conserved. The strict selectivity of Cdc14 may be useful in identifying novel substrates and thereby elucidating biological functions in humans and other eukaryotes, including defining and clarifying roles in regulating cell division.

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REFERENCES

FOOTNOTES

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8The abbreviations used are: Acm1-S4T, an Acm1 mutant in which Thr replaces four Ser-containing consensus Cdk phosphorylation sites; Cdk, cyclin-dependent kinase; Clp1, fission yeast ortholog of budding yeast Cdc14; GST, glutathione S-transferase; MS, mass spectrometry; pSer, phosphoserine; pThr, phosphothreonine; PTP, protein tyrosine phosphatase; rRNA, ribosomal RNA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

FIGURE LEGENDS

**FIGURE 1.** *Budding yeast Cdc14 is highly selective for pSer at Cdk phosphorylation sites in phosphopeptide substrates.* (A) Time-dependent dephosphorylation of phosphopeptide substrates Acm1pS3 (●), Acm1pT161 (○), and Cdh1pT157 (×) by budding yeast Cdc14. The concentration of all substrates was 300 µM. (B) Same as (A) showing pSer-containing peptide substrates Acm1pS3 (●), Acm1pS31 (■), Acm1pS48 (▲), Cdh1pS42 (●), Cdh1pS169 (▼), and Cdh1pS239 (○). All substrates were 100 µM. Peptide sequences are shown in Table 1 and supplemental Table S1. (C) The rate of dephosphorylation of peptides Acm1pS31 (■) and Acm1pT31 (○) and (D) peptides Cdc6pS7 (■) and Cdc6pT7 (●) was measured as a function of peptide concentration. Data are the average of three independent experiments, and using nonlinear regression, were fit with a form of the Michaelis-Menten equation containing a substrate inhibition term. The amino acid sequence and kinetic parameters for peptides in (C) and (D) are given in Table 1 or supplemental Table S2.
FIGURE 2. Budding yeast Cdc14 preferentially dephosphorylates pSer-containing Cdk phosphorylation sites in physiologic protein substrates. Dephosphorylation of the indicated residues from full-length recombinant (A) GST-Acm1, (B) GST-Fin1, and (C) GST-Cdc6 by budding yeast Cdc14 was measured over time (black bars, 0 min; white bars, 5 min; gray bars, 30 min) using a quantitative mass spectral assay. Data are means of three trials with standard errors. Remaining phosphorylation at each site is plotted relative to 0 min, which was set at 100%.

FIGURE 3. The activity of Cdc14 phosphatases is dependent on basic residues C-terminal to pSer at Cdk sites. The complete sequences of the wild type Acm1 pS31 (A) and Cdh1pS169 (C) peptides are shown in black on the first line with the residues of the +3 to +5 region (relative to pSer, “pS”) in each variant shown below in red. Rates of dephosphorylation of Acm1 pS31 and its variants (B) and Cdh1pS169 and its variants (D) by budding yeast Cdc14 were compared at a single substrate concentration of 300 µM. The amino acid sequence of the +3 to +5 region in each peptide is shown below the x axis. Data represent the mean of three independent experiments with standard errors.

FIGURE 4. Cdc14 pSer selectivity exists in vivo. (A) Schematic of Acm1 protein showing the location and sequence of the 4 pSer-containing Cdk consensus sites and the corresponding amino acid substitutions made to create the Acm1-S4T mutant. (B) Extracts from asynchronous cultures of cells expressing Acm1 or Acm1-S4T from the natural ACM1 promoter before and after galactose-induced overexpression of 3HA-Cdc14 were analyzed by SDS-PAGE and immunoblotting. G6PD is a loading control. “pAcm1” represents a slow mobility Cdk-phosphorylated form of Acm1. (C) Anti-HA antibody resin was used to isolate 3HA-Cdc14-C283S and interacting proteins from soluble extracts of asynchronous cultures expressing either wild-type Acm1 or Acm1-S4T from the natural ACM1 promoter. Immunoblotting was used to detect the indicated proteins in the initial extracts and after anti-HA IP. G6PD is a loading control.

FIGURE 5. Human Cdc14A, human Cdc14B and fission yeast Clp1 phosphatases exhibit selectivity for pSer. (A) The rate of dephosphorylation of the indicated phosphopeptides by the hCdc14A(1-379) catalytic domain was compared at single substrate concentrations (250 µM for pSer peptides, Acm1 pT31 and Cdc6 pT7; 1 mM for all other pThr-containing peptides). (B) The rate of dephosphorylation of the indicated phosphopeptides by the hCdc14B(1-418) catalytic domain was compared at single substrate concentrations (500 µM for Acm1 pS3 and Acm1 pS31, 1 mM for all others). Data in (A) and (B) are averages of four trials with standard errors. (C) Dephosphorylation of Acm1 pS31 (●) and Acm1 pT31 (■) by fission yeast Clp1 was measured as a function of peptide concentration. Data were fit with the Michaelis-Menten equation containing a substrate inhibition term. Rates are expressed per pmol Cdc14.

FIGURE 6. Cdc14 selectivity for pSer arises from the structure of the Cdc14 active site. (A) The active site of human Cdc14B (PDB ID: 1OHE) (17) with the bound peptide substrate modified in silico to contain a pThr side chain. A surface representation of the protein and peptide is depicted with purple indicating the pThr side chain methyl group and the mesh delineating the surface of Ala316. The distance between the carbon atoms of the methyl groups on Ala 316 and the pThr side chain is 2.3 Å, a value that is substantially less than the sum of the Van der Waals radii of the two atoms indicating severe steric clash. The surface after a Gly (orange) substitution at 316 is also shown. The side chain of Lys315 was hidden for optimal visualization of the active site pocket. MacPyMOL (The PyMOL Molecular Graphics System, Schrodinger, LLC) was used to visualize the hCdc14B structure, mutate the bound phosphopeptide substrate by utilizing the site mutagenesis function, and to measure distances between atoms. (B) Relative rates of Cdc6 pT7 dephosphorylation by wild-type budding yeast Cdc14 (●) and the Cdc14 A285G mutant (■) were measured as a function of peptide concentration. Activities were normalized to activity on Cdc6 pS7 to directly compare differences in selectivity. Data are averages of
three trials and were fit with the Michaelis-Menten equation. (C) Catalytic efficiencies ($k_{\text{cat}}/K_m$) determined from (B) and similar experiments on Cdc6pS7. Selectivity ($pS/pT$) is $k_{\text{cat}}/K_m$ for Cdc6pS7 divided by $k_{\text{cat}}/K_m$ for Cdc6pT7.
**TABLE 1**

The catalytic efficiency ($k_{\text{cat}}/K_m$) of budding yeast Cdc14 for pSer- and pThr-containing peptide substrates

To calculate catalytic efficiencies, steady state kinetic parameters were determined by measuring initial velocity as a function of peptide substrate concentration at pH 8.0 and 30 °C. The $k_{\text{cat}}$ and $K_m$ values were determined by fitting the Michaelis-Menten equation to the data using nonlinear regression and are given in supplemental Tables S1 and S2.

<table>
<thead>
<tr>
<th>Peptide Name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$k_{\text{cat}}/K_m$ ($M^{-1} s^{-1}$)</th>
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<tbody>
<tr>
<td>Acm1pS31</td>
<td>VKGNELR&lt;sup&gt;b&lt;/sup&gt;SPSKRRSQI</td>
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<tr>
<td>Acm1pT31&lt;sup&gt;*&lt;/sup&gt;</td>
<td>VKGNELR&lt;sup&gt;b&lt;/sup&gt;TSPSKRRSQI</td>
<td>8</td>
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<tr>
<td>Cdc6pT7</td>
<td>MSAIPIT&lt;sup&gt;b&lt;/sup&gt;TPTKRIRRN</td>
<td>5</td>
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<tr>
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<td>MSAIPISPTKRIRRN</td>
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<td>Cdh1pS239</td>
<td>DSKQLLL&lt;sup&gt;b&lt;/sup&gt;SPGKQFRQI</td>
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<td>SSASLLS&lt;sup&gt;b&lt;/sup&gt;SPRSRPS</td>
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<td>Acm1pT161</td>
<td>ISLPSFIT&lt;sup&gt;b&lt;/sup&gt;PPRNSKIS</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Cdh1pT176</td>
<td>SPHSTPV&lt;sup&gt;b&lt;/sup&gt;TPRRLFTSQ</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

<sup>a</sup>The name of the parent protein from budding yeast is followed by the identity (pS for pSer or pT for pThr) and residue number of the phosphorylated residue.

<sup>b</sup>Peptide sequences are given in single-letter code with phosphorylated residues underlined in bold and are identical to those of the parent phosphoprotein with the exception of the Acm1pT31 and Cdc6pS7 variants (*asterisks*) in which the phosphoamino acid was replaced as indicated.

<sup>c</sup>ND, in reactions containing from 0.2-1.0 μM Cdc14 and 1-5 mM substrate, rates were at or below the limit of detection (about 1.0 pmol min<sup>-1</sup>), thus steady state kinetic parameters were not determined.
Figure 1  Bremmer et al.
Figure 2  Bremmer et al.
Figure 3  Bremmer et al.

A

Acm1pS31 – V K G N E L R pS P S K R R S Q I

B

Cdh1pS169 – A A G L E E F pS P H S T P V T P

C

D

Velocity (pmol min⁻¹)

10 20

5

0

A A A K AA K R A K R R

Velocity (pmol min⁻¹)

10 20

5

0

STP K TP K R P K RR

Acm1pS31 and variants

Cdh1pS169 and variants
Figure 4  Bremmer et al.

A.

B.

C.

Galactose:

Galactose:

Galactose:
Figure 5  Bremmer et al.

A

Velocity (pmol min⁻¹)

Acm1pS3  Acm1pS31  Cdh1pS239  Cdc6pT7  Acm1pT31  Acm1pS31  Cdh1pT173  Cdh1pT176  Cdh1pT189

B

Velocity (pmol min⁻¹)

Acm1pS3  Acm1pS31  Cdh1pS239  Cdc6pT7  Acm1pT31  Acm1pS31  Cdh1pT173  Cdh1pT176  Cdh1pT189

C

Velocity (pmol min⁻¹)

[Peptide] (µM)

0 1000 2000 3000 4000 6000
Figure 6  Bremmer et al.

A

B

C

<table>
<thead>
<tr>
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<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>Selectivity pS/pT</th>
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<tr>
<td></td>
<td>Cdc6pT7</td>
<td>Cdc6pS7</td>
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