Characterization of the CTD Phosphatase Fcp1 from Fission Yeast

PREFERENTIAL DEPHOSPHORYLATION OF SERINE 2 VERSUS SERINE 5

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The C-terminal domain (CTD) of RNA polymerase II undergoes extensive phosphorylation and dephosphorylation at positions Ser5 and Ser2 during the transcription cycle. A single CTD phosphatase, Fcp1, has been identified in yeast and metazoans. Here we conducted a biochemical characterization of Fcp1 from the fission yeast Schizosaccharomyces pombe. The 723-amino acid Fcp1 protein was expressed at high levels in bacteria. Recombinant Fcp1 catalyzed the metal-dependent hydrolysis of para-nitrophenyl phosphate with a pH optimum of 5.5 (kcat = 2 s−1; Km = 19 mM). Deletion analysis showed that 139- and 143-amino acid segments could be deleted from the N and C termini of Fcp1, respectively, without affecting phosphatase activity. A segment containing amino acids 487–580, deletion of which abolished activity, embraces a BRCT domain present in all known Fcp1 orthologs. Mutations of residues Asp170 and Asp172 abrogated Fcp1 phosphatase activity; the essential aspartates are located within a DDXDTX2 motif that defines a superfamily of metal-dependent phospho-transferases. We exploited defined synthetic CTD phosphopeptide substrates to show for the first time that: (i) Fcp1 CTD phosphatase activity is not confined to native polymerase II and (ii) Fcp1 displays an inherent preference for a particular CTD phosphorylation array. Using equivalent concentrations (25 μM) of CTD peptides of identical amino acid sequence and phosphoserine content, which differed only in the positions of phosphoserine within the heptad, we found that Fcp1 was 10-fold more active in dephosphorylating Ser−PO4 than Ser−PO4−.

The C-terminal domain (CTD) of the largest subunit of RNA polymerase II (pol II) serves as a landing pad for macromolecular assemblies that regulate mRNA synthesis and processing (1). The CTD is composed of a tandemly repeated heptad motif that regulates assemblies that regulate mRNA synthesis and processing (1). The CTD is composed of a tandemly repeated heptad motif.

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1 The abbreviations used are: CTD, C-terminal domain; pol, polymerase; pNp0, p-nitrophenyl phosphate; pN0, p-nitrophenol.

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phosphatase function. Several groups have demonstrated the presence of Fcp1 in a complex with pol II (7, 9; Kimura et al., 10) recently identified a direct contact between S. pombe Fcp1 and the Rpb4 subunit of pol II. Fcp1 also binds to the RAP74 subunit of TFIIH, which results in a stimulation of CTD phosphatase activity (6, 18). Recombinant S. cerevisiae Fcp1 per se was capable of dephosphorylating pol II that was labeled with 32P in vitro with the TFIIH kinase; however, the reaction required stoichiometric concentrations of Fcp1 (15). Reombinant S. cerevisiae Fcp1 also catalyzed dephosphorylation of p-nitrophenyl phosphate (pN\textsubscript{OP}), albeit at high substrate concentrations and with a low turnover number (15).

Here we conducted a biochemical characterization of the Fcp1 ortholog of the fission yeast S. pombe. The S. pombe Fcp1 cDNA encodes a predicted 723-amino acid polypeptide related with a low turnover number (15).

The amino acid sequence of S. pombe Fcp1 from residues 140 to 326 is aligned to the sequences of Fcp1 from S. cerevisiae (Sce), Xenopus laevis (Xla), Drosophila melanogaster (Dme), and Homo sapiens (Hsa). Gaps in the alignment are indicated by dashes. The positions of side chain identity/similarity in all five Fcp1 proteins are denoted by dots. The signature motif of the DXDXDT family of phosphatases and phosphotransferases is indicated by the shaded box. The conserved Asp\textsuperscript{77} and Asp\textsuperscript{79} residues of S. pombe Fcp1 that were targeted for mutational analysis in the present study are indicated by vertical lines.

**EXPERIMENTAL PROCEDURES**

Reombinant S. pombe Fcp1—S. pombe Fcp1 was produced in Escherichia coli as a His\textsubscript{6}-tagged fusion as follows. The fcp1 coding sequence (GenBank\textsuperscript{TM} accession AL390814) was PCR-amplified from a S. pombe cDNA library using primers designed to introduce an NcoI site at the translation start codon, a glycine codon following the start codon, and a BamHI site 3' of the stop codon. The PCR product was ligated into the cloning vector pET-Fcp1 and the construct was transformed into E. coli BL21(DE3)-RIL (Stratagene). A 500-ml culture was grown at 37 °C to an A\textsubscript{600} of 6.5. The culture was adjusted to 0.2 mM isopropyl-1-thio-

buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithi-
soluble bacterial lysates using the same protocol described for the wild-type Fcp1.

N-terminal deletion mutants were constructed by PCR amplification with mutagenic sense-strand primers that introduced an NcoI restriction site and a methionine codon in lieu of the codon for Gly⁴⁰⁰ or Gly⁴⁰⁴. C-terminal deletion mutants were constructed by PCR amplification with mutagenic antisense-strand primers that introduced a stop codon in lieu of the codons for Pro⁵⁸⁵, Lys⁴⁸⁶, or Glu⁴⁰⁰ and a BamHI site 3’ of the new stop codon. The PCR products were digested with NcoI and BamHI and then inserted into pET16m. The inserts of the resulting pET-Fcp1A plasmids were sequenced completely to confirm the desired mutations and exclude the acquisition of unwanted changes. The pET-Fcp1A plasmids were introduced into E. coli BL21(DE3)-RIL, and the truncated Fcp1 proteins were purified from soluble bacterial lysates as described for the wild-type Fcp1.

Velocity Sedimentation—An aliquot (50 μg) of the wild-type Fcp1 preparation was mixed with catalase (45 μg), bovine serum albumin (45 μg), and cytochrome c (45 μg), and the mixture was applied to a 4.8-ml 15–50% glycerol gradient containing 50 mM Tris-HCl (pH 7.4), 0.1 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 0.05% Triton X-100. The gradient was centrifuged in a SW50 rotor at 50,000 rpm for 15 h at 4 °C. Fractions (0.2 ml) were collected from the bottom of the tube. Aliquots (20 μl) of odd-numbered gradient fractions were analyzed by SDS-PAGE. Aliquots (5 μl) of every fraction were assayed for hydrolysis of pNØP.

Phosphatase Assay—Reaction mixtures (100 μl) containing 50 mM Tris acetate (pH 5.5), 10 mM MgCl₂, 10 mM pNØP, and Fcp1 as specified were incubated for 30 min at 37 °C. The reactions were quenched by adding 900 μl of 1 M sodium carbonate. Release of p-nitrophenol (pNØ) was determined by measuring A₄₁₀ and extrapolating the value to a pNØ standard curve.

CTD Phosphatase Assay—N-terminal biotinylated CTD phosphopeptides composed of four tandem YSPTSPS repeats containing phosphoserine at either position 2, position 5, or positions 2 and 5 of each repeat were synthesized and purified as described previously (11, 21). CTD phosphatase reaction mixtures (25 μl) containing 50 mM Tris acetate (pH 5.5), 10 mM MgCl₂, 25 μM CTD peptide, and Fcp1 as specified were incubated for 60 min at 37 °C. The reactions were quenched by adding 0.5 or 1 ml of malachite green reagent (BIOMOL green reagent, pur-}

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\text{FIG. 2. Phosphatase activity of recombinant Fcp1. A, Fcp1 purification. Aliquots (7 μg) of the nickel-agarose preparations of wild-}
\text{type (WT) Fcp1 and mutants D170A, D170E, D170N, D172A, D172E, and D172N were analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS. The polypeptides were visualized by staining with Coomassie Blue dye. The positions and sizes (in kDa) of marker proteins are indicated on the left. (Panel B) Phosphatase activity. Reaction mixtures containing 50 mM Tris acetate (pH 5.5), 10 mM MgCl₂, 10 mM pNØP, and WT or mutant Fcp1 proteins as specified were incubated for 30 min at 37 °C. pNØ release is plotted as a function of input protein.}
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activate Fcp1 is in agreement with the findings for the human CTD phosphatase (5). In contrast, S. cerevisiae CTD phosphatase can be activated by calcium (6).

The kinetic parameters were gauged by measuring the production of pNØ as a function of substrate concentration in the range of 5–40 mM pNØ. A double-reciprocal plot of the data fit well to a linear function (Fig. 3D). From these data, we calculated a $K_m$ of 19 mM pNØ and a $k_{cat}$ of 2 s$^{-1}$. The turnover number of S. pombe Fcp1 is 1000-fold higher than the $k_{cat}$ value of 2 s$^{-1}$ reported for the hydrolysis of pNØ by recombinant S. cerevisiae Fcp1 (15). The $K_m$ value of the S. cerevisiae enzyme for pNØ (60 mM) is 3-fold higher than that of S. pombe Fcp1.

Increasing ionic strength exerted a modest inhibitory effect on the hydrolysis of pNØ by S. pombe Fcp1. The extents of pNØ formation in the presence of 125, 250, and 500 mM NaCl were 75, 48, and 33% of the activity in the absence of added salt (data not shown).

**Sedimentation of S. pombe Fcp1 in a Glycerol Gradient**—The native size of Fcp1 was gauged by zonal velocity sedimentation through a 15–30% glycerol gradient. Marker proteins catalase (248 kDa), bovine serum albumin (66 kDa), and cytochrome c (13 kDa) were included as internal standards. After centrifugation, the polypeptide compositions of the odd-numbered gradient fractions were analyzed by SDS-PAGE (Fig. 4A). The Fcp1 polypeptide (calculated size, 84 kDa) sedimented as a single discrete peak coincident with bovine serum albumin. The phosphatase activity profile paralleled exactly the sedimentation profile of the Fcp1 polypeptide (Fig. 4B). The peak of Fcp1 activity was one fraction lighter than the bovine serum albumin peak. Centrifugation of the recombinant Fcp1 protein in a parallel gradient without marker proteins resulted in an identical single-peak profile of phosphatase activity that coincided with the distribution of the Fcp1 polypeptide (not shown). These results suggest that S. pombe Fcp1 is a monomeric protein in solution, probably with an elongated shape.

**Fcp1 Residues Asp\textsuperscript{170} and Asp\textsuperscript{172} Are Essential for Catalysis**—
domain is a short conserved peptide motif \(^{167LIVLDQTI176}\) in \(S.\textit{pombe}\) Fcp1 that corresponds to the signature sequence of a family of metal-dependent phosphohydrolases and phosphotransferases (22, 23). The two aspartates in the \(\text{DxDxT}\) element were found to be essential for phosphoryl transfer by human phosphomannomutase and 1-3-phosphoserine phosphatase (22, 24). Several family members have been shown to act via an acyl-phosphoenzyme intermediate in which the phosphate is linked to the first aspartate in the \(\text{DxDxT}\) motif (25). Kobor \textit{et al.} (15) recognized the presence of the \(\text{DxDxT}\) motif in \(S.\textit{cerevisiae}\) Fcp1 and found that mutation of either aspartate abolished Fcp1 function.

To assess the role of the \(\text{DxDxT}\) motif in \(S.\textit{pombe}\) Fcp1, we replaced Asp\(^{170}\) and Asp\(^{172}\) with alanine, asparagine, and glutamate and then purified the recombinant mutant proteins from bacteria (Fig. 2A). The D170A, D170N, and D170E proteins were apparently inert in hydrolyzing \(\text{pNpO}\) at up to 5 \(\mu\)g of input protein (Fig. 2B). Comparison with the titration profile of wild-type Fcp1 (which released 21 nmol of \(\text{pNpO}/\mu\)g of protein in 30 min) indicated that the specific activities of the three Asp\(^{170}\) mutants were \(<0.2%\) of the activity of wild-type Fcp1. The D172A, D172N, and D172E mutants were also catalytically defective, with their respective specific activities being 1.8, 4, and 1% of the wild-type value (Fig. 2B). We conclude that: (i) Asp\(^{170}\) and Asp\(^{172}\) are likely constituents of the active site of \(S.\textit{pombe}\) Fcp1; (ii) catalysis is dependent on a carboxylate functional group at both positions; and (iii) there is a steric constraint on the main chain to carboxylate distance both positions that precludes functional substitution of either aspartate by glutamate.

**Deletion Analysis Defines a Phosphatase Catalytic Domain**—Two N-terminal deletion mutations Fcp1(107–723) and Fcp1(140–723) were designed to progressively truncate the Fcp1 protein up to the margins of the conserved region shown in Fig. 1. The N-terminal deletion mutants were expressed in bacteria as His-tagged fusions and purified from soluble lysates by nickel-agarose chromatography (Fig. 5A). The Fcp1(107–723) and Fcp1(140–723) proteins migrated more rapidly during SDS-PAGE than the wild-type Fcp1, as expected, although their mobility was still anomalously slow compared with their calculated sizes of 72 and 66 kDa, respectively. Fcp1(107–723) and Fcp1(140–723) both retained activity in the hydrolysis of \(\text{pNpO}\) to \(\text{pNO}\) (Fig. 5B); their respective specific activities were 83 and 120% of the wild-type activity. We conclude that the N-terminal 140 amino acids of \(S.\textit{pombe}\) Fcp1 are not required for catalysis.

Three C-terminal deletion mutants, Fcp1(1–580), Fcp1(1–486), and Fcp1(1–399), were also expressed in bacteria and purified in parallel. The mobility of the Fcp1(1–580), Fcp1(1–486), and Fcp1(1–399) proteins during SDS-PAGE was fairly consistent with their calculated sizes of 68, 57, and 47 kDa (Fig. 5A). These findings suggest that the C-terminal segment of Fcp1 is responsible for its aberrant electrophoretic mobility. The Fcp1(1–580) protein retained full activity in the hydrolysis of \(\text{pNpO}\) to \(\text{pNO}\) (specific activity 130% of wild type), whereas the more extensively truncated derivatives Fcp1(1–486) and Fcp1(1–399) were apparently inert (Fig. 5B). We concluded that the C-terminal 143 amino acids of \(S.\textit{pombe}\) Fcp1 are not required for catalysis. The segment from amino acids 487–580, deletion of which abolished Fcp1 function, embraces a BRCT domain that is present in all known Fcp1 orthologs.

**Fcp1 Dephosphorylates a Defined CTD Phosphopeptide**—A caveat inherent in the use of pol II as a substrate for CTD phosphatases is that the structure of the phosphorylation array is not known and is likely to consist of a heterogeneous mixture of different arrays. A rigorous treatment of the substrate specificity of Fcp1 requires a defined CTD molecule in which the number and position of the phosphates is known and, if possible, subject to manipulation by the investigator. In the present study, we examined the activity of recombinant \(S.\textit{pombe}\) Fcp1 with a synthetic 28-amino acid phosphopeptide (Fig. 6) consisting of four tandem repeats of the CTD heptad sequence (YSP(T)SP(S)) in which all Ser\(^2\) and Ser\(^5\) residues are Ser-PO\(_4\).

Fcp1 was incubated for 60 min at 37 °C with 25 \(\mu\)M of the Ser\(^5\)-PO\(_4\)/Ser\(^5\)-PO\(_4\) CTD peptide and 10 \(\mu\)M \(\text{MgCl}_2\). Release of inorganic phosphate from the CTD was measured colorimetrically using the malachite green method. We found that wild-type Fcp1 was active in dephosphorylating the synthetic CTD phosphate peptide; the extent of \(P_i\) release was proportional to input Fcp1 in the range of 40–620 ng (Fig. 6A). \(P_i\) release plateaued at 2.5–5 \(\mu\)g of input Fcp1, at which point \(\sim60\%\) of the input phosphoserine residues had been hydrolyzed. The specific activity on this substrate in the linear range of the Fcp1 titration curve was 7 nmol of \(P_i\)/\(\mu\)g of protein in 60 min, which corresponds to a turnover number of \(\sim0.16\) s\(^{-1}\). \(P_i\) release was ablated when magnesium was omitted from the reaction mixture (data not shown). The activity of the recombinant Fcp1 preparation with the synthetic CTD phosphopeptide substrate was evidently intrinsic to Fcp1, insofar as the recombinant D170A mutant preparation catalyzed no detectable \(P_i\) release up to 5 \(\mu\)g of input protein (Fig. 6A).

Fcp1 activity on the Ser\(^5\)-PO\(_4\)/Ser\(^5\)-PO\(_4\) CTD peptide displayed a bell-shaped pH profile with an acidic optimum at pH 5.5 (Fig. 6B). As we observed earlier with Fcp1 activity on the pNpO substrate, its CTD phosphatase activity was also abol-
characterization of Fcp1 from fission yeast

Fig. 6. S. pombe Fcp1 dephosphorylates a defined CTD phosphopeptide. The synthetic CTD peptide composed of four tandem heptad repeats containing phosphoserine at positions 2 and 5 of each heptad is shown. A, CTD phosphatase activity. Reaction mixtures containing 50 mM Tris acetate (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5), 10 mM MgCl₂, either 25 µM CTD Ser²-Po₄/Ser⁵-Po₄, peptide and either wild-type (WT) Fcp1 or the D170A mutant as specified were incubated for 60 min at 37 °C. Phosphorylation release is plotted as a function of pH. B, pH dependence. Reaction mixtures containing 50 mM Tris acetate (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5), 10 mM MgCl₂, 25 µM of the Ser²-Po₄/Ser⁵-Po₄ peptide, and 0.3 µg of Fcp1 were incubated for 60 min at 37 °C. Phosphate release is plotted as a function of pH.

Displacement at a pH level of ≤4.5. The CTD phosphatase activity diminished as the pH was increased toward neutrality. Pᵢ release from the CTD at pH 7.5 was 20% of the activity at pH 5.5.

Fcp1 Is More Active on a CTD Ser²-Po₄ Substrate than a CTD Ser⁵-Po₄ Substrate—The preceding experiment using a doubly phosphorylated Ser²-Po₄/Ser⁵-Po₄ peptide substrate showed clearly that Fcp1 is capable of dephosphorylating the CTD outside the context of pol II. To gauge whether Fcp1 has any intrinsic preference for the position of phosphoserine within the CTD heptad, we employed synthetic 28-amino acid peptides consisting of four tandem repeats phosphorylated exclusively at Ser² or Ser⁵ of each heptad. Reaction of Fcp1 with 25 µM of the CTD Ser²-Po₄ peptide resulted in Pᵢ release proportional to input Fcp1 (Fig. 7). Dephosphorylation of Ser² plateaued at 0.6–5 µg of input Fcp1, at which point 98% of the input phosphorysine residues had been hydrolyzed. The specific activity was 16 nmol of Pᵢ release/µg of protein in 60 min, which corresponds to a turnover number of ~0.37 s⁻¹. The instructive finding was that Fcp1 was much less active in dephosphorylation of Ser⁵. The titration profile with 25 µM of the CTD Ser⁵-Po₄ substrate displayed a clear shift to the right (Fig. 6). The specific activity of 1.6 nmol of Pᵢ release/µg of protein in 60 min was 1 order of magnitude lower than the activity of Fcp1 in dephosphorylating the Ser²-Po₄ substrate. We conclude that S. pombe Fcp1 displays an intrinsic preference for dephosphorylation of Ser² versus Ser⁵ of the CTD heptad.

The specificity of Fcp1 for the CTD peptide was underscored by an assay of the ability of Fcp1 to dephosphorylate free phosphoserine. A 60-min reaction of 10 µg of Fcp1 with 2 mM phosphoserine (a level 20-fold higher than the total concentration of phosphoserine present in 25 µM of the 28-mer CTD Ser²-Po₄ peptide) resulted in the release of 0.3 nmol of Pᵢ. The specific activity with 2 mM phosphoserine was 0.2% of the activity with 25 µM CTD Ser²-Po₄ peptide. We surmise that the CTD itself and the position of the phosphoserine within the heptad are recognized by Fcp1.

Discussion

Our studies of the physical and enzymatic properties of recombinant S. pombe Fcp1 complement and extend prior studies of CTD phosphatases from other sources; they also provide important new insights to the intrinsic ability of Fcp1 to dephosphorylate the isolated CTD (divorced from the body of pol II) and an inherent preference of Fcp1 for dephosphorylation of CTD Ser²-Po₄ versus CTD Ser⁵-Po₄.

Fission yeast Fcp1 provides an excellent model system for biochemical and structural analysis, insofar as the soluble recombinant protein is expressed at very high levels in bacteria and is easily purified. We surmised via velocity sedimentation that S. pombe Fcp1 has a monomeric native structure, most likely with an asymmetric shape to account for the fact that it sediments slightly lighter than expected for a globular protein of ~84 kDa. Our data agree with the findings of Chambers and Kane (6) for the S. cerevisiae CTD phosphatase, which they judged via sedimentation and gel filtration to be an elongated monomer. Human Fcp1 is a 961-amino acid polypeptide, but it chromatographs during gel filtration with an apparent size of ~300 kDa (9). It is not clear whether the native mammalian enzyme is a monomer or an oligomer of the Fcp1 polypeptide. The human Fcp1 polypeptide is 30% larger than the fungal Fcp1 polypeptides (723–732 amino acids), and the human protein contains two long internal structural modules that are
Characterization of Fcp1 from Fission Yeast

missing from the fungal orthologs. Thus, it is conceivable that the segments unique to the human enzyme might either extend the conformation of a human Fcp1 monomer or else mediate its oligomerization.

One of our notable findings, with practical and mechanistic implications, is that Fcp1 activity displays an acidic pH optimum (at pH 5.5) for the hydrolysis of pNp0P and the phosphorylated CTD. Practically, it is conceivable that prior studies of CTD phosphatases underestimated Fcp1 catalytic activity because the assays were performed at a pH level of ≈7.5. Mechanistically, the pH profile suggests the existence of at least two different functional groups at which the protonation state has a strong impact on phosphatase activity. We speculate that the sharp decrease in catalysis between pH 5.5/5.0 and pH 4.5 is a consequence of protonation of one or both of the essential Asp residues of the 170DXD 172 active site motif. We entertain two possible explanations for the decline in activity as the pH is increased above neutrality: (i) activity requires a protonated functional group on the Fcp1 enzyme (e.g., a histidine, with a presumptive pK of ~6.5) or (ii) Fcp1 is more active when the phosphate group of the substrate is in the monoanionic state (protonated) rather than the dianionic state; this transition would have a presumptive pK of ~6.5–7.0.

Our demonstration that the Asp 170 side chain of the 170DXD 172 motif of S. pombe Fcp1 is strictly essential for catalysis is consistent with the mutational studies of the equivalent side chain (Asp 166) of S. cerevisiae Fcp1 (15). However, whereas we found that replacement of Asp 172 of S. pombe Fcp1 with either Asn or Glu caused a severe decrease in hydrolysis of pNp0P, the activity of the corresponding S. cerevisiae Fcp1 mutant D182E with pNp0P was 42% of the wild-type activity. The different residual activity of the glutamate mutants (1% for S. pombe versus 42% for S. cerevisiae) may reflect the different conditions used for the phosphatase assay. Alternatively, different enzymes in the DXD phosphatase family might vary in their sensitivity to changes in the main-chain-carboxylate distance at the distal Asp of the motif. We found recently that both Asp residues of the DXD motif are strictly required for the activity of T4 polynucleotide 3′ phosphatase, i.e. neither could be substituted functionally by glutamate (26).

Deletion analysis showed that large segments could be deleted from the N and C termini of S. pombe Fcp1 without loss of phosphatase activity. The apparent margins of the catalytic domain (amino acids 140–580) are in accord with the results of in vivo deletion analysis of S. cerevisiae Fcp1 (27). In both fission and budding yeast, the BRCT domain is apparently critical for Fcp1 function along with the Fcp1 homology domain. The complete loss of S. pombe Fcp1 phosphatase activity in vitro with deletion of the BRCT domain implicates this segment either in ensuring the proper tertiary structure of the protein or in supplying one or more functional groups to the phosphatase active site.

Ser2 and Ser5 are both extensively phosphorylated in vivo, and various CTD serine kinases differ in their site preference (28–30). If the enzymes that add phosphates to the CTD can discriminate position within the heptad, then it is reasonable to think that the enzymes that dephosphorylate the CTD might also have inherent site specificity. This issue had not been addressed previously. Here we found that, contrary to prior reports, Fcp1 was able to dephosphorylate an isolated CTD substrate divorced from the rest of pol II. Indeed, Fcp1 was capable of releasing a large molar excess of phosphate from the CTD peptide substrate during the in vitro reaction (~1300-fold excess in the case of the CTD Ser2-P04 peptide). It is worth emphasizing that the CTD phosphatase activity was observed at 25 μM concentration of the synthetic CTD phosphopeptide, i.e. nearly 3 orders of magnitude lower than the Km of Fcp1 for the nonspecific substrate pNp0P. The inactivation of the CTD phosphatase by the D170A mutation dispelled any doubt that the CTD phosphatase function was intrinsic to Fcp1. Earlier studies of Fcp1 activity on the isolated CTD may not have been optimally sensitive because of the use of higher pH reaction conditions than those used presently and, perhaps more significantly, the use of a recombinant CTD fusion protein substrate that had been phosphorylated on Ser5 (see below).

We exploit the synthetic CTD peptide substrates to show for the first time Fcp1 displays an inherent preference for a particular CTD phosphorylation array. Using equivalent concentrations of CTD peptides of identical amino acid sequence and phosphoserine content, which differed only in the positions of phosphoserine within the heptad, we found that Fcp1 was 10-fold more active in dephosphorylating Ser2-P04 than Ser5-P04. Preferential action of Fcp1 at Ser2-P04 in vitro provides an explanation for the observation of Cho et al. (14) that conditional mutations of S. cerevisiae Fcp1 lead to increased levels of CTD Ser5 phosphorylation in vivo at the semipermissive temperature. Their finding that Ser5 phosphorylation was apparently not affected in the fcp1 mutants led to the suggestion that a phosphatase other than Fcp1 is likely to be responsible for dephosphorylating Ser5 in vivo (14). Schroeder et al. (12) reported that Ser5 phosphate levels did increase on transcribed genes in fcp1 mutants at restrictive temperature, and they inferred that Fcp1 is involved in dephosphorylating Ser5 in vivo. Here we show that recombinant Fcp1 can dephosphorylate CTD Ser2-P04, albeit less efficiently than Ser2-P04. It is conceivable that the Ser2 and Ser5 dephosphorylation activities of conditional mutants of Fcp1 display differential sensitivity to thermal inactivation.

The finding that S. pombe CTD phosphatase has inherent specificity for phosphate position raises numerous interesting questions about the potential for regulation of Fcp1 action, e.g. via variations in the complexity of the phosphorylation array, changes in CTD structure or accessibility caused by proteins (or other regulators) that interact with the CTD, and changes in Fcp1 catalytic activity or substrate specificity elicited by proteins (or other regulators) that interact with Fcp1. We anticipate that synthetic CTD phosphopeptides will provide powerful tools to dissect the structural requirements for substrate recognition and catalysis by Fcp1 and the effects of potential regulatory factors.

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