**cis-Proline-mediated Ser(P)⁵ Dephosphorylation by the RNA Polymerase II C-terminal Domain Phosphatase Ssu72**

Received for publication, October 22, 2010, and in revised form, December 2, 2010 Published, JBC Papers in Press, December 15, 2010, DOI 10.1074/jbc.M110.197129

Jon W. Werner-Allen¹, Chul-Jin Lee¹, Pengda Liu¹, Nathan I. Nicely¹, Su Wang², Arno L. Greenleaf³, and Pei Zhou³*

From the ¹Department of Biochemistry and the ³X-ray Crystallography Shared Resource, Duke University Medical Center, Durham, North Carolina 27710

RNA polymerase II coordinates co-transcriptional events by recruiting distinct sets of nuclear factors to specific stages of transcription via changes of phosphorylation patterns along its C-terminal domain (CTD). Although it has become increasingly clear that proline isomerization also helps regulate CTD-associated processes, the molecular basis of its role is unknown. Here, we report the structure of the Ser(P)⁵ CTD phosphatase Ssu72 in complex with substrate, revealing a remarkable CTD conformation with the Ser(P)⁵–Pro⁶ motif in the *cis* configuration. We show that the *cis*-Ser(P)⁵–Pro⁶ isomer is the minor population in solution and that Ess1-catayzed *cis*-trans-proline isomerization facilitates rapid dephosphorylation by Ssu72, providing an explanation for recently discovered *in vivo* connections between these enzymes and a revised model for CTD-mediated small nuclear RNA termination. This work presents the first structural evidence of a *cis*-proline-specific enzyme and an unexpected mechanism of isomer-based regulation of phosphorylation, with broad implications for CTD biology.

The C-terminal domain (CTD)² of the largest subunit of RNA polymerase II (RNAPII) consists of multiple tandem heptad repeats, with the consensus sequence Y¹S²P³T⁴S⁵P⁶S⁷, that serve as a flexible binding platform for nuclear factors (1). CTD-binding partners influence the initiation, elongation, and termination of transcription as well as a myriad of co-transcriptional processes (2). The recruitment of these activities is tied to the progress of the polymerase by cyclic phosphorylation and dephosphorylation of the CTD repeats. For example, phosphorylation at the Ser⁵ position (Ser(P)⁵) predominates at the 5'-end of genes, attracting CTD-binding partners that influence initiation complex formation, mRNA capping, and the transition into elongation (3). As the polymerase moves toward the 3'-end of genes, the level of Ser(P)⁵ declines, whereas phosphorylation at Ser² (Ser(P)²) increases, recruiting nuclear factors responsible for elongation, termination, and 3'-end formation (3). The variable phosphorylation patterns within each heptad repeat, and distributions of these patterns across the full domain create a "CTD code" with a staggering potential complexity (4).

In addition to phosphorylation, proline isomerization provides a second mechanism for regulating the association of CTD-binding partners (5). Due to its cyclic side chain, proline can adopt both *cis* and *trans* conformations about its peptide bond, creating distinct and interconvertible backbone structures with the *cis* isomer being energetically disfavored and therefore less populated (6). Each CTD heptad contains 2 proline residues, and both are preceded by serine residues that are critical targets of phosphorylation. Phosphorylation of Ser-Pro motifs in non-CTD peptides has been shown to modestly stabilize the *cis* form and decrease the rate of isomerization (7). A study with a Ser(P)⁵ CTD peptide reported a *cis* population of <30% for the Ser(P)⁵–Pro⁶ motif with very slow interconversion of *cis* and *trans* isomers, on the order of s⁻¹ (8). Relative to the short timescale of transcriptional events, this slow intrinsic exchange presents two structurally distinct and kinetically isolated binding epitopes for each proline residue in the CTD, adding an additional layer of complexity to the CTD code (4).

The biological importance of CTD proline isomerization is unclear, but hints have been provided by studies of the yeast peptidyl prolyl isomerase Ess1 (Pin1 in humans). Peptidyl prolyl isomerases speed the interconversion of proline isomers by several orders of magnitude to restore *cis*-trans equilibria at a biologically relevant timescale (6). Regulation of the proline "conformational switch" has been proposed to give these enzymes control over the duration and amplitude of a variety of cellular processes (6). Ess1/Pin1 specifically targets phosphorylated Ser-Pro motifs and influences transcription by RNA polymerase II, likely by regulating the phosphorylation state of the CTD (9). Ess1/Pin1 acts on pCTD peptides *in vitro*, preferentially binding to the Ser(P)²–Pro⁶ site over the Ser(P)⁵–Pro⁶ site (10, 11). Numerous genetic links between Ess1 and CTD kinases and phosphatases have been reported (5), and abnormal levels of Pin1 activity cause the accumulation of aberrantly phosphorylated forms of the CTD (12). This latter phenotype may be due in part to the influence of Pin1 on the activity of Ser(P)⁵ CTD phosphatase Fcp1 (12–14). Despite these intriguing findings, there is no detailed understanding of how catalyzed proline isomerization modulates the CTD phosphorylation state.

---

¹This work was supported, in whole or in part, by National Institutes of Health Grants GM079376 (to P. Z.) and GM040505 (to A. L. G.). This work was also supported by a Kamin Fellowship (to J. W. W.-A.). The atomic coordinates and structure factors (code 3P9Y) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

²The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures and Figs. S1–S3.

1 To whom correspondence should be addressed: 220 Sands Bldg., Research Dr., Durham, NC 27710. Tel.: 919-668-6400; Fax: 919-684-8885; E-mail: peizhou@biochem.duke.edu.

2 The abbreviations used are: CTD, C-terminal domain; pCTD, phosphorylated CTD; RNAPII, RNA polymerase II; LMW PTP, low molecular weight protein tyrosine phosphatase; snRNA, small nuclear RNA.
Structure of the Ssu72-pCTD Complex

One of the enzymes linked to Ess1 activity is Ssu72, a CTD phosphatase with specificity for the Ser(P)\(^5\) position (15). Despite its distinct substrate, Ssu72 shares many characteristics of the low molecular weight subfamily of protein tyrosine phosphatases (LMW PTPs) including the signature catalytic motif (CX\(_2\)R) and a similar predicted arrangement of secondary structure elements (16, 17). Biologically, Ssu72 influences all three stages of transcription. Ssu72 interacts genetically and physically with initiation factor TFIIB (18, 19) and has been implicated in gene looping, a proposed mechanism for transcription reinitiation that tethers the promoter and terminator regions of a gene (20–22). During elongation, impaired Ssu72 activity leads to increased RNAPII pausing (23). Finally, Ssu72 is a component of the cleavage and polyadenylation factor complex through its association with Pta1 and is essential for the proper termination of small nuclear RNA (snRNA) transcripts (16, 24–27).

Recently, two studies illustrated an in vivo connection between Ess1 and Ssu72 (28, 29). Impairing Ess1 catalytic activity in yeast cells resulted in a temperature-sensitive phenotype and the accumulation of Ser(P)\(^5\) CTD. Both defects were ameliorated by overexpression of Ssu72 (29). A genomewide analysis of mRNA expression in these cells showed readthrough transcription for a set of genes, mainly snRNAs, with marked similarity to those previously identified in cells with impaired Ssu72 activity (28). The prevailing model explains these results in the following way (28, 29). Phosphorylation at the Ser\(^5\) position would cause the CTD to adopt pre-dominantly the cis form of the Ser(P)\(^5\)-Pro\(^6\) motif. Near the end of snRNA transcripts, dephosphorylation of Ser(P)\(^5\) would coordinate the exchange of Nrd1 and Pcf11, two factors involved in Nrd1-dependent transcription termination. However, Ssu72 would have isomeric specificity for the less populated trans form of the Ser(P)\(^5\)-Pro\(^6\) motif and would require Ess1 activity to efficiently process the entire pool of available substrate. Consequently, reduced catalysis by Ess1 would lead to an increased level of Ser(P)\(^5\) CTD, causing an improper localization of Ser(P)\(^5\)-CTD-bound Nrd1 and a concomitant blocking of Ser(P)\(^2\) CTD-mediated Pcf11 recruitment and resulting in readthrough transcription. This model is supported by the fact that all reported enzymes that target Ser(P)-Pro motifs with isomeric specificity recognize the trans configuration. On the other hand, there is no evidence that the cis-Ser(P)\(^5\)-Pro\(^6\) isomer of the CTD becomes the major population upon phosphorylation (7, 8).

To investigate the mechanism of proline isomer-based regulation of CTD phosphorylation states, we determined the structure of Ssu72 in complex with its Ser(P)\(^5\) CTD substrate. The structure reveals an enzyme fold that contains the conserved LMW PTP scaffold and unique additions and a surprising substrate conformation with the Ser(P)\(^5\)-Pro\(^6\) motif of the CTD adopting the cis configuration. We show that the cis isomer is a minor population of substrate in solution and that Ess1 significantly enhances Ssu72 activity by catalyzing rate-limiting cis-trans interconversion. Together, this work presents structural and kinetic evidence for a unique cis-proline-specific enzymatic activity and a fascinating explanation for the in vivo relationship of Ess1 and Ssu72, with broad implications for the regulation of the CTD code and its role in coordinating co-transcriptional events.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Protein Purification—The full-length WT Drosophila melanogaster Ssu72 gene was PCR-amplified from cDNA (Open Biosystems, clone ID: RE29729), digested, and ligated into a pET15b vector (EMD Biosciences, Inc.) between the NdeI and BamHI restriction sites. Point mutants of Ssu72 and Ess1 were prepared using the QuikChange site-directed mutagenesis kit (Stratagene). The correct sequences for all constructs were verified by DNA sequencing. Protein expression and purification procedures are described in the supplemental material.

NMR—Isotopically enriched proteins for NMR studies were overexpressed in M9 minimal media with \([^{15}N]NH_4Cl\) and \([^{13}C]glucose\) (Cambridge Isotope Laboratories, Inc.) as the sole nitrogen and carbon sources. NMR experiments were conducted at 30 °C using Varian INOVA 600- or 800-MHz spectrometers. Backbone resonances for C13D/D144N D. melanogaster Ssu72 (dSsu72) were assigned using a \(^2H/^{13}C/^{15}N\)-labeled sample with standard three-dimensional triple-resonance experiments.

Crystallization and Structure Determination—The Ssu72-pCTD complex was prepared by incubating a 5:1 molar ratio of pCTD peptide (4.17 mM) to C13D/D144N dSsu72 (0.83 mM) for 30 min on ice. The complex was crystallized by hanging drop vapor diffusion; 1 μl of protein sample was mixed with 1 μl of 22% (w/v) PEG monomethyl ether 550, 100 mM imidazole, pH 6.5, and 150 mM DL-malic acid and suspended over a reservoir of the same solution. Equilibration at 4 °C for ~5 days produced 50 × 50 × 200-μm rhombic-shaped crystals, which were cryoprotected by dipping into a solution of mother liquor with 25% ethylene glycol before flash-cooling in liquid nitrogen. Methods for data collection, phasing, and model refinement are provided in the supplemental material.

Enzyme Kinetics—Reactions with synthetic pCTD substrate (see Fig. 4, A–D) were followed by measuring phosphate release with a standard Malachite Green reagent (30). For experiments with WT Ess1, 350 μM reactions were set up in 50 mM HEPES, 20% glycerol, 1 mM EDTA, 2 mM DTT buffer at pH 6.5 with a SpectraMax microplate reader (Molecular Devices). Absorbance values were converted to phosphate content using a standard curve made with NaPO\(_4\) solutions of known concentrations. The same protocol was followed for experiments with Ess1 mutants, with reactions containing 1 μM of each mutant. Preparation and characterization of the substrate peptide are described in the supplemental material, along with a description of kinetic assays with full-length pCTD substrate (see Fig. 4, E and F).
RESULTS

*Ssu72 Adopts the Conserved LMW PTP Fold with Unique Additions*—Previous kinetic experiments suggest that Ssu72 shares the catalytic mechanism of protein tyrosine phosphatases (17, 31), a two-step reaction that requires: 1) the C\(\text{X}_5\)R catalytic motif with the cysteine side chain as a negatively charged thiolate group and 2) an aspartate residue on a flexible loop positioned near the active site (“the aspartate loop”). In the first step, the thiolate of the catalytic cysteine attacks the substrate phosphorous atom to generate a phosphoenzyme intermediate, and in the second step, the aspartate protonates the leaving phosphate to regenerate the enzyme. By screening orthologs, we identified dSsu72 as a highly stable and soluble enzyme with significant sequence similarity to *Saccharomyces cerevisiae* Ssu72 (Fig. 1A). Mutation of both the catalytic cysteine (C13D) and the catalytic aspartate
Table 1: Data collection and refinement statistics

| Data collection | | | |
|---|---|---|---|
| Resolution range (Å) | 50–2.10 (2.18–2.10) | | |
| Space group | R3 | | |
| Unit cell dimensions (Å) | 157.5, 157.5, 118.8 | | |
| Completeness (%) | 99.9 (99.8) | | |
| Unique reflections | 63898 (6361) | | |
| Redundancy | 3.9 | | |
| Rmerge (%) | 6.1 (32.6) | | |
| I/σ(|I|) | 23.7 (41) | | |

| Refinement | | | |
|---|---|---|---|
| Rmerge/Rfree (%) | 20.90/24.11 | | |
| Number of atoms | Protein 6360 | Peptide 236 | Water 658 |
| | Other molecules 31 | | |
| B-factors (Å²) | Protein 33.38 | Peptide 36.53 | Water 34.55 |
| | Other molecules 32.83 | | |
| r.m.s.d. from ideal geometry | Bond lengths (Å) 0.003 | Bond angles (°) 0.748 | Ramachandran plot |
| | Allowed (%) 99.9 | Favored (%) 97.2 | |

* Rmerge was calculated with the 5% of the data randomly omitted from refinement.
* Nonhydrogen atoms. Riding hydrogens were used in refinement and are included in the deposited structure.
* Imidazole and a PEG fragment.
* Only nonhydrogen atoms included.
* r.m.s.d., root mean square deviation.

(D144N) fully abolished the activity of dSsu72, and an NMR titration of [15N]labeled C13D/D144N dSsu72 with Ser(P)5 CTD peptide revealed tight binding (slow exchange on the NMR timescale) and significant perturbations of residues near the active site loop and the aspartate loop (supplemental Fig. S1).

To obtain a high resolution model of the Ssu72-pCTD interaction, we used x-ray crystallography to determine the structure of C13D/D144N dSsu72 in complex with a synthetic, 7-residue Ser(P)5 CTD peptide containing the minimal binding epitope (32) with an additional residue at each end (Ac-PTpSYS-NH2, where the pS indicates Ser(P)). Phasing was carried out by molecular replacement using the apoWT dSsu72 structure deposited by the Northeast Structural Genomics Consortium (Protein Data Bank (PDB) code 3FDF), and the model was refined at 2.1 Å resolution (Table 1).

The Ssu72 structure contains the LMW PTP fold and two sizable additions (Fig. 1, A–C, PDB code 3PYV). The conserved scaffold consists of a central, four-stranded, parallel β-sheet flanked by two α-helices on one side and a third α-helix on the other and is represented in Fig. 1 by the structure of the LMW PTP Lpt1 in complex with its substrate p-nitrophenyl phosphate (33). The first addition to the Ssu72 fold is an ~60-residue insertion after the second β-strand that forms a subdomain of two β-strands packed against three α-helices. This insert structure sits immediately adjacent to the active site loop and contributes to substrate binding (see below). The corresponding region in the LMW PTP fold contains ~25 residues and forms a single α-helix that packs against the back of the central β-sheet and is tethered by long loops that interact with substrate. The second addition is an ~30-residue C-terminal extension that forms a helix-turn-strand motif with the β-strand bonding in an anti-parallel fashion to the central β-sheet. Another distinguishing characteristic of Ssu72 is the length of its aspartate loop. The α-helix following the aspartate loop is one turn shorter in Ssu72, which allows a commensurate shortening of the aspartate loop and increases active site accessibility from the side opposite the insert subdomain.

The active site architecture of Ssu72 is very similar to LMW PTPs, providing structural support for a common mechanism of dephosphorylation. Fig. 1, D and E, show a close-up view of phosphate binding for the Ssu72-pCTD complex and the LMW PTP Lpt1-p-nitrophenyl phosphate complex (33). The conformations of active site loops in these structures are nearly identical, with the backbone amide groups forming a series of hydrogen bonds to the substrate phosphate group and the guanidinium group of the catalytic arginine extending this hydrogen bonding to form a complete circle. Despite the different length of aspartate loops, the positions of the catalytic aspartate side chains (D144N in the Ssu72 structure) are very similar. Although the carboxylate of the Lpt1 aspartate is turned away from the active site, presumably due to electrostatic repulsion with the substrate phosphate group, in the Ssu72 structure, the catalytic D144N side chain is pointed toward the phosphate. Another difference between the active sites is the position of the substrate phosphate. In the Lpt1 structure, the catalytic cysteine is mutated to alanine, and this loss of negative charge allows the phosphate to bind deeper in the active site, close to the position predicted for the phosphoenzyme intermediate (33), whereas the phosphate group in the Ssu72 structure is held ~0.7 Å higher by the negative charge of the C13D side chain.

Ssu72 Recognizes a cis-Proline Substrate—The electron density for the pCTD peptide is excellent and extends almost completely through both termini (Fig. 2A). While the substrate phosphate group is anchored in the active site with strong hydrogen bonds, the insert subdomain creates a deep and narrow recognition cleft that forces the C-terminal end of the CTD to extend away immediately from the phosphate in a nearly opposite direction. This constrained conformation is made possible by a cis isomer of the Ser(P)5–Pro6 peptide bond (ω = −2.42°) that provides an abrupt turn in the substrate backbone. The uniqueness of the cis-Ser(P)5–Pro6 isomer and its pronounced effect on the substrate conformation are readily apparent by comparison with the structure of Ser(P)5 CTD bound to the CTD phosphatase Scp1 (PDB code 2GHT) in Fig. 2, C and D (34). In the Scp1 complex, the trans isomer of the Ser(P)5–Pro6 bond provides an extended backbone configuration, in contrast to the sharp kink produced by the cis-Ser(P)5–Pro6 isomer.

To achieve its unusual isomeric specificity, Ssu72 must stabilize the high energy cis-proline substrate conformation (Fig. 2B). This is accomplished in part by facilitating the formation...
of an intramolecular hydrogen bond between the substrate Thr$^4$ side-chain hydroxyl and the Pro$^6$ backbone carbonyl. The Thr$^4$ side chain is positioned by its methyl group binding in a hydrophobic pocket formed by the side chains of Lys$^{44}$, Pro$^{46}$, and Pro$^{53}$ and the backbone of Leu$^{45}$. Hydrogen bonding with the Thr$^4$ hydroxyl forces the Pro$^6$ residue to adopt the cis isomer, which is further stabilized by strong van der Waal interactions between the Pro$^6$ ring and a hydrophobic patch of Ssu72 formed by the side chains of Pro$^{46}$, Leu$^{82}$, and Met$^{85}$ and the backbone of Asn$^{145}$. Between the Thr$^4$ and Pro$^6$ residues, the Ser(P)$^5$ backbone amide forms a hydrogen bond with the Lys$^{44}$ backbone carbonyl of Ssu72, while the Ser(P)$^5$ side chain sits in the active site as described above. At the N-terminal end of the substrate peptide, the Pro$^3$ residue makes minor van der Waal contacts with the Lys$^{44}$ side chain of Ssu72. Toward the C-terminal end, additional isomer-specific binding energy is provided by an intermolecular hydrogen bond between the substrate Ser$^7$ main-chain amide group and the Asn$^{144}$ backbone carbonyl. While the side chains of the Ser$^7$ and Ser$^2$ residues are largely solvent-exposed, the phenol ring of the intervening Tyr$^1$ side chain forms an aromatic-amide stacking interaction with the Ala$^{49}$--Phe$^{50}$ peptide bond of Ssu72 (35), causing a reorientation of the loop between the two $\beta$-strands of the insert subdomain in the complex when compared with the apo structure (supplemental Fig. S2).

Ess1-catalyzed Proline Isomerization Stimulates Ssu72 Activity—A previous study of peptides with Ser(P)-Pro motifs showed that the cis isomer is less favored than the trans form, with populations of 12--20% depending on the adjacent residues (7). Similar work with a Ser(P)$^2$ CTD peptide reported a cis population under 30% for the Ser(P)$^2$--Pro$^3$ isomer (8). Although these results suggest that phosphorylation of the Ser$^5$--Pro$^6$ CTD motif is unlikely to change the minority status of the cis isomer, no direct measurements have been reported. To address this point, we measured the fractions of cis- and trans-Ser(P)$^5$--Pro$^6$ in our substrate, a synthetic peptide with the sequence Ac-PtpSPSYS-NH$_2$ (where the pS indicates Ser(P)), which is the same peptide used for crystallization. A natural abundance $^{13}$C-heteronuclear single quantum correlation spectrum shows two sets of signals for each proline C$\alpha$/C$\beta$ atom, corresponding to the cis and trans forms (Fig. 3A). However, the substrate peptide contains 2 prolines, making it difficult to unambiguously assign peaks to the Pro$^3$ and Pro$^6$ residues. By collecting the same experiment with a shorter peptide containing only the Pro$^6$ residue (Fig. 3B), we were able to assign the Pro$^6$ resonances and readily map them to the longer peptide. Populations were calculated using the

![FIGURE 2. Binding of cis-proline pCTD substrate by Ssu72.](image-url)
average ratio of cis and trans peak volumes for the Pro6 Cβ and Cγ atoms. This gave a fractional population of 12.4% for the cis isomer, verifying that the Ser(P)5–Pro6 motif is predominantly in the trans configuration.

Given that Ssu72 recognizes the minor population of cis-Ser(P)5–Pro6 isomers, the extremely slow interconversion of cis-trans-proline (s⁻¹) may present a rate-limiting step for dephosphorylation. To test this possibility, we examined the effect of catalyzed proline isomerization on Ssu72 activity. Reactions were monitored by measuring phosphate release with a Malachite Green-based assay. In a reaction with 60 μM pCTD and 4 μM WT dSsu72, less than half of the input substrate was dephosphorylated in 5 min (Fig. 4A). To speed the interconversion of proline isomers, we added increasing amounts of Ess1, a CTD proline isomerase that acts on Ser(P)5–Pro6 motifs. Ess1 significantly stimulated the activity of Ssu72, with a 2 μM Ess1 concentration causing a 2-fold increase in reaction completion over 5 min. The increase in Ssu72 activity was saturable (Fig. 4B), and a concentration of 10 μM Ess1 was sufficient to maximize the completion percentage at all time points (data not shown). To ensure that this result is not caused by reduced thermostability, we measured melting temperatures for each Ess1 mutant by circular dichroism (data not shown). Only the H164R mutation caused a large decrease in stability (melting temperature of 32 °C) that may contribute to its impaired stimulation. Together, these studies demonstrate that Ess1 enhances Ssu72 activity by catalyzing proline isomerization of the pCTD substrate.

To confirm that catalyzed isomerization is the cause of Ssu72 stimulation, we performed identical experiments with four catalytically impaired Ess1 mutants. Three of these mutations are in the catalytic domain of Ess1 (C120S, S122P, and H164R), whereas the fourth disrupts phosphate binding in the WW domain (K68A). All four mutations significantly decreased the enhancement of Ssu72 activity (Fig. 4D). To ensure that this result is not caused by reduced thermostability, we measured melting temperatures for each Ess1 mutant by circular dichroism (data not shown). Only the H164R mutation caused a large decrease in stability (melting temperature of 32 °C) that may contribute to its impaired stimulation. Together, these studies demonstrate that Ess1 enhances Ssu72 activity by catalyzing proline isomerization of the pCTD substrate.

Finally, to evaluate the effect of Ess1 on Ssu72 activity toward its natural substrate, we performed in vitro reactions using the full-length, 26-repeats CTD from S. cerevisiae. A GST-S. cerevisiae CTD-His fusion construct was hyperphosphorylated with the yeast CTD kinase CTDK-1 (36), and dephosphorylation by Ssu72 was monitored by measuring substrate depletion with a Ser(P)5-specific antibody in reactions with and without 100 μM Ess1 (Fig. 4E and F). As in the reactions with synthetic pCTD substrate, Ess1 greatly facilitates dephosphorylation by Ssu72, decreasing the time required to reach ~90% completion by 20-fold (from 20 to 1 min).
DISCUSSION

Ssu72 as a New Family of LMW Phosphatases—The CTD phosphatase Ssu72 is an intriguing enzyme. Despite being built on the scaffold of a protein tyrosine phosphatase, its activity is directed at phosphorylated serine residues in the 5th position of the CTD heptad, with isomeric specificity for the cis configuration of the Ser(P)5–Pro6 peptide bond. Our complex structure also suggests a conserved catalytic mechanism, which agrees well with prior kinetic studies (17). Of the two additions to the LMW PTP scaffold, the insert subdomain plays a major role in the unique substrate specificity of Ssu72, contributing nearly all of the residues that form the non-phosphate substrate recognition surface and severely restricting access to the active site. Very recently, Xiang et al. (37) reported a crystal structure of human Ssu72 in complex with pCTD substrate and symplekin (homologous to Pta1 in yeast), a scaffold protein in the cleavage and polyadenylation complex, which shows that the C-terminal extension of Ssu72 forms the symplekin-binding site. The Ssu72 fold, pCTD conformation, and effect of catalyzed proline isomerization on dephosphorylation are consistent with the data presented in this study.

Substrate Specificity of Ssu72—Prior kinetic work on Ssu72 has demonstrated a strict specificity for the Ser(P)5 position of the CTD heptad (15, 32), in contrast to CTD phosphatases Scp1 and Fcp1, which have only preferential activity for Ser(P)5 and Ser(D)5, respectively (38, 39). Interestingly, both potential sites are Ser(P)-Pro motifs, meaning that 2 of the 4

FIGURE 4. Ess1 stimulates pCTD dephosphorylation by Ssu72. A, the activity of Ssu72 was monitored with various concentrations of the proline isomerase Ess1. The maximum stimulation was ~2-fold under these conditions. In A–D, each point represents the average from three independent reactions, and error bars denote S.D. B, Ess1 enhancement of Ssu72 activity is saturable. The last points of the reactions in A are plotted versus Ess1 concentration, with black circles representing reactions omitted from A for clarity. C, pCTD dephosphorylation by Ssu72 reaches completion without Ess1. The no Ess1 reaction in A was monitored over 25 min and reached ~90% completion in 20 min; 4-fold slower than the reaction with 2 μM Ess1. D, four catalytically impaired Ess1 mutants fail to significantly stimulate Ssu72. Mutants contained 1 μM of each Ess1 protein. Residual enhancement corresponds to a 50–100-fold reduction of WT Ess1 activity (compare with A). E and F, Ssu72 stimulates dephosphorylation of full-length pCTD. The 26-repeat S. cerevisiae CTD was hyperphosphorylated in vitro and used as a substrate in reactions monitored by Western blotting with Ser(P)5 (SSP)-specific antibodies. As a control, Ser(P)2 (S2P) levels were also measured; the apparent increase in the S2 signal is likely due to a higher affinity of the S2 antibody for singly phosphorylated Ser(P)5 heptads over doubly phosphorylated Ser(P)2/Ser(P)5 heptads, as reported for other Ser(P)2-specific antibodies (44). Ser(P)5 CTD levels were quantified with infrared imaging, and the resulting substrate depletion curves are shown in F. Each point represents the average of three independent reactions, and error bars denote S.D.
residues recognized by Ssu72 are identical in the Ser(P)⁷ and Ser(P)⁵ substrates. Therefore, discrimination must be based on the Thr⁴ and distal Tyr⁴ positions. For a Ser(P)⁷ substrate, the Tyr⁴ position would be substituted by serine, causing a decrease in binding energy from the loss of the aromatic amide stacking interaction. However, the Thr⁴ position is likely the more important determinant of substrate specificity. The Thr⁴ residue would be replaced by tyrosine in a Ser(P)⁷ substrate, which would disrupt substrate recognition in two major ways. First, the favorable hydrophobic interactions with the insert subdomain would be lost, and the accommodation of the large phenol group would require a significant reconfiguration of the substrate backbone to prevent steric clashes. Second, this substrate would be unable to replicate the intramolecular hydrogen bond we observe between the Thr⁴ hydroxyl and the Pro⁶ backbone carbonyl, making the already unfavorable cis conformation even less energetically stable.

Although the exact degree of isomeric specificity of Ssu72 is unclear, our structural and kinetic studies demonstrate a strong preference for the cis-Ser(P)⁵–Pro⁶ conformation. First, modeling the trans-Ser(P)⁷–Pro⁶ isomer in the Ssu72-pCTD complex structure leads to large sterical clashes between the pCTD backbone and the side chains of the C-terminal α-helix of the insert subdomain. Also, if the activity toward cis and trans isomers were comparable, there would be no stimulation of dephosphorylation by Ess1. Finally, additional support for strong cis-proline specificity comes from a kinetic study that measured activity toward a set of Ser(P)³ CTD substrates with an alanine substitution at each heptad position (32). These experiments showed that CTD positions Thr⁴, Pro⁶, and Tyr⁴ of the following heptad were critical for substrate recognition, a result that is consistent with the intermolecular contacts in our complex structure. Importantly, the largest decrease in Ssu72 activity was observed with the P6A mutant substrate, an ~30-fold reduction when compared with wild type. This likely reflects not only the loss of binding energy for the proline ring but also the reduced propensity of the Ser(P)⁵–Ala⁶ peptide bond to adopt a cis configuration.

**Regulation of Isomer-specific Enzymes—**To the best of our knowledge, Ssu72 is the first example of an enzyme with specificity for the cis isomer of proline. Isomeric specificity for trans-Ser(P)-Pro motifs has been previously established for two enzymes. The serine/threonine phosphatase PP2A dephosphorylates trans-Ser(P)-Pro motifs in its substrate Cdc25C and Tau proteins and shows increased activity in reactions that include Pin1 (40). Likewise, the serine/threonine kinase ERK2 phosphorylates trans-Ser-Pro motifs in the RNase T1 substrate and is also stimulated by proline isomerase activity (41). Importantly, catalyzed proline isomerization only increases the activity of these trans-specific enzymes toward Ser(P)-Pro motifs that adopt the cis conformation in the structure of their protein. For example, ERK2 phosphorylates two sites in RNase T1, cis-Ser⁵⁴–Pro⁵⁵ and trans-Ser⁷₂–Pro⁷₃; however, only phosphorylation at Ser⁵⁴–Pro⁵⁵ is enhanced by the addition of peptidyl prolyl isomerases. This illustrates a crucial point: catalyzed proline isomerization is an effective regulatory mechanism of phosphorylation/dephosphorylation only when the target motif exists predominantly in the opposite isomeric configuration required for catalysis. In this case, the isomer-specific enzyme can only process a small fraction of the total substrate, and the extremely slow, uncatalyzed cis-trans isomerization prevents re-equilibration of the non-processed fraction. In the opposite scenario, the majority of substrate is already in the correct isomeric form and can be readily processed, with catalyzed proline isomerization having little influence.

The effect of proline isomerization on CTD-associated activities depends greatly on the CTD conformation during the transcription cycle. All structural studies of CTD-binding proteins have revealed binding that is, in fact, isomer-specific but preferential for trans-Ser(P)-Pro motifs. In the case of Pcf11, the CTD-interacting domain discriminates in favor of 3 consecutive trans-prolines, including one Ser(P)²–Pro³ motif (8). In the structure of CTD phosphatase Scp1 in complex with Ser(P)²/Ser(P)³ CTD, both the Ser(P)²–Pro³ and the Ser(P)³–Pro⁶ motifs adopt the trans configuration, with binding to either cis form seemingly prevented by steric clashes (34). For both of these proteins, the regulation of binding or catalysis by proline isomerization would demand a CTD conformation with a minority trans population. This is especially true for non-enzymatic proteins such as Pcf11, which may only require one correctly configured binding site out of dozens of CTD repeats. However, our measurements with a Ser(P)³ CTD peptide and prior work with a Ser(P)² CTD peptide (8) show that cis-proline is the less populated isomer. Given the lack of evidence for any stable full-length CTD structure, it seems likely that cis-prolines constitute the isomeric minority in vivo as well and that proteins with specific recognition of cis-proline are the major targets of regulation by isomerization.

The timescale of transcriptional events is another critical determinant of the effect of Ess1 on CTD-associated activities. We observed only an ~2-fold enhancement of Ssu72-catalyzed product accumulation in reactions with synthetic pCTD substrate as a significant amount of non-catalyzed proline isomerization occurs over the course of the 5-min reactions. A similar level of stimulation was reported for the PP2A phosphatase described above in reactions on the same timescale (40). However, at a faster timescale, the effect of Ess1 on Ssu72 activity is greater, as shown in supplemental Fig. S3 in 1.25-min reactions monitored at 15-s increments. Although the exact in vivo timescale of Ssu72 activity is unknown, the effect of impaired Ess1 activity on Nrd1 localization and Pcf11 recruitment is largely confined to the 3′-end of genes (28), suggesting that Ser(P)⁵ CTD dephosphorylation by Ssu72 occurs mainly during termination, which may take only tens of seconds (42). This scenario is well illustrated by the kinetic assays with full-length pCTD in Fig. 4, E and F, where the reaction without Ess1 takes 20 min to dephosphorylate ~90% of the initial pCTD substrate. In contrast, the reaction with Ess1 reaches ~90% completion in 1 min, on par with the estimated timescale of transcription termination.

**A Revised Model for Ess1-regulated Ssu72 Activity—**Our kinetic experiments provide an excellent interpretation of recent in vivo studies of Ess1 (28, 29). We show that the stimulation of Ssu72 activity by Ess1-catalyzed cis-trans intercon-
version of Ser(P)$^5$–Pro$^6$ motifs in the CTD heptad is severely reduced with catalytically impaired Ess1 mutants, which explains the in vivo accumulation of Ser(P)$^5$ CTD in ess1 cells. Interestingly, overexpression of Ssu72 suppressed this phenotype in an A144T ess1 strain, but not an H164R ess1 strain (29). It is possible that different levels of residual activity or thermostability of the Ess1 mutants are responsible for this discrepancy. In contrast, overexpression of Ssu72 rescues the temperature sensitivity of both ess1 strains (29), suggesting that improper regulation of CTD phosphorylation is the main cause of this phenotype. As described in the Introduction, the proposed proline isomer-based regulatory scheme that explains these results requires trans specific activity by Ssu72 (28, 29). Our results compel a simple but unexpected adjustment of this model: reversing the isomeric specificity of Ssu72 (Fig. 5). Interestingly, genetic evidence suggests that this model may be partially bypassed by overexpression of Ser(P)$^5$ CTD phosphatase Fcp1 in yeast (16).

Implications of Proline Isomerization in CTD Biology—Ssu72 provides an exciting validation of proline isomers as a critical component of the CTD code but also raises many new questions about how this regulatory mechanism operates. For example, it is unclear whether the regulation of Ssu72 is a general property of RNAPII transcription or a gene-specific one. Although impaired Ess1 and Ssu72 activity causes readthrough transcription of a small subset of genes consisting mainly of snRNA that undergo Nrd1-dependent termination (16, 28), it is possible this phenotype is only apparent for short transcripts that require a sudden drop in Ser(P)$^5$ levels for proper termination. It will also be interesting to see what other CTD-binding partners specifically recognize cis-proline and how their functions are regulated by catalyzed isomerization. Although these proteins may only regulate the CTD phosphorylation state, there may also be non-enzymatic CTD-binding factors with cis-proline recognition whose association with the transcribing polymerase is regulated by Ess1/Pin1 to fine-tune the recruitment of their co-transcriptional activities.

Acknowledgments—Crystal screening was performed at the Duke University Medical Center X-ray Crystallography Shared Resource. Data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found on the SER-CAT web page. Use of the Advanced Photon Source was supported by the United States Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract Number W-31-109-Eng-38. We thank Louis Metzger and Jeffrey Boyles for enlightening discussions.

REFERENCES
1. Meinhart, A., Kamenski, T., Hoepfner, S., Baumli, S., and Cramer, P. (2005) Genes Dev. 19, 1401–1415
2. Phatnani, H. P., and Greenleaf, A. L. (2006) Genes Dev. 20, 2922–2936
3. Buratowski, S. (2009) Mol. Cell 36, 541–546
4. Buratowski, S. (2003) Nat. Struct. Biol. 10, 679–680
5. Shaw, P. E. (2007) EMBO Rep. 8, 40–45
6. Lu, K. P., Finn, G., Lee, T. H., and Nicholson, L. K. (2007) Nat. Chem. Biol. 3, 619–629
7. Schutkowski, M., Bernhardt, A., Zhou, X. Z., Shen, M., Reimer, U., Rahfeld, J. U., Lu, K. P., and Fischer, G. (1998) Biochemistry 37, 5566–5575
8. Noble, C. G., Hollingworth, D., Martin, S. R., Ennis-Adeniran, V., Smerdon, S. J., Kelly, G., Taylor, I. A., and Ramos, A. (2005) Nat. Struct. Mol. Biol. 12, 144–151
9. Xu, Y. X., and Manley, J. L. (2004) Cell Cycle 3, 432–435
10. Gemmill, T. R., Wu, X., and Hanes, S. D. (2005) J. Biol. Chem. 280, 15510–15517
11. Verdecia, M. A., Bowman, M. E., Lu, K. P., Hunter, T., and Noel, J. P. (2000) Nat. Struct. Biol. 7, 639–643
12. Xu, Y. X., Hirose, Y., Zhou, X. Z., Lu, K. P., and Manley, J. L. (2003) Genes Dev. 17, 1588–1598
13. Palancade, B., Marshall, N. F., Tremeau-Bravard, A., Bensaude, O., Dahmus, M. E., and Dubois, M. F. (2004) J. Biol. Chem. 278, 15917–15921
14. Wu, W. H., Pinto, I., Chen, B. S., and Hampsey, M. (1999) Genetics 153, 643–652
15. Singh, B. N., and Hampsey, M. (2007) Mol. Cell 27, 806–816
16. Ansari, A., and Hampsey, M. (2005) Genes Dev. 19, 2969–2978
17. O’Sullivan, J. M., Tan-Wong, S. M., Morillon, A., Lee, B., Coles, J., Mello, J., and Proudfoot, N. J. (2004) Nature 433, 1014–1018
18. Reyes-Reyes, M., and Hampsey, M. (2007) Mol. Cell 27, 926–936
19. Steinmetz, E. J., and Brow, D. A. (2003) Mol. Cell. Biol. 23, 6339–6349
20. Kim, M., Vasiljeva, L., Rando, O. J., Zhelkovsky, A., Moore, C., and Buratowski, S. (2006) Mol. Cell 24, 723–734
21. Ghazy, M. A., He, X., Singh, B. N., Hampsey, M., and Moore, C. (2009) Mol. Cell 29, 2296–2307
22. Neder, E., He, X., Kim, M., Pootoolal, J., Zhong, G., Canadien, V., Hughes, T., Buratowski, S., Moore, C. L., and Greenblatt, J. (2003) J. Biol. Chem. 278, 33000–33010
23. Xu, Y. X., and Manley, J. L. (2004) Cell Cycle 3, 432–435
24. Gemmill, T. R., Wu, X., and Hanes, S. D. (2005) J. Biol. Chem. 280, 15510–15517
25. Verdecia, M. A., Bowman, M. E., Lu, K. P., Hunter, T., and Noel, J. P. (2000) Nat. Struct. Biol. 7, 639–643
26. Xu, Y. X., Hirose, Y., Zhou, X. Z., Lu, K. P., and Manley, J. L. (2003) Genes Dev. 17, 1588–1598
27. Palancade, B., Marshall, N. F., Tremeau-Bravard, A., Bensaude, O., Dahmus, M. E., and Dubois, M. F. (2004) J. Biol. Chem. 278, 15917–15921
28. Wu, W. H., Pinto, I., Chen, B. S., and Hampsey, M. (1999) Genetics 153, 643–652
29. Singh, B. N., and Hampsey, M. (2007) Mol. Cell 27, 806–816
30. Ansari, A., and Hampsey, M. (2005) Genes Dev. 19, 2969–2978
31. O’Sullivan, J. M., Tan-Wong, S. M., Morillon, A., Lee, B., Coles, J., Mello, J., and Proudfoot, N. J. (2004) Nature 433, 1014–1018
32. Reyes-Reyes, M., and Hampsey, M. (2007) Mol. Cell 27, 926–936
33. Steinmetz, E. J., and Brow, D. A. (2003) Mol. Cell. Biol. 23, 6339–6349
34. Kim, M., Vasiljeva, L., Rando, O. J., Zhelkovsky, A., Moore, C., and Buratowski, S. (2006) Mol. Cell 24, 723–734
35. Ghazy, M. A., He, X., Singh, B. N., Hampsey, M., and Moore, C. (2009) Mol. Cell 29, 2296–2307
36. Neder, E., He, X., Kim, M., Pootoolal, J., Zhong, G., Canadien, V., Hughes, T., Buratowski, S., Moore, C. L., and Greenblatt, J. (2003) J. Biol. Chem. 278, 33000–33010
37. Xu, Y. X., and Manley, J. L. (2004) Cell Cycle 3, 432–435
38. Gemmill, T. R., Wu, X., and Hanes, S. D. (2005) J. Biol. Chem. 280, 15510–15517
39. Verdecia, M. A., Bowman, M. E., Lu, K. P., Hunter, T., and Noel, J. P. (2000) Nat. Struct. Biol. 7, 639–643
40. Xu, Y. X., Hirose, Y., Zhou, X. Z., Lu, K. P., and Manley, J. L. (2003) Genes Dev. 17, 1588–1598
41. Palancade, B., Marshall, N. F., Tremeau-Bravard, A., Bensaude, O., Dahmus, M. E., and Dubois, M. F. (2004) J. Biol. Chem. 278, 15917–15921
42. Wu, W. H., Pinto, I., Chen, B. S., and Hampsey, M. (1999) Genetics 153, 643–652
43. Singh, B. N., and Hampsey, M. (2007) Mol. Cell 27, 806–816
44. Ansari, A., and Hampsey, M. (2005) Genes Dev. 19, 2969–2978
45. O’Sullivan, J. M., Tan-Wong, S. M., Morillon, A., Lee, B., Coles, J., Mello, J., and Proudfoot, N. J. (2004) Nature 433, 1014–1018
46. Reyes-Reyes, M., and Hampsey, M. (2007) Mol. Cell 27, 926–936
47. Steinmetz, E. J., and Brow, D. A. (2003) Mol. Cell. Biol. 23, 6339–6349
48. Kim, M., Vasiljeva, L., Rando, O. J., Zhelkovsky, A., Moore, C., and Buratowski, S. (2006) Mol. Cell 24, 723–734
49. Ghazy, M. A., He, X., Singh, B. N., Hampsey, M., and Moore, C. (2009) Mol. Cell. Biol. 29, 2296–2307
50. Neder, E., He, X., Kim, M., Pootoolal, J., Zhong, G., Canadien, V., Hughes, T., Buratowski, S., Moore, C. L., and Greenblatt, J. (2003) J. Biol. Chem. 278, 33000–33010