Characterization of the Schizosaccharomyces pombe Cdk9/Pch1 Protein Kinase

Sp5 PHOSPHORYLATION, AUTO PHOSPHORYLATION, AND MUTATIONAL ANALYSIS*

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Characterization of the Schizosaccharomyces pombe Cdk9/Pch1 protein kinase is a functional ortholog of the essential Saccharomyces cerevisiae Bur1/Bur2 kinase and a putative ortholog of metazoan P-TEFb (Cdk9/cyclin T). SpCdk9/Pch1 phosphorylates of the carboxyl-terminal domain (CTD) of the S. pombe transcription elongation factor Sp5, which consists of 18 tandem repeats of a nonapeptide of consensus sequence TPAPWSGSK. We document the divalent cation dependence and specificity of SpCdk9/Pch1, its NTP dependence and specificity, the dependence of Sp5-CTD phosphorylation on the number of tandem nonamer repeats, and the specificity for phosphorylation of the Sp5-CTD on threonine at position 1 within the nonamer element. SpCdk9/Pch1 also phosphorylates the CTD heptadepptide repeat array of the largest subunit of S. pombe RNA polymerase II (consensus sequence YSPTSPS) and does so exclusively on serine. SpCdk9/Pch1 catalyzes autophosphorylation of the kinase and cyclin subunits of the kinase complex. The distribution of phosphorylation sites on SpCdk9 (88% Ser(P), 11% Thr(P), 3% Tyr(P)) is distinct from that on Pch1 (94% Ser(P), 6% Thr(P)). We conducted a structure-guided mutational analysis of SpCdk9, whereby a total of 29 new mutations of 12 conserved residues were tested for in vivo function by complementation of a yeast bur1Δ mutant. We identified many lethal and conditional mutations of side chains implicated in binding ATP and the divalent cation cofactor, phosphoacceptor substrate recognition, and T-loop dynamics. We surmise that the lethality of the of T212A mutation in the T-loop reflects an essential phosphorylation event, insofar as the conservative T212S change rescued growth at 30 °C; and the effects of mutat-

mRNA synthesis by RNA polymerase (pol) II is regulated by phosphorylation of several of the protein components of the transcription apparatus (1). Phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of pol II has been the focus of much attention because the CTD acts as an essential scaffold for the binding of macromolecular assemblies that regulate mRNA synthesis and cotranscriptional mRNA processing (2). The pol II CTD is composed of a heptapeptide motif of consensus sequence YSPTSPS repeated in tandem. The mammalian pol II CTD has 52 heptad repeats, the fission yeast Schizosaccharomyces pombe CTD has 29 repeats, and the budding yeast Saccharomyces cerevisiae CTD has 26 copies. The pol II CTD undergoes a cycle of extensive phosphorylation and dephosphorylation at positions Ser5 and Ser2, which is coordinated with the transcription cycle (3). Multiple CTD kinases are present in eukaryotic cells, e.g. budding yeast has four CTD kinases, two of which (Kin28 and Bur1) are essential (3). CTD kinases are heteromeric enzymes consisting of a cyclin subunit plus a cyclin-dependent kinase (Cdk) subunit.

The essential Bur1 Cdk of budding yeast and its cyclin partner Bur2 are putative orthologs of the Cdk9 and cyclin T subunits of the metazoan protein kinase P-TEFb (4, 5). P-TEFb is a transcription elongation factor that overrides the negative actions of Sp5 and its associated factors. Metazoan Sp5, with its binding partner Spt4 and a second factor NELF, arrests pol II elongation at promoter-proximal positions (6–8). Escape from the elongation delay depends on the kinase activity of P-TEFb, which phosphorylates both the pol II CTD and Sp5 (5–13). Sp5 was initially suggested to have negative and positive effects on elongation (6, 14), but several recent studies underscore a negative role for Sp5 during transcription elongation in vivo which is reversed by Cdk9/cyclin T (8, 15–17).

One potential role for phosphorylation of Sp5 by Cdk9 may be to modulate Sp5 function, e.g. converting it from a negative elongation factor into a positive factor. Although the rationale for the arrest and Cdk9-dependent restart of pol II elongation is still not clear, recent studies suggest a connection to mRNA 5’-capping, which is coupled to transcription elongation via physical and functional interactions among the cap-forming enzymes, the phosphorylated pol II CTD, and Sp5 (18–28). Sp5 binds directly to the triphosphatase and guanylyltransferase components of the capping apparatus in mammals and in the fission yeast S. pombe (27, 28). Connections between capping enzymes and Sp5 have also been reported in S. cerevisiae (29). The interactions of the S. pombe triphosphatase (Pct1) and guanylyltransferase (Pce1) enzymes have been studied in vivo and in vitro using two-hybrid assays and purified recombinant proteins, respectively (28). Pce1 and Pct1 bind directly and independently to the unmodified CTD domain of Sp5, which consists of tandem repeats of a nonapeptide motif of consensus sequence TPAPWSGSK (28).

We recently described a physical interaction between S. pombe RNA triphosphatase and SpCdk9, a fission yeast

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‡ The abbreviations used are: pol, polymerase; Cdk, cyclin-dependent kinase; CTD, carboxyl-terminal domain; DTT, dithiothreitol; 5-FOA, 5-fluoroorotic acid; GST, glutathione S-transferase; TEF, transcriptional elongation factor.
homolog of metazoan Cdk9 and *S. cerevisiae* Bur1 (30). Initial studies of SpCdk9 included the identification of the essential *S. pombe* cyclin Peh1 as its binding partner. Peh1 is a homolog of metazoan cyclin T and *S. cerevisiae* cyclin Bur2. Complementation of the *S. cerevisiae bur1Δ* and *bur2Δ* mutants by coexpression of SpCdk9 and Peh1 showed that the fission yeast proteins are genuine orthologs of Bur1/Bur2, a putative fungal P-TEFb. Analysis of the recombinant SpCdk9/Peh1 complex produced in baculovirus-infected insect cells showed that the *S. pombe* proteins comprise a bona fide protein kinase with a heterodimeric quaternary structure (30). The capacity of SpCdk9/Peh1 to phosphorylate the CTD arrays of both pol II and Spt5 in *vitro* echoed the substrate specificity of metazoan P-TEFb (11, 12, 31, 32). These findings suggested a model whereby Spt5-induced arrest of early elongation ensures a temporal window for recruitment of the capping enzymes, which, in turn attract Cdk9 to alleviate the arrest via phosphorylation of one or more components of the pol II elongation complex (30).

Here, we present a biochemical and molecular characterization of the fission yeast SpCdk9/Peh1 kinase complex. The biochemical characterization focuses on delineation of the reaction requirements, optima, and kinetic parameters for SpCdk9/Peh1-mediated phosphorylation of the Spt5-CTD. This event has received less attention from the enzymatic perspective than pol II CTD phosphorylation and is of interest in light of the unique structure of the CTD nonamer array of the active site and a requirement for phosphorylation of the T-loop for SpCdk9 function in yeast.

**EXPERIMENTAL PROCEDURES**

Protein Kinase Assay—The SpCdk9/His-Peh1 kinase complex was isolated from SF9 insect cells coinfected with recombinant baculoviruses expressing SpCdk9 or His-Peh1 by nickel-nitrilotriacetic acid-agarose affinity chromatography as described previously (30). Kinase reaction mixtures (20 μl) containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 2.5 mM MnCl₂ or 10 mM MgCl₂, 5 μM [γ-32P]ATP, 4 μg of GST-Sp5 (5801–909) containing the U-terminal nonapeptide repeat array of S. pombe Spt5, and SpCdk9/His-Peh1 were incubated for 60 min at 22 °C. The reactions were halted by adding SDS to 1% final concentration. The products were analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS. Phosphorylated polypeptides were visualized by autoradiographic exposure of the dried gel and quantified by scanning the gel with a Fujix BAS2500 PhosphorImager.

Phosphoamino Acid Analysis—A kinase reaction mixture (200 μl) containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 2.5 mM MnCl₂ or 10 mM MgCl₂, 50 μM [γ-32P]ATP, 60 μg of GST-Sp5 (5801–898), and 3 μg of SpCdk9/Peh1 complex was incubated for 2 h at 22 °C, then supplemented with 300 μl of binding buffer A (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM DTT, 5% glycerol, 0.03% Triton X-100) and 50 μl of OSE-Sepharose (30). The 32P-labeled GST-Sp5 (5801–898) was adsorbed to the beads during a 1.5-h incubation at 4 °C. The beads were then washed three times with 1 ml of binding buffer A to remove the free [γ-32P]ATP. After the third wash, the bound protein was eluted with 100 μl of 10 mM glutathione. The eluted protein was hydrolyzed by adding 100 μl of concentrated HCl and then heating the mixture at 110 °C for 10 min in a sealed tube. The acid hydrolysate was evaporated to dryness in a vacuum centrifuge. The sample was reconstituted in 10 μl of water. An aliquot was mixed with unlabeled Tyr(P), Thr(P), and Ser(P) markers (Sigma) and spotted onto a cellulose thin layer plate. The phosphoamino acids were separated by high voltage electrophoresis in pyridine acetate (pH 3.5) (24). The unlabeled phosphoamino acid standards were visualized by spraying the plate with ninyhdrin; 32P-labeled material was visualized by autoradiography.

32P-labeled phosphoamino acid analysis of autophosphorylated SpCdk9 and Peh1 was performed as follows. Reaction mixtures (200 μl) containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 2.5 mM MnCl₂, 50 μM [γ-32P]ATP, and 10 μg of SpCdk9/Peh1 complex were incubated for 2 h at 22 °C, then supplemented with 300 μl of binding buffer B (50 mM NaH₂PO₄ (pH 8.0), 50 mM NaCl, 7.5 mM imidazole, and 0.0025% Tween 20) and 50 μl of nickel-agarose beads. The phosphorylated products were absorbed to the beads during a 1.5-h incubation at 4 °C. The beads were washed three times with 1 ml of binding buffer B. After the third wash, the bound protein was eluted with 30 μl of binding buffer B containing 250 mM imidazole. The eluted phosphoproteins were resolved by SDS-PAGE and then transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane-bound radio-labeled SpCdk9 and Peh1 polypeptides were localized by autoradiography and excised. The membrane slices were incubated in 6 N HCl for 2 h at 110 °C and then analyzed by high voltage thin layer electrophoresis.

Test of SpCdk9 Activity in Vivo by Plasmid Shuffle in *S. cerevisiae*—The BUR1 gene was deleted in the *S. cerevisiae* diploid strain W303 and replaced with a cassette specifying kanamycin resistance as described previously (30). Sporulation of the BUR1 bur1Δ:kan cassette with plasmid p360-BUR1 (BUR1 URA3 CEN) yielded viable bur1Δ:kan haploids that were incapable of growth in the presence of 5-fluoroorotic acid (5-FOA), a drug that selects against the BUR1 URA3 plasmid. A MinA haploid of the bur1Δ strain was cotransformed with pYX-SpCDK9 (CEN TRP1) and pYX-SpCDK9 (BUD1 TRP1) containing wild-type or mutant SpCdk9 alleles and with plasmid pYX-PCH1 (CEN ADE2 PCH1) containing the full-length cDNA encoding Peh1 driven by the TRP1 promoter (30). Individual transformants were selected on medium lacking tryptophan and adenine. Colonies were patched on drop-out medium and then streaked on agar plates containing 0.75 mg/ml 5-FOA to select for mutations which failed to support growth after incubation on 5-FOA agar for 7 days 30 °C. The viable 5-FOA-resistant colonies containing mutated SpCdk9 genes were patched to YPD agar at 30 °C and then tested for growth on YPD agar at 18, 23, 30, and 37 °C. Growth was assessed as follows: + + + indicates colony size indistinguishable from strains bearing wild-type SpCdk9; + + denotes reduced colony size; + indicates that only pinpoint colonies were formed; − indicates no growth.

**RESULTS**

Sp5-CTD Kinase Activity of the Fission Yeast Cdk9/Peh1 Complex—Recombinant SpCdk9/Peh1 catalyzed the transfer of 32P from [γ-32P]ATP to the CTD of *S. pombe* Spt5, which spans amino acids 801–990 and consists of 18 tandem repeats of a nonapeptide motif (consensus sequence TPAPNSGSK) (Fig. 1A). SpCdk9/Peh1 phosphorylated the nonamer array of Spt5 in the context of a GST-Spt5 fusion protein (Fig. 1C) or as tag-free Spt5(801–990) (30). No phosphoryl transfer was detected to GST alone (Fig. 1C) or to histone H1 (30). The radio-labeled GST-Spt5-CTD-PO₄ product is detectable by SDS-PAGE analysis and can be quantified by scanning the gel with a PhosphorImager. Initial experiments showed that the extent of the kinase reaction was severalfold higher in the presence of MnCl₂ than MgCl₂ (Fig. 1C).

**Divalent Cation Dependence and Specificity—**Phosphorylation of Spt5-CTD by SpCdk9/Peh1 required a divalent cation cofactor (Fig. 2A). Protein kinase activity was proportional to magnesium concentration in the range of 0.6–2.5 mM and plateaued at 5–10 mM. Manganese was a superior cofactor at all concentrations tested; activity was optimal at 0.6–10 mM MnCl₂ (Fig. 2A). Cobalt (10 mM) supported kinase activity, albeit less effectively than manganese or magnesium (Fig. 2B). Other divalent cations were tested for cofactor activity at 10 mM concentration (Fig. 2B) and for their effects on activity in reactions containing 2.5 mM manganese (Fig. 2C). Whereas calcium neither activated nor inhibited SpCdk9/Peh1, copper

*P. pombe* Cdk9/Peh1 Protein Kinase
and zinc were unable to support activity and were profoundly inhibitory in the presence of manganese.

Effect of pH—The phosphorylation of Spt5-CTD by SpCdk9/Pch1 in the presence of manganese was optimal at pH 6.0–7.0 in Tris acetate buffer and at pH 6.0–8.0 in Tris-HCl buffer (Fig. 3A). We calculated that 0.6 pmol of phosphate was incorporated per pmol of input Spt5 protein at optimal pH. The manganese-dependent kinase activity was abolished at pH ≤ 4.5 or ≥ 9.0. The activity of SpCdk9/Pch1 in magnesium was also optimal at pH 6.0–7.0 in Tris acetate buffer and declined as the pH was incrementally acidified (Fig. 3B), just as was seen in the manganese-dependent reaction. However, the magnesium-dependent reaction was apparently insensitive to inhibition at alkaline pH, insofar as activity in Tris-HCl buffer at pH 9.5 was ∼ 65% of the activity in this buffer at the optimal pH of 6.0–6.5 (Fig. 3B).

Kinetic Parameters, Nucleotide Specificity, and Inhibition by Salt—The transfer of \( ^{32}\text{P} \) from \([\gamma-^{32}\text{P}]\text{ATP}\) to the Spt5-CTD increased with reaction time up to 80 min; the initial rate was severalfold higher in manganese than magnesium, and the extent of product formation remained severalfold higher in manganese at all times tested (Fig. 4A). Kinase activity was proportional to the amount of Spt5(801–990) phosphate acceptor protein included in the reaction in the range of 0.06–2 μM of Spt5-CTD and began to level off at higher Spt5-CTD concentrations (Fig. 4B). From a double-reciprocal plot of the data in Fig. 4B, we calculated a \( K_a \) value of 3 μM Spt5-CTD. The kinetic activity was proportional to the ATP concentration in the range of 2–12 μM and saturated at 40–50 μM (Fig. 5A). A double-reciprocal plot of the data yielded a \( K_a \) of 10 μM ATP (Fig. 5B). SpCdk9/Pch1 was also capable of catalyzing the transfer of \( ^{32}\text{P} \) from \([\gamma-^{32}\text{P}]\text{GTP}\) to the Spt5-CTD (Fig. 5C). The extent of \( ^{32}\text{P} \) incorporation was comparable with 50 μM GTP and 50 μM ATP in the presence of manganese. Although both the GTP- and ATP-dependent kinase activities were reduced when magnesium replaced manganese, the GTP-dependent activity was more sensitive to the switch in metal cofactor (Fig. 5C). Phosphorylation of Spt5 was inhibited by increasing the ionic strength of the reaction mixture with either NaCl or NH₄Cl (Fig. 4C). Activity was inhibited by 50% at ∼150 mM concent-

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**Fig. 1.** SpCdk9/Pch1 phosphorylates the C-terminal nonamer repeat domain of Spt5 on threonine. A, the amino acid sequence of the Spt5-CTD is displayed with the nonamer repeats aligned vertically. The consensus sequence TPANWNSGSK is shown below the alignment. Only modules containing a proline at position 2 are counted as CTD repeats. The six repeats from amino acids 845 to 898 are shaded. B, 4-μg aliquots of affinity-purified GST, GST-Spt5(801–990), GST-Spt5(801–888), GST-Spt5(845–885) and GST-Spt5(845–880) were analyzed by SDS-PAGE. A Coomassie Blue-stained gel is shown. The positions and sizes (in kDa) of marker proteins are indicated on the left. C, protein kinase reaction mixtures containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 50 μM \([\gamma-^{32}\text{P}]\text{ATP}\), either 10 mM MgCl₂ (Magnesium) or 2.5 mM MnCl₂ (Manganese), 4 μg of GST or GST-Spt5, and 100 ng of SpCdk9/Pch1 complex were incubated for 60 min at 22 °C. The reaction products were resolved by SDS-PAGE, an autoradiograph of the dried gel is shown. The extents of protein phosphorylation (pmol of \( ^{32}\text{P} \) incorporated) are indicated below each lane. The positions and sizes (in kDa) of marker proteins are indicated on the left. D, \( ^{32}\text{P} \)-Labeled phosphoamino acid analysis of GST-Spt5(801–889) phosphorylated in vitro by SpCdk9/Pch1 was performed as described under “Experimental Procedures.” An autoradiograph of the cellulose plate is shown. The positions of unlabeled phosphoamino acid standards and the electrophoretic origin are indicated on the right.

**Fig. 2.** Divalent cation dependence of SpCdk9/Pch1 kinase activity. A, 20-μl reaction mixtures containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 50 μM \([\gamma-^{32}\text{P}]\text{ATP}\), 4 μg of GST-Spt5(801–990), 100 ng of SpCdk9/Pch1, and either MnCl₂ or MgCl₂ as specified were incubated for 60 min at 22 °C. The extent of Spt5(801–990) phosphorylation is plotted as a function of divalent cation concentration. B, 20-μl reaction mixtures containing 50 mM Tris acetate (pH 6.0), 50 μM \([\gamma-^{32}\text{P}]\text{ATP}\), 4 μg of GST-Spt5(801–990), 100 ng of SpCdk9/Pch1, and 10 mM divalent cation as specified were incubated for 60 min at 22 °C. Magnesium, manganese, cobalt, and calcium were added as chloride salts; copper and zinc were added as sulfates. C, 20-μl reaction mixtures containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 50 μM \([\gamma-^{32}\text{P}]\text{ATP}\), 4 μg of GST-Spt5(801–990), 100 ng of SpCdk9/Pch1, and 2.5 mM MnCl₂ were supplemented with 10 mM concentrations of the indicated divalent cations. Magnesium and calcium were added as chloride salts; copper and zinc were added as sulfates. The mixtures were incubated for 60 min at 22 °C.
Six Nonamer Repeats of the Spt5-CTD Suffice for Effective Phosphorylation—We tested the kinase activity of SpCdk9/Pch1 with a series of GST-Spt5 fusion proteins containing either the full-length array of 18 nonamer repeats (amino acids 801–898) or truncated segments containing 10 repeats (amino acids 845–898), 6 repeats (amino acids 845–880), or 4 repeats (amino acids 845–880). SDS-PAGE analysis of the purified GST-Spt5 substrates is shown in Fig. 1B and reveals the expected decrements in electrophoretic mobility as the CTD was truncated incrementally. The extent of manganese-dependent phosphorylation of the 10-repeat substrate (43 pmol) was similar to that of the full CTD array (45 pmol), which implies that the amino-terminal half of the CTD sufficed as a phosphate acceptor (Fig. 1C). The extent of phosphorylation of the 6-repeat array (22 pmol) was half that of the 10-repeat array. Taking into account that the number of potential phosphorylation sites within the 10-repeat CTD (10 threonines and 11 serines) is nearly twice that of the 6-repeat array (6 threonines and 6 serines), we surmise that the 6-repeat CTD is also an effective phosphate acceptor on a molar basis. The steep decline in the extent of phosphorylation of the 4-repeat CTD substrate (4 pmol of $^{32}$P incorporation) was disproportionate to the reduction in the number of potential phosphorylation sites (4 threonines and 5 serines) compared with the 6-repeat CTD. Thus, we conclude that 6 nonamer repeats suffice for effective phosphorylation of the SpCdk9/Pch1 kinase in the presence of manganese. A similar relationship between kinase activity and CTD length was observed with manganese as the cofactor, except that the decrement in activity between the 10-repeat and 6-repeat substrates was 3-fold instead of 2-fold as with manganese (Fig. 1C).

SpCdk9/Pch1 Phosphorylates Spt5 on Threonine within the CTD Nonamer Repeat—GST-Spt5(801–898) phosphorylated in vitro by SpCdk9/Pch1 in the presence of 2.5 mM manganese was isolated free of [$\gamma$-$^{32}$P]ATP and the kinase by adsorption to GSH-Sepharose and elution with GSH. The protein was subjected to acid hydrolysis, and the $^{32}$P-labeled phosphoamino acid content of the hydrolysate was gauged by high voltage electrophoresis under conditions designed to separate Tyr(P), Thr(P), and Ser(P) (Fig. 1D). This analysis showed that SpCdk9/Pch1 phosphorylated Spt5 exclusively on threonine and not on serine. SpCdk9/Pch1 also phosphorylated Spt5 exclusively on threonine when the kinase reactions were performed with 10 mM magnesium as the cofactor (data not shown). As noted above, the CTD substrate composed of 10 nonamer repeats contains 10 threonines and 11 serines, but no tyrosines. We surmise that Thr$^1$ of the nonamer consensus sequence $^3$TPAWNSGSK$^9$ is the target of the SpCdk9/Pch1 kinase.

SpCdk9/Pch1 Phosphorylation of the S. pombe pol II CTD on Serine—We compared the kinase activity of SpCdk9/Pch1 with GST-fused phosphate acceptors containing either the CTD nonamer array of the S. pombe Spt5 or the complete CTD of S. pombe pol II, which consists of 29 heptapeptide repeats. The extent of phosphorylation of the SpCdk9/Pch1 pol II CTD was 5–8-fold higher than that of the S. pombe pol II CTD in the presence of manganese or magnesium (data not shown). Phosphorylation of the S. pombe pol II CTD by SpCdk9/Pch1 was 2-fold more effective with 2.5 mM manganese than with 10 mM magnesium (data not shown). GST-pol2CTD phosphorylated in vitro by SpCdk9/Pch1 in the presence of either 2.5 mM manganese or 10 mM magnesium was isolated by adsorption to GSH-Sepharose and elution with GSH. The protein was subjected to acid hydrolysis, and the $^{32}$P-phosphoamino acid content was analyzed by high voltage electrophoresis, which revealed that the substrate was phosphorylated exclusively on serine, independent of the choice of divalent cation cofactor (data not shown).

SpCdk9/Pch1 Autophosphorylation—The peak fractions of the recombinant SpCdk9/Pch1 complex isolated by glycerol sedimentation contain two predominant polypeptides: a 70-kDa species corresponding to SpCdk9 and a 43-kDa species corresponding to His-Pch1 (Fig. 6A). (We had previously established the identity of these polypeptides by amino-terminal Edman sequencing and matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Ref. 30.) Incubation of the peak kinase fractions with [$\gamma$-$^{32}$P]ATP in the absence of an exogenous phosphate acceptor protein resulted in autophosphorylation of the SpCdk9 and His-Pch1 polypeptides (Fig. 6B). $^{32}$P labeling of both polypeptides required a divalent cation. Manganese and magnesium supported similar autophosphorylation activity at their respective optima: 0.6–2.6 mM manganese and 2.5–10 mM magnesium (not shown). The His-Pch1 polypeptide contains a TEV protease cleavage site at the junction between the amino-terminal His tag and the start of the native Pch1 protein. Treatment of the autophosphorylated kinase complex with TEV protease resulted in conversion of the 43-kDa $^{32}$P-labeled polypeptide to a 41-kDa $^{32}$P-labeled.
brane slices containing the 32P-labeled SpCdk9 and 32P-labeled proteins, and these are highlighted by dots in Fig. 7. The alignment encompasses a 233-amino acid segment from SpCdk9 residues 36–268, corresponding to the Cdk2 segment from residues 4–216 (Fig. 7). 96 side chains are identical or structurally similar in all six Cdk proteins, and these are highlighted by dots in Fig. 7.

Previously, we used budding yeast as a surrogate model to test the in vivo effects of mutations in several residues of SpCdk9 which were predicted, based on the crystal structure of the activated Cdk2/cyclin A-substrate complex, to be either constituents of the kinase active site (Lys65, Glu 83, and Asp184) or a potential site for regulation of SpCdk9 function. The products were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membrane slices containing the 32P-labeled SpCdk9 and 32P-labeled His-Pch1 polypeptides were subjected to acid hydrolysis, and the 32P-phosphoamino acid content of the hydrolysates were analyzed by high voltage electrophoresis (Fig. 6C). SpCdk9 was phosphorylated predominantly on serine (86%) and to a lesser extent on threonine (11%) and tyrosine (3%). In contrast, Pch1 was phosphorylated predominantly on threonine (98%) and minimally on serine (2%). No tyrosine phosphorylation of Pch1 was detected.

Structure-guided Mutational analysis of SpCdk9—The amino-terminal kinase domain of SpCdk9 is closely related to the sequences of the HsCdk9 and DmCdk9 subunits of human and Drosophila P-TEFb, to the essential S. cerevisiae cyclin-dependent kinase Bur1, and to the Crk1 kinase of Candida albicans (35). We aligned the sequences of these Cdk-like kinases to each other and to the sequence of human Cdk2, which has been the subject of intensive structural analysis via x-ray diffraction (33, 36, 37, 49). The alignment encompasses a 233-amino acid segment from SpCdk9 residues 36–268, corresponding to the Cdk2 segment from residues 4–216 (Fig. 7). 96 side chains are identical or structurally similar in all six Cdk proteins, and these are highlighted by dots in Fig. 7.

Previously, we used budding yeast as a surrogate model to test the in vivo effects of mutations in several residues of SpCdk9 which were predicted, based on the crystal structure of the activated Cdk2/cyclin A-substrate complex, to be either constituents of the kinase active site (Lys65, Glu83, and Asp184) or a potential site for regulation of SpCdk9 function by phosphorylation (Thr215). We found that the K65A,
D184N, and T212A mutations were lethal in vivo, i.e. they abolished the ability of SpCdk9 to complement bur1/H9004 when coexpressed with Pch1 (30). The E83A mutation elicited a severe temperature-sensitive and cold-sensitive growth phenotype on rich medium, i.e. SpCDK9-E83A yeast cells were unable to form colonies at either 23 or 37 °C, although they were viable at 30 °C (Table I).

Here, we used the crystal structure of the human Cdk2/cyclin A-substrate complex (33) to guide a more comprehensive mutational analysis of the active site of SpCdk9, targeting FIG. 6. **Autophosphorylation of the SpCdk9/Pch1 complex.** A, polypeptide composition of SpCdk9/Pch1 glycerol gradient fractions 13 and 15. Aliquots (20 μl) of the fractions were analyzed by SDS-PAGE. A Coomassie Blue-stained gel is shown (fraction 13 in left lane; fraction 15 in right lane). The polypeptides corresponding to SpCdk9 and His-tagged Pch1 are denoted by arrowheads on the right. The positions and sizes (in kDa) of marker polypeptides are indicated on the left. B, autophosphorylation. Reaction mixtures containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 2.5 mM MnCl₂, 50 μM γ-[³²P]ATP, and 4 μl of SpCdk9/Pch1 glycerol gradient fraction 13 or 15 were incubated for 60 min at 22 °C. The phosphorylated products were resolved by SDS-PAGE. An autoradiograph of the dried gel is shown. The ³²P-labeled polypeptides corresponding to SpCdk9 and His-tagged Pch1 are denoted by arrowheads on the left. The positions and sizes (in kDa) of prestained marker polypeptides are indicated on the right. C, identification of the ~43-kDa phosphoprotein as Pch1. Three reaction mixtures containing 50 mM Tris acetate (pH 6.0), 1 mM DTT, 2.5 mM MnCl₂, 50 μM γ-[³²P]ATP, and 1 μg of SpCdk9/Pch1 complex were incubated for 60 min at 22 °C. One reaction was halted by the addition of SDS-PAGE loading buffer (left lane). The other two mixtures were continued for another 60 min in the absence (–, middle lane) or presence (+, right lane) of 10 units of TEV protease (Invitrogen). The ³²P-labeled products were resolved by SDS-PAGE and visualized by autoradiography. The identities of the autophosphorylated polypeptides are indicated on the right. D, ³²P-labeled phosphoamino acid analysis of autophosphorylated SpCdk9 and Pch1 was performed as described under “Experimental Procedures.” An autoradiograph of the cellulose plate is shown. The positions of unlabeled phosphoamino acid standards are indicated on the right.

**FIG. 7.** Alignment of SpCdk9 to homologous yeast and metazoan Cdns. The amino acid sequence of the kinase domain of SpCdk9 from residues 36 to 268 is aligned to the sequences of Drosophila Cdk9 (DmCdk9), human Cdk9 (HsCdk9), S. cerevisiae Bur1 (ScBur1), C. albicans Crk1 (CaCrk1), and human Cdk2 (HcCdk2). Positions of side chain identity and similarity in all six Cdk proteins are indicated by dots. Conserved residues subjected to mutational analysis are shaded.
The in vivo phenotypes of the indicated SpCDK9 mutants were assessed by plasmid shuffle in a yeast bur1Δ strain as described under “Experimental Procedures.” Lethal mutations were those that failed to support growth after incubation on 5-FOA agar for 7 days at 30 °C. The viable 5-FOA-resistant strains containing mutated SpCDK9 genes were tested for growth on YPD agar at 18, 23, 30, and 37 °C. Growth was scored as follows: +++, indicates colony size indistinguishable from strains bearing wild-type SpCDK9; +, denotes reduced colony size; −, indicates no growth. The residues in human Cdk2 corresponding to the mutated SpCDK9 positions are specified under Cdk2 equivalent. The atomic contacts of the corresponding SpCdk9 residues are surmised from the contacts made by Cdk2 side chains in the crystal structure of activated Cdk2/cyclin A-substrate complex (33). SpCdk9 mutations reported previously (30) are indicated by asterisks (*).

| Mutant  | 5-FOA | Growth on YPD | Cdk2 equivalent | Contacts |
|---------|-------|---------------|----------------|---------|
|         |       | 18 | 23 | 30 | 37 |                   | |
| T46A    | Viable | + | + | ++ | ++ | ++ | Thr14 | ATP β-PO4 |
| K65A*   | Lethal | − | − | −  | −  | −  | Lys33 | ATP α-PO4 |
| K65R    | Lethal | + | ++ | ++ | ++ | ++ | Glu51 | Lys65 |
| K65Q    | Lethal | − | − | −  | −  | −  | Asp127 | SerOγ, Thr217, ATP γ-PO4 |
| E83A*   | Viable | + | ++ | ++ | ++ | ++ | Asp127 | SerOγ, Asn171, Thr217 |
| E83D    | Viable | + | ++ | ++ | ++ | ++ | Asn132 | Mg, Asp166 |
| E83Q    | Lethal | − | − | −  | −  | −  | Lys129 | SerOγ, Thr217, ATP γ-PO4 |
| K168A   | Viable | + | + | ++ | ++ | ++ | Lys129 | SerOγ, Thr217, ATP γ-PO4 |
| K168R   | Lethal | − | − | −  | −  | −  | Lys129 | SerOγ, Thr217, ATP γ-PO4 |
| K168Q   | Lethal | − | − | −  | −  | −  | Lys129 | SerOγ, Thr217, ATP γ-PO4 |
| D166A   | Lethal | − | − | −  | −  | −  | Lys129 | SerOγ, Thr217, ATP γ-PO4 |
| D166E   | Lethal | − | − | −  | −  | −  | Lys129 | SerOγ, Thr217, ATP γ-PO4 |
| D166N   | Lethal | − | − | −  | −  | −  | Lys129 | SerOγ, Thr217, ATP γ-PO4 |
| N171A   | Lethal | − | − | −  | −  | −  | Asn132 | Mg, Asp166 |
| N171D   | Lethal | − | − | −  | −  | −  | Asn132 | Mg, Asp166 |
| N171Q   | Viable | + | + | ++ | ++ | ++ | Asp145 | Mg |
| D184N*  | Lethal | − | − | −  | −  | −  | Asp145 | Mg |

Functional groups implicated in binding ATP and the essential divalent cation cofactor, the network of contacts between SpCdk9 and the phosphate acceptor substrate peptide, and the intramolecular contacts to the phosphorylated threonine of the T-loop. Nine new residues were initially subjected to alanine scanning. This approach removes the side chain beyond the β-carbon and provides a simple means of gauging whether the missing side chain atoms are functionally relevant. The positions at which alanine substitution elicited a growth defect in vivo were then targeted for further analysis by the introduction of conservative side chain substitutions. A total of 29 new mutations of 12 conserved residues were constructed and tested for in vivo function by plasmid shuffle in a yeast bur1Δ strain. The results are summarized in Tables I and II and discussed in detail below.

To perform the plasmid shuffle assay, mutant SpCDK9 alleles were cloned into a yeast CEN TRP1 plasmid so as to place their expression under the control of a constitutive promoter. The plasmids were then cotransformed with a CEN ADE2 PCH1 plasmid into a S. cerevisiae bur1Δ strain in which the chromosomal BUR1 gene was deleted. Growth of bur1Δ is contingent on maintenance of a wild-type BUR1 allele on a CEN URA3 plasmid. Therefore, the bur1Δ strain is unable to grow on agar medium containing 5-FOA (a drug that selects against the wild-type SpCDK9 yeast strain); ++ signifies smaller than wild-type colonies; + denotes pinpoint colonies; − indicates no growth. Five of the mutants displayed conditional phenotypes. K168A cells were both cold-sensitive (failing to grow at 18 or 23 °C) and temperature-sensitive (no growth at 37 °C), and they formed smaller than wild-type colonies at the permissive temperature of 30 °C (Table I). R82A and R221A cells were also cold-sensitive (no growth at 18 °C and + growth at 23 °C) and slow growing at the permissive temperature (+ + at 30 °C). Mutants R189A and T217A grew normally at 30 °C but formed pinpoint colonies at 18 and 37 °C (Table II). Thus, we surmise that Lys129, Arg50, Arg221, Arg189, and Thr217 are important for SpCdk9 function. Lys129 of SpCdk9 is the equivalent of Cdk2 Lys129, which makes a bridging contact between the γ-phosphate of ATP and the SerOγ of the phosphate acceptor peptide (Fig. 8). The equivalents of SpCdk9 side chains Arg32 and Arg189 in Cdk2 (Arg50 and Arg150) Makes bidentate contacts with the activated Thr14 side chain of Cdk2 coordinates the nonbridging oxygens of the β-phosphate of ATP (Fig. 8). We surmise that this contact is not important for SpCdk9 function in vivo.

Structure-Activity Relationships at Essential and Important Residues of SpCdk9—We tested the effects of conservative substitutions at the SpCdk9 positions defined here and previously as essential or important by alanine scanning. Arginines 82, 165, 189, and 221 were replaced by lysine and glutamine and lysines 65 and 168 were changed to arginine and glutamine. Glu63 was replaced by glutamine and aspartate, Asp166 by asparagine and glutamate, and Asn171 by glutamine and aspartate. The T-loop threonine was changed to serine and...
glutamate, the latter being a putative mimetic of phosphothreonine. The 20 conservative mutants were tested by plasmid shuffle for bur1Δ complementation; the results are shown in Tables I and II. Insights into the structural requirements for ATP and metal binding, phosphoryl transfer chemistry and the importance of T-loop phosphorylation emerge when the mutational data are interpreted in light of the atomic contacts seen in the Cdk2 crystal structure.

The kinase reaction entails the nucleophilic attack of the substrate SerO on the γ-phosphorus of ATP via an in-line mechanism in which the attacking nucleophile is apical to the β-phosphate of the ADP leaving group. Indeed, this orientation is seen in the crystal structure of the activated Cdk2/cyclin A-substrate complex, in which the SerOγ is 3.7 Å from the γ-phosphorus of ATP (Fig. 8) (33). The proper conformation of the triphosphate moiety of ATP is apparently attained via contacts to the divalent cation. Three of the six constituents of the octahedral coordination complex of magnesium are: a non-bridging oxygen of the octahedral coordination complex of magnesium are: a non-bridging oxygen of the metal complex (33). The other constituents of the metal complex are: Asp145, which is equivalent to SpCdk9 Asp184; Asn171, which corresponds to SpCdk9 Asn171; and a water molecule. Based on the earlier finding that the SpCdk9 D184N mutation was lethal in vivo (30) plus the lack of other atomic contacts by this Asp in the Cdk2 structure, we inferred an essential function for the Asp184 carboxylate in metal binding and therefore eschewed making additional mutations at this position. However, Asn171 contacts not only the magnesium via its Oδ, but also makes a hydrogen bond to Asp166 via its Nδ. The latter contact appears to be essential in SpCdk9, insofar as replacement of the Asn171 amide functional group with aspartate was lethal in vivo (N171D in Table I), whereas the N171Q mutant was viable at 30 °C (++) growth), albeit unable to grow at either 18 or 37 °C. We infer that the amide is critical and that there is a steric constraint at the active site which does not easily accommodate the extra methylene group of glutamine.

Lys65 of SpCdk9 is predicted to contact the nonbridging oxygen of the α-phosphorus of ATP (i.e. the oxygen that is not part of the metal coordination complex). Conservative substitutions of Lys65 with arginine or glutamine were lethal in vivo, just like the K65A mutation (Table I). We conclude that there is a strict requirement for lysine at this position for SpCdk9 function. Glu83 of SpCdk9 corresponds to the conserved glutamate carboxylate by glutamine was lethal, whereas a conservative replacement of the Glu83 carboxyl group with aspartate was lethal (N171D in Table I), whereas the N171Q mutant was viable at 30 °C (++) growth), albeit unable to grow at either 18 or 37 °C. We infer that the amide is critical and that there is a steric constraint at the active site which does not easily accommodate the extra methylene group of glutamine.

The in vivo phenotypes of the indicated SpCdk9 mutants were assessed by plasmid shuffle in a yeast bur1Δ strain. The equivalent residues in human Cdk2 are indicated. The atomic contacts of the corresponding SpCdk9 residues are surmised from the contacts made by Cdk2 side chains in the crystal structure of activated Cdk2/cyclin A-substrate complex (PDB ID 1QMZ). SpCdk9 mutation T212A reported previously (30) is indicated by an asterisk (*).

**TABLE II**

| Mutant      | 5-FOA | Growth on YPD | Cdk2 equivalent | Contacts |
|-------------|-------|---------------|-----------------|----------|
|             |       | 18            | 23              | 30       | 37       |
| T212A*      | Lethal|                |                 |          |
| T212S       | Viable| ++            | ++              | ++       | ++       |
| T212E       | Viable| +             | ++              | +        | ++       |
| R82A        | Viable| +             | +               | +        | +        |
| R82K        | Viable| +             | +               | +        | +        |
| R82Q        | Viable| +             | +               | +        | +        |
| R165A       | Lethal|                |                 |          |
| R165K       | Lethal|                |                 |          |
| R165Q       | Lethal|                |                 |          |
| R189A       | Viable| +             | +               | ++       | +        |
| R189K       | Viable| +             | +               | ++       | ++       |
| R189Q       | Viable| +             | +               | ++       | ++       |
| T217A       | Viable| +             | +               | ++       | +        |
| R221A       | Viable| +             | +               | ++       | +        |
| R221K       | Viable| +             | +               | ++       | +        |
| R221Q       | Viable| +             | +               | ++       | +        |

![Fig. 8. Active site of activated Cdk-cyclin complex with bound nucleotide and phosphate acceptor peptide. A stereo view of the structure of the activated Cdk20-cyclin A-substrate complex (PDB ID 1QMZ) was prepared using SETOR (50).](image)
of the SpCdk9 PITAIRE helix. Apparently, the kinase can accommodate the shortening of the main chain to carboxylate distance in the E83D mutant.

The SerO nucleophile of the substrate peptide is contacted by Cdk2 side chains Asp127, Lys129, and Thr165, equivalent to Asp166, Lys168, and Thr217 in SpCdk9. It is suggested that the Asp residue functions as a general base catalyst of phosphoryl transfer by SpCdk9. Lys168 of SpCdk9 is predicted to interact with the substrate Ser-O and the ATP γ-phosphate. Whereas the conservative K168R mutation resulted in a severe cs and ts growth defect similar to that seen with the K168A change, the K168Q mutation was lethal (Table I). These results are consistent with a role for Asp166 as a general base catalyst of phosphoryl transfer by SpCdk9. Lys168 of SpCdk9 is predicted to interact with the substrate Ser-O and the ATP γ-phosphate. Whereas the conservative K168R mutation resulted in a severe cs and ts growth defect similar to that seen with the K168A change, the K168Q mutation was lethal (Table I). These results, together with the Cdk2 crystal structure, suggest that Lys168 plays a role in facilitating phosphoryl transfer chemistry, either by stabilizing the serine anion or the γ-phosphate of the transition state.

We found previously that alanine mutation of Thr212, a potential activating phosphorylation site in the T-loop of SpCdk9, abolished SpCdk9 complementation of bar1Δ and did not affect the interaction of the kinase domain with Pch1 in a two-hybrid assay (30). These results suggest that SpCdk9 function may be activated by phosphorylation of the T-loop. Here we found that replacing Thr212 with serine restored wild-type yeast growth at all temperatures tested (Table II). Thus, a hydroxyamino acid at position 212 is critical for SpCdk9 function. These effects do not reveal whether the hydroxyl is critical per se or whether its function is simply to serve as a target for a requisite phosphorylation event. An instructive finding was that replacing Thr212 by glutamate, which can be viewed as an electrostatic mimic of phosphothreonine, also restored viability at 30 °C, albeit with accompanying cs and ts growth defects (Table II). These results suggest that phosphorylation of the T-loop is critical for SpCdk9 function in yeast. The conditional growth defect of the T212E mutant may be because glutamate cannot recapitulate all of the interactions of phosphoserine (e.g., with the three arginines in Fig. 8) or because SpCdk9 must be transiently dephosphorylated to function normally in vivo.

Arginines 82, 165, and 189 are predicted to comprise a network of contacts to the activated phosphothreonine of the T-loop, with each guanidinium group contributing two hydrogen bonds to two different phosphate oxygens. Conservative mutations of Arg165 to lysine or glutamine were lethal in vivo, as was the alanine mutation (Table II). These results highlight the strict requirement for the bidentate interaction of Arg165 with the T-loop phosphate. Conservative changes of Arg221 to lysine or glutamine resulted in the same severe cs and ts growth defects seen for the R82A mutant (Table II). Again, these results indicate that the contribution of Arg221 to SpCdk9 function requires the bidentate hydrogen bonding capacity of arginine. The cs and ts phenotypes of the R189A mutant were alleviated partially by lysine substitution, whereas the glutamine change restored +/+ growth at 23 and 37 °C (Table II). Apparently, Arg165 is the least critical of the three side chains that are predicted to chelate the phosphothreonine of the T-loop.

Finally, replacing Arg221 of SpCdk9 with lysine relieved the cs and ts growth defects of the R221A mutant, whereas the change to glutamine had no salutary effect (Table II). We surmise that a positive charge at this position is important for SpCdk9 function. Arg221 is conserved in Cdkks and is likely to play a structural role at the phosphate acceptor binding site of SpCdk9, insofar as the equivalent Arg169 side chain in Cdk2 makes hydrogen bonds to the main chain carbonyls of Val163 and Val166; these contacts promote the formation of a specific binding pocket for the proline flanking the hydroxyamino acid phosphate acceptor of the peptide substrate (33). The Val-Val dipeptide is conserved among Cdks (Fig. 7); the predicted contacts of Arg221 in SpCdk9 would be to the backbone carbonyls of Val215 and Val216.

**DISCUSSION**

**Kinase Activity of S. pombe Cdk9/Pch1**—Here we conducted a biochemical and molecular genetic analysis of the S. pombe Cdk9/Pch1 kinase, a functional ortholog of the essential S. cerevisiae Bur1/Bur2 kinase and a putative ortholog of metazoan P-TEFb. The biochemical characterization focused on the requirements for phosphorylation of the CTD nonamer array of the S. pombe ortholog of transcription elongation factor Sp5t. We document the divergent cation dependence and specificity of SpCdk9/Pch1, its NTP dependence and specificity, the dependence of Sp5t phosphorylation on the number of tandem nonamer repeats, and the specificity for phosphorylation of the Sp5t-CTD on threonine within the nonamer consensus sequence "TPAWNSGSK".

The regularity and specific amino acid composition of the S. pombe nonamer array are unique among eukaryotes (28). Although the fission yeast and metazoan Sp5t proteins contain a series of Thr-Pro and Ser-Pro dipeptides, the intervals between the dipeptide repeats are different among metazoan species, and they also differ from the regular nonamer spacing in S. pombe Sp5t. The metazoan repeats do not adhere to the S. pombe consensus sequence TPAPWSNSGSK. In particular, although the residue located two positions downstream of the Thr-Pro dipeptide in S. pombe Sp5t is typically a tryptophan (Fig. 1A), it is never a tryptophan in the human, nematode, or zebrafish proteins; instead, this position is usually occupied by tyrosine or histidine (see Ref. 28). Ivanov et al. (11) showed that mammalian Cdk9/Cyclin T (P-TEFb) phosphorylates mammalian Sp5t with the carboxyl-terminal repeat 1 consensus sequence TPMYGSQ/R). They found that changing the carboxyl-terminal repeat 1 threonines to alanine abolished phosphorylation of carboxyl-terminal repeat 1, whereas changing the serines to alanine did not. This result suggested that threonine within a Thr-Pro dipeptide was the target for Sp5t phosphorylation by mammalian Cdk9. In a separate study (31), Kim and Sharp showed that human P-TEFb phosphorylates the C-terminal segment of human Sp5t: phosphoamino acid analysis documented phosphorylation on both threonine and serine residues, with the labeled products distributed as 75% Thr(P) and 25% Ser(P). We find that that phosphorylation of the fission yeast Sp5t-CTD by SpCdk9/Pch1 is exclusively on threonine.

The S. pombe pol II CTD consists of 29 heptad repeats preceded by two pseudorepeats (47). The S. pombe pol II CTD contains exclusively Ser2-Pro3 or Ser3-Pro4 dipeptides; there are no Thr-Pro dipeptides within the S. pombe CTD array. The SpCdk9/Pch1 complex phosphorylates a GST fusion protein containing the complete S. pombe CTD, and it does so exclusively on serine. These findings indicate that SpCdk9/Pch1 is a proline-directed kinase that recognizes a (Ser/Thr)-Pro target site in the phosphoacceptor substrate, be it Sp5t or pol II.

SpCdk9/Pch1 displays optimum activity at pH 6.0 with manganese as the cofactor. Magnesium supports lower kinase activity but affords a broader activity profile in the alkaline pH range. The ability of SpCdk9/Pch1 to utilize GTP as a phosphate donor is also enhanced with manganese compared with magnesium. These metal effects were not specific to the use of Sp5t-CTD as a substrate, insofar as phosphorylation of the pol
II CTD by SpCdk9/Pch1 was also more effective in manganous than magnesium. The specificity of SpCdk9/Pch1 for threonine phosphorylation of the Sp5-CTD and serine phosphorylation of the pol II CTD was independent of the choice of divalent cation cofactor.

SpCdk9/Pch1 Autophosphorylation—We find that SpCdk9/Pch1 catalyzes autophosphorylation of the kinase and cyclin subunits of the kinase complex and that the distribution of phosphorylation sites on Cdk9 (86% Ser(P), 11% Thr(P), 3% Tyr(P)) is quite distinct from that on Pch1 (2% Ser(P), 98% Thr(P)). We presume that autophosphorylation entails reaction in trans of two SpCdk9/Pch1 complexes rather than an intramolecular process. Autophosphorylation in vitro has also been documented for mammalian P-TEFb, resulting in 

In vivo labeling of both the Cdk9 and cyclin T subunits (31, 32, 39). The mammalian Cdk9 subunit was phosphorylated on both serine and threonine. Garber et al. (32) localized the major in vitro phosphorylation sites to a peptide derived from the C terminus of mammalian Cdk9. This peptide is not conserved in SpCdk9.

The autophosphorylation pattern of S. pombe Cdk9/Pch1 is broadly consistent with the predicted distribution of proline-directed Cdk phosphorylation sites. For example, SpCdk9 contains seven potential sites: three Ser-Pro, three Thr-Pro, and one Tyr-Pro. The predominance of Thr(P) over Ser(P) in the acid hydrolysate reflects the distribution of potential proline-directed sites. The Pch1 Tyr-Pro dipeptide is apparently not subject to autophosphorylation in vitro. Future studies entailing direct mapping of the phosphorylation sites and the analysis of the effects of mutating these sites in vivo will await the development of additional genetic tools to study SpCdk9 in S. pombe.

Mutational Analysis of SpCdk9—Our inferences about which features of the individual side chains of SpCdk9 are required for activity in vivo have been discussed in detail above in light of the Cdk2/cyclin A-substrate complex structure. Thus, we focus discussion here on how our findings relate to structure-function data reported for other Cdks. Most studies have focused on mutating the conserved lysine that contacts ATP (Lys65 in SpCdk9), the conserved aspartate that binds the phosphate-mimicking glutamate substitution. Remarkably, the T-loop threonine is not essential in Bur1, the budding yeast ortholog of SpCdk9. Bur1 T-loop mutant alleles T240A and T240E were able to sustain growth of a bur1Δ strain; however, these mutant strains did have nonlethal pleiotropic transcriptional phenotypes (42). It is conceivable that the SpCdk9/Pch1 complex is more acutely dependent than Bur1/Bur2 on a phosphorylation event to attain the threshold level of kinase activity required for growth of S. cerevisiae. We surmise that the in vivo requirements for SpCdk9 Thr212 reflect a requisite phosphorylation event, given that: (i) the T212S change rescues wild-type growth; (ii) the glutamate change rescues growth at 30 °C; and (iii) the effects of mutating the T-loop threonine are phenocopied by mutations in the three conserved arginines that chelate the phosphate on the T-loop threonine (Table II). Specifically, we found that alanine and conservative mutations in Arg186 were lethal, Arg282 mutants were severely cs and ts, and Arg186 mutations also displayed conditional growth phenotypes. Chelation of the T-loop phosphate by the arginines promotes a conformational change in the Cdk/cyclin complex which is critical to form the substrate binding site (33, 36). The notion that mutations in the phosphate-binding arginines exert effects concordant with those in the T-loop threonine is borne out by available data for yeast Cdc28. In Cdc28, where the T-loop threonine is essential, at least one of the three arginines predicted to coordinate the activating phosphate moiety is also essential, i.e. the Cdc28 R159G mutation was lethal in vivo (45). Arg186 in Cdc28 corresponds to Arg150 in Cdc2 and Arg189 in SpCdk9. The effects of the glycine mutation in Cdc28 are more severe than the cs and ts effects of the R189A change in SpCdk9. This difference may reflect protein context effects or the fact that glycine is more prone than alanine to affect the main chain conformation of the polypeptide.

The other mutational effects we describe for SpCdk9 generally support the inferences about Cdk mechanism derived from the crystal structures. Although many of the side chains that contact the nucleotide or divalent cation are essential for function, there are exceptions (e.g. Thr46 in SpCdk9). Our findings that Arg271 is important for SpCdk9 function provides evidence in support of the structural data implicating Cdk2 Arg271 in the formation of a proline-specific substrate binding pocket (33). Cross and Levine (45) reported that a serine mutation of the equivalent arginine in Cdc28 was lethal in vivo.

In summary, the mutant collection we have constructed sheds light on the structural requirements for SpCdk9 function in vivo. Using yeast as a surrogate genetic system, we were able to identify many conditional mutations predicted to affect different aspects of Cdk function, including ATP binding, phosphoacceptor substrate recognition, and T-loop dynamics. These alleles will prove useful for further biochemical analyses and genetic studies of SpCdk9/Pch1 function in the native S. pombe background.
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