Analysis of the Large Inactive P-TEFb Complex Indicates That It Contains One 7SK Molecule, a Dimer of HEXIM1 or HEXIM2, and Two P-TEFb Molecules Containing Cdk9 Phosphorylated at Threonine 186*

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Positive transcription elongation factor b (P-TEFb) regulates eukaryotic gene expression at the level of elongation, and is itself controlled by the reversible association of 7SK RNA and an RNA-binding protein, HEXIM1 or HEXIM2. To further understand how P-TEFb is regulated, we analyzed the stoichiometry of all the known components of the large, inactive P-TEFb complex. Mutational analyses of a putative coiled coil region in the carboxyl-terminal portion of HEXIM1 revealed that the protein is a dimer in solution and remains a dimer after binding to 7SK. Although a HEXIM1 dimer contains two potential RNA binding motifs and ultimately recruits two P-TEFb molecules, it associates with only one molecule of RNA. The first 172 nucleotides of the 330-nucleotide 7SK are sufficient to bind HEXIM1 or HEXIM2, and then recruit and inhibit P-TEFb. Deletion of the first 121 amino acids of HEXIM1 allowed it to inhibit P-TEFb partially in the absence of 7SK RNA. Mutation of a conserved tyrosine (Tyr271 in HEXIM1) to alanine or glutamate or mutation of a conserved phenylalanine (Phe208) to alanine, aspartate, or lysine, resulted in loss of inhibition of P-TEFb, but did not affect formation of the 7SK-HEXIM-P-TEFb complex. Analysis of T-loop phosphorylation in Cdk9 indicated that phosphorylation of Thr186, but not Ser175, was essential for kinase activity and for recruitment of P-TEFb to the 7SK-HEXIM complex. A model illustrates what is currently known about how HEXIM proteins, 7SK, and P-TEFb assemble to maintain an activated kinase in a readily available, but inactive form.

Cyclin-dependent kinases (Cdks)1 are key regulators of a variety of cellular processes, such as cell cycle progression, transcription, and neuronal differentiation. The kinase activity of Cdks in turn is tightly regulated. Association with a cyclin partner and phosphorylation of the T-loop is needed for activation of Cdks. They are also subject to negative regulation via phosphorylation or through interaction with a family of Cdk inhibitory proteins (1–4).

P-TEFb plays a key role in RNA polymerase II elongation control (5–7). It is comprised of one of two isoforms of Cdk9 (8, 9) and one of three cyclins, T1, T2 (10), or K (11) in human. One of the major targets of the kinase activity of P-TEFb is the carboxyl-terminal domain of the largest subunit of RNA polymerase II (12), and this phosphorylation of the carboxyl-terminal domain by P-TEFb occurs during transcription elongation (13). P-TEFb controls gene expression by regulating the fraction of RNA polymerase II molecules that generate full-length mRNAs (6). In addition to its normal cellular role, P-TEFb has been shown to be recruited by the viral transactivator Tat to the promoter to enhance viral transcription, which is required for efficient HIV-1 replication (8, 14–18).

P-TEFb is uniquely regulated by the reversible association of a small nuclear RNA, 7SK (19, 20), and HEXIM proteins (21–24). Glycerol gradient analyses of cell lysates indicate that two forms of P-TEFb exist in the cell: a large inactive form containing 7SK and HEXIM proteins, and a smaller active form comprised of just P-TEFb subunits (9, 19, 21, 23, 24). When cells are treated with P-TEFb inhibitors, such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, or other agents that block transcription elongation, the large form is converted into the small active form (21). This form of P-TEFb regulation is physiologically significant, because it has been shown that all signals that trigger cardiac hypertrophy converge at the critical step of activating P-TEFb through dissociation of 7SK and HEXIM. This activation causes increased cellular transcription, and an increase in the size of cardiomyocytes (25–27). Several studies have uncovered some of the important interactions in the 7SK-HEXIM1-P-TEFb complex. Two regions of HEXIM1 have been characterized. The region centered upon KHRR (amino acids 152–155) is involved in binding of 7SK, and contains nuclear localization signals (21, 28, 29). A second region centered upon PYNT (amino acids 202–205) is involved in interaction with P-TEFb (23, 29). In addition, regions involved in interactions have been narrowed down to amino acids 1–254 of 726 of cyclin T1, all of Cdk9, and nucleotide 1–175 of 7SK (19, 21, 22). Furthermore, phosphorylation of the T-loop of Cdk9 has been implicated in activation of P-TEFb, and is required for the formation of the 7SK-HEXIM1-P-TEFb complex (30).

In this report, we analyzed the stoichiometry of the 7SK-HEXIM1-P-TEFb complex. We identified residues critical for inhibition of the kinase activity of P-TEFb, and formation of the 7SK-HEXIM1-P-TEFb complex. These findings provide...
mechanistic insight into how P-TEFb kinase activity is controlled by 7SK RNA and HEXIM proteins.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Mutagenesis of HEXIM and P-TEFb Proteins**—The His-tagged HEXIM1 wild type and mutant proteins described under “Results” were expressed in *Escherichia coli* BL21(DE3) cells by overnight induction with 0.1 mM isopropyl-1-thio-β-D-galacto-pyranoside at 18 °C. Purification on Ni-NTA resin was carried out as previously described (23). PCR-based site-directed mutagenesis was carried out with Pfu Ultra HF DNA polymerase (Stratagene) according to the manufacturer's instructions. Baculoviruses expressing human cyclin T1, wild type and mutant Cdk9s, as described under “Results,” were generated using the BaculoDirect Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. Purification of wild type and mutant P-TEFb was carried out as previously described (23). For the co-expression experiment, FLAG-tagged, wild type HEXIM1 was cloned into pACYCDuet-1 vector (Novagen), and His-tagged ΔN was cloned into pET21a (Novagen). Glycerol Gradient Analysis—HeLa cells stably expressing FLAG-Cdk9 (provided by Dr. Zhou, University of California at Berkeley) or transiently transfected with pFLAG-CMV2-HEXIM1 were cultured in Dulbecco’s modified Eagle’s medium-F12 with 10% fetal bovine serum under standard conditions. Recombinant P-TEFb (His-Cdk9/cyclin T1, previously described (23)) was expressed in *Escherichia coli* BL21(DE3) cells by overnight induction with 0.1 mM isopropyl-1-thio-β-D-galacto-pyranoside at 18 °C. Purification on Ni-NTA resin was carried out as previously described (23). PCR-based site-directed mutagenesis was carried out with Pfu Ultra HF DNA polymerase (Stratagene) according to the manufacturer's instructions. Purification of wild type and mutant P-TEFb was carried out as previously described (23). For the co-expression experiment, FLAG-tagged, wild type HEXIM1 was cloned into pACYCDuet-1 vector (Novagen), and His-tagged ΔN was cloned into pET21a (Novagen). Glycerol Gradient Analysis—HeLa cells stably expressing FLAG-Cdk9 (provided by Dr. Zhou, University of California at Berkeley) were transfected with pFLAG-CMV2-HEXIM1 (Invitrogen). After 6 h the transfection media was removed, and the cells were cultured in Dulbecco’s modified Eagle’s medium-F12 with 10% fetal bovine serum under standard conditions for 48 h. Cells were scraped, spun down at 2,000 rpm, and then lysed for 15 min on ice in 150 mM NaCl, 2 mM MgCl₂, 10 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1% phenylmethylsulfonyl fluoride, EDTA-free Complete protease inhibitor mixture from Roche, and 0.5% Nonidet P-40. The lysates were treated with centrifugation for 10 min at 14,000 rpm prior to fractionation on 5-ml 5-45% glycerol gradients in the same buffer conditions used during lysis, except that Nonidet P-40 was omitted. Gradients were run at 45,000 rpm for 16 h in a Beckman SW-Ti55 rotor before being fractionated.

**Immunoprecipitations and Western Blotting**—HeLa cells stably expressing FLAG-Cdk9 (provided by Dr. Zhou, University of California at Berkeley) were transfected with pFLAG-CMV2-HEXIM1 (Invitrogen). After 6 h the transfection media was removed, and the cells were cultured in Dulbecco’s modified Eagle’s medium-F12 with 10% fetal bovine serum under standard conditions (37 °C in 5% CO₂). Cell lysates and glycerol gradient analyses were performed as described above. Gradient fractions 4 and 9 (out of 16 fractions) were incubated with EZview™ Red Anti-FLAG® M2 Affinity Gel (Sigma) for 2 h at 4 °C. The beads were washed 3 times with 10 bead volumes of phosphate-buffered saline and 0.2% Tween 20 prior to suspension in SDS-PAGE loading buffer. Western blotting was carried out as previously described (23).

**Electrophoretic Mobility Shift Assay (EMSA) and Native Gel Analysis**—12-μl Reactions were carried out in 25 mM HEPES, pH 7.6, 15% glycerol, 60 mM KCl, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Nonidet P-40, 1 μg of bovine serum albumin, 300 ng of poly(I)-poly(C) (Amersham Biosciences), and included 500 pg of radiolabeled 7SK RNA, recovered P-TEFb, and cyclin T1, as indicated. 7SK and poly(rI)-poly(rC) were incubated with EZview™ Red Anti-FLAG® M2 Affinity Gel (Sigma) for 2 h at 4 °C. The beads were washed 3 times with 10 bead volumes of phosphate-buffered saline and 0.2% Tween 20 prior to suspension in SDS-PAGE loading buffer. Western blotting was carried out as previously described (23).

**Identification of Cdk9 Phosphorylation Sites by Mass Spectrometry**—Recombinant P-TEFb (His-Cdk9/cyclin T1, ~2 μg) was purified using Ni-NTA resin, separated by 12.5% SDS-PAGE, and stained with Coomassie Blue G-250. The Cdk9-containing band was excised from the gel and split into two pieces, one of which was fully trypsinized as described (31), and the other was incubated with trypsin for only 1 h at room temperature to allow partial digestion. The tryptic peptides were extracted and analyzed as previously reported (32) using an LCQ-DECA XP-Plus ion trap mass spectrometer (Thermo Finnigan, San Jose, CA).

**RESULTS**

**HEXIM1 Is a Dimer**—Sequence alignment of HEXIM1 and HEXIM2 proteins from different species revealed a relatively conserved region at their COOH termini, containing several invariant leucine residues (23). When this region of human HEXIM1 (amino acids 284–318) was plotted on a helical wheel, a pattern of leucine residues characteristic of a leucine zipper coiled coil motif emerges (Fig. 1A). Because this motif is frequently involved in protein-protein interactions, we first examined if HEXIM1 could oligomerize via this region. Two proteins containing single mutations with either leucine 287 or 294 substituted with arginine (L287R, L294R), and a double mutant with both Leu²⁸⁷ and Leu²⁹⁴ replaced by arginine (2LR) were produced. All three mutant proteins had relatively similar mobility compared with wild type HEXIM1 when analyzed by SDS-PAGE (Fig. 1B). However, when analyzed on a native gel, L287R and 2LR had higher mobility than wild type and L294R (Fig. 1C). It is unlikely that the differences are because of the small fractional changes in charge. This is supported by two observations. Wild type and L294R have similar mobilities, but different charge, and L287R and L294R have the same charge, but different mobilities. It is likely that the differences in mobility are because of the disruption of oligomerization in the L287R and 2LR mutants. To confirm the hypothesis that the carboxyl-terminal domain allowed oligomerization, two truncation mutants were generated. A control protein, ΔN, lacked the amino-terminal 170 amino acids, whereas ΔC, lacking the carboxyl-terminal domain, did not contain the entire leucine zipper motif (Fig. 1A). ΔN had much higher mobility than ΔC on the SDS-PAGE gel (Fig. 1B). In contrast, this difference in mobility was reversed on the native gel (Fig. 1C), strongly suggesting the leucine zipper motif mediates oligomerization of HEXIM1.

The function of these mutants was compared with wild type HEXIM1 using a mobility shift assay and a kinase assay. HEXIM1 formed the expected complex with 7SK that was further shifted by association with P-TEFb (Fig. 1D). The ΔC mutant, assayed at two concentrations, formed a complex with double the mobility of the wild type HEXIM1 complex, but was still able to recruit P-TEFb. Because ΔC cannot dimerize, we assume that the predominant shift seen at the highest concentration contains a HEXIM1 monomer. The less severe mutant, 2LR, formed a 7SK complex with slightly lower mobility than ΔC, and at a higher concentration also formed a complex with mobility similar to the wild type complex (Fig. 1D). Evidently, it binds as a monomer at low concentration, but can dimerize at higher concentrations. The 2LR dimer may be stabilized by interactions with 7SK. Both 2LR-7SK complexes were able to recruit P-TEFb (Fig. 1D). The effects of the HEXIM1 proteins on the kinase activity of P-TEFb were assayed in the absence and presence of 7SK. As expected, without 7SK the wild type and leucine zipper mutants had no effect on P-TEFb kinase activity (Fig. 1E). Supporting an idea stated earlier that removing the amino-terminal region might unmask P-TEFb binding or inhibitory regions of HEXIM1 (29), ΔN inhibited P-TEFb activity down to 40% without 7SK. In the presence of 7SK all proteins inhibited P-TEFb, but the inhibition by proteins with reduced oligomerization (L287R, 2LR, and ΔC) was less robust (Fig. 1E). The ability of ΔN to inhibit P-TEFb was further stimulated by 7SK. Evidently the unmasking of the inhibitory regions by deletion of the first 120 amino acids is not complete, and is further stimulated by binding of 7SK.
FIG. 1. Putative COOH-terminal leucine zipper coiled coil of HEXIM1 mediates its oligomerization. A, diagram of recombinant HEXIM1 proteins. WT, wild type; ΔN, 121–359 amino acids of HEXIM1; ΔC, 1–282 amino acids of HEXIM1. On the right is a helical wheel representation of amino acids 283–318 of HEXIM1. B, SDS-PAGE analysis followed by silver staining of the indicated recombinant HEXIM1 proteins. C, native gel analysis followed by silver staining of recombinant proteins. D, EMSA analysis with 7SK of the indicated HEXIM1 proteins in the absence and presence of P-TEFb as indicated. E, inhibition of P-TEFb activity by 0.5 pmol of the indicated HEXIM1 proteins in the absence or presence of 0.5 pmol of 7SK RNA. Details of methods are found under “Experimental Procedures.”
Evidence that HEXIM1 is a dimer came from a co-expression experiment. A FLAG-tagged, wild type HEXIM1 and a His-tagged ΔN were co-expressed in E. coli using two different plasmids with compatible replication origins (Fig. 2A). Cell lysates were first subjected to Ni-NTA chromatography and FLAG-tagged wild type HEXIM1 co-eluted with His-tagged ΔN, demonstrating that the two HEXIM1 proteins could stably interact (Fig. 2B). The material that eluted from the Ni-NTA column was further purified by FLAG affinity chromatography. Again, His-tagged ΔN and FLAG-tagged wild type HEXIM1 co-eluted. Unlike the material that eluted from the Ni column, which contained more His-tagged protein than FLAG-tagged protein, equal amounts of each protein eluted from the FLAG antibody column (Fig. 2B). This 1 to 1 ratio indicated that HEXIM1 was at least a dimer. Because similar results would have been obtained if HEXIM1 formed a trimer, tetramer, or higher order oligomer, the oligomerization state was determined by comparing the mobilities of the two homo-oligomers with the hetero-oligomer. If HEXIM1 dimerizes, then only one band with intermediate mobility should be given with the hetero-oligomer on a native gel. If HEXIM1 trimerizes, then two bands with intermediate mobility should be seen (one with 2 large and 1 small protein and one with 2 small and one large protein). Silver staining of the native gel demonstrated that the hetero-oligomer gave only one band of intermediate mobility between the two homo-oligomers, clearly indicating that HEXIM1 forms dimers (Fig. 2C). To determine whether HEXIM1 binds to 7SK as a dimer, the same logic was applied to the results of a mobility shift assay. The mobility of 7SK complex with the heterodimer gave a single band that was intermediate between the two homodimers (Fig. 2C). Thus we conclude that HEXIM1 oligomerizes regardless of its association with 7SK and P-TEFb in vivo. If the 7SK-HEXIM1-P-TEFb complex (GGF9) was used in immunoprecipitations by anti-FLAG antibodies. The top panel was probed with antibodies to HEXIM1, and the lower panel by antibodies to Cdk9. B, glycerol gradient analysis of a lysate from HeLa cells transfected with a construct expressing FLAG-tagged HEXIM1 2LR mutant. Fractions were analyzed by Western blotting using cyclin T1 (Cyc T1) and HEXIM1 antibodies (top panel) or Cdk9 antibodies (bottom panel). C, there are two P-TEFb in the 7SK-HEXIM1-P-TEFb complex. Cell lysates from a stable cell line expressing FLAG-tagged Cdk9 were fractionated on a glycerol gradient, and the indicated fractions subjected to anti-FLAG immunoprecipitation and probing with the indicated antibodies.
Although we have not rigorously proven that HEXIM1 is a dimer, rather than a trimer or higher oligomer in vivo, the sedimentation of wild type free HEXIM1 (40 kDa) is consistent with it being a dimer (80 kDa) because it sediments slower than free P-TEFb (120 kDa).

A similar immunoprecipitation experiment was carried out on cells expressing FLAG-tagged Cdk9. Anti-FLAG antibodies brought down only FLAG-tagged Cdk9 from a glycerol gradient fraction containing the free form of P-TEFb (Fig. 3C). However, FLAG-tagged Cdk9 and untagged, endogenous Cdk9, as well as HEXIM1, were pulled down from a fraction containing the large form of P-TEFb (Fig. 3C). These results suggest that free P-TEFb does not dimerize in the cell, but that the large complex contains two P-TEFb molecules.

The 7SK-HEXIM1-P-TEFb Complex Contains One 7SK Molecule—We examined the stoichiometry of 7SK in complexes formed with HEXIM proteins. A 330-nucleotide full-length 7SK and a truncated RNA containing the first 172 nucleotides of 7SK were synthesized by in vitro transcription. The abilities of the full-length and truncated 7SK to support HEXIM1-mediated inhibition of P-TEFb in vitro were analyzed. Neither RNA inhibited P-TEFb alone, but when mixed with equal molar amounts of HEXIM1 both RNAs produced a dose-dependent inhibition down to about 10% (Fig. 4A). Evidently, only the first half of 7SK is needed for a functional interaction with HEXIM1 and P-TEFb. Both RNAs were able to form complexes with HEXIM1 and subsequently recruit P-TEFb (Fig. 4B). To determine whether more than one 7SK would bind to a HEXIM1 dimer, the two versions of 7SK were mixed together before adding HEXIM1 and then analyzed by EMSA. The shifts that were generated were identical to the shifts seen with the individual RNAs (Fig. 4B). Identical results were obtained when HEXIM1 was replaced by a functionally similar protein, HEXIM2 (23) (Fig. 4C). Because there was no detectable intermediate complex we conclude that there is only one 7SK in the complex.

Tyrosine 271 and Phenylalanine 208 of HEXIM1 Are Critical for Inhibition of P-TEFb Kinase Activity—The sequence alignment of HEXIM proteins across many species revealed five invariant tyrosine residues corresponding to amino acids 167, 203, 271, 274, and 291 of human HEXIM1. Previously, we showed that Tyr<sup>203</sup> is located in the region of HEXIM1 important for the recruitment of P-TEFb to the 7SK-HEXIM1 complex (29). To examine potential roles of the other tyrosines, mutant HEXIM1 proteins containing Y167E, Y271E, Y274E, or Y291E were generated and tested for their ability to inhibit P-TEFb kinase activity in the presence of 7SK. Only Y271E lost most of its inhibitory properties (data not shown). To examine the role of Tyr<sup>271</sup> in detail, Y271A and Y271F mutants were also generated, and the abilities of the three mutants to inhibit P-TEFb were compared with wild type HEXIM1. Y271A and Y271E did not support robust inhibition of P-TEFb, but Y271F behaved the same as the wild type (Fig. 5A). Interestingly, all four proteins were able to form complexes with 7SK and recruit P-TEFb (Fig. 5B). These results indicate that it is critical for inhibition of P-TEFb to maintain an aromatic residue at position 271, but that this residue is not essential for the recruitment of P-TEFb to the 7SK-HEXIM1 complex. A similar analysis of a conserved phenylalanine, Phe<sup>208</sup>, was carried out. F208A, F208D, and F208K mutants were generated, and the purified proteins were used in a kinase assay and electrophoretic mobility shift assay to determine their ability to inhibit P-TEFb and to recruit P-TEFb to a 7SK-HEXIM1 complex. All three mutants displayed a significantly reduced ability to inhibit P-TEFb in the presence of 7SK, but all mutants, except F208K, maintained their ability to recruit P-TEFb to the 7SK-HEXIM1 complex (Fig. 5, C and D). We conclude that both Tyr<sup>271</sup> and Phe<sup>208</sup> are critical for inhibition of P-TEFb, but have only a minimal influence on the ability of HEXIM1 to associate with P-TEFb.

Phosphorylation of Threonine 186 of Cdk9 Is Required for P-TEFb Kinase Activity and Formation of the 7SK-HEXIM1-P-TEFb Complex—To examine the role of phosphorylation of the T-loop of Cdk9, recombinant P-TEFb proteins containing cyclin T1 and wild type, S175A, S175D, T186A, or T186E mutations in Cdk9 were purified individually (Fig. 6A). Because Cdk9, but not cyclin T1 was His-tagged, and equal molar amounts of Cdk9 and cyclin T1 were purified, none of the mutations had an effect on association of the cyclin. Increasing amounts of each purified protein were subjected to a direct kinase assay using the large subunit of DSIF (SPT5) as a substrate. The wild type kinase and the two S175 mutants had similar high levels of activity (Fig. 6B). P-TEFb containing Cdk9 with a T186A mutation had less than 10% of the activity of the wild type kinase. This leads to the assumption that Thr<sup>186</sup> phosphorylation occurs to some extent during expression of P-TEFb in baculovirus-infected insect cells. Introduction of a negative charge to mimic phosphorylation (T186E) increased the kinase activity about 3-fold from what was seen with the T186A mutant (Fig. 6B). This suggests that phosphorylation of Thr<sup>186</sup> of Cdk9 is required for efficient kinase activity of P-TEFb. The mobility shift assay was then used to look at association of the P-TEFb
mutants with 7SK-HEXIM1 (Fig. 6C) or 7SK-HEXIM2 (Fig. 6D). A dose-dependent shift was seen for the wild type and Ser\textsuperscript{175} mutants (Fig. 6, C and D). P-TEFb mutants containing the T186A or T186E displayed a greatly reduced affinity for 7SK-HEXIM1 and 7SK-HEXIM2 (Fig. 6, C and D). At the highest level of P-TEFb added, a second P-TEFb shift was visible with 7SK-HEXIM1 complexes and was clearly present with 7SK-HEXIM2 complexes. This supports the finding that two P-TEFb molecules were found in the large 7SK-HEXIM complex, and that the 7SK-HEXIM1-P-TEFb complex contains Cdk9 and cyclin T1 as indicated. C, inhibition of P-TEFb by 0.5 pmol of HEXIM1 proteins containing mutations F208A (F/A), F208D (F/D), and F208K (F/K) in the absence or presence of 0.5 pmol of 7SK. D, effects of phenylalanine mutations on the formation of the 7SK-HEXIM1-P-TEFb complex were analyzed by EMSA. 10 ng of HEXIM proteins (same level of purity as those shown in Fig. 1B) were used and P-TEFb contained Cdk9 and cyclin T1.

FIG. 5. Tyr\textsuperscript{271} and Phe\textsuperscript{208} residues of HEXIM1 are important for inhibition of P-TEFb. A, inhibition of P-TEFb by 0.5 pmol of HEXIM1 proteins containing mutations Y271A (Y/A), Y271E (Y/E), and Y271F (Y/F) in the absence or presence of 0.5 pmol of 7SK. B, effects of tyrosine mutations on the formation of the 7SK-HEXIM1-P-TEFb complex. EMSA with 7SK was carried out in the presence of 10 ng of HEXIM proteins, and P-TEFb contained Cdk9 and cyclin T1 as indicated. C, inhibition of P-TEFb by 0.5 pmol of HEXIM1 proteins containing mutations F208A (F/A), F208D (F/D), and F208K (F/K) in the absence or presence of 0.5 pmol of 7SK. D, effects of phenylalanine mutations on the formation of the 7SK-HEXIM1-P-TEFb complex were analyzed by EMSA. 10 ng of HEXIM proteins (same level of purity as those shown in Fig. 1B) were used and P-TEFb contained Cdk9 and cyclin T1.

In this report, we provide evidence that HEXIM1 is a dimer both when it is free and when it is complexed with 7SK and P-TEFb, and that the 7SK-HEXIM1-P-TEFb complex contains one 7SK molecule and two P-TEFb molecules. We also identified conserved residues Tyr\textsuperscript{271} and Phe\textsuperscript{208} as critical for the inhibition of P-TEFb kinase activity, but not the formation of the 7SK-HEXIM1-P-TEFb complex. Furthermore, we demonstrated that phosphorylation of Thr\textsuperscript{186} is a major phosphorylation site on Cdk9. Although negative MS/MS data is not conclusive, we did not detect any phosphopeptide containing modified Ser\textsuperscript{175}, suggesting that it may not contribute significantly to Cdk9 activation.

**DISCUSSION**

Functional Interactions in the Large P-TEFb Complex
HEXIM2 can oligomerize when HEXIM2 is overexpressed in HeLa cells. This hetero-oligomerization cannot be merely driven by the concentrations of the two proteins, because we found that HEXIM1 and HEXIM2 partition differently into free and large P-TEFb complexes in two cell lines (23) and this would not be expected if homo- and hetero-oligomerization were equally favored. Oligomerization may play a role in stabilizing HEXIM1 in vivo. During transient expression of HEXIM1 proteins in HeLa cells, the levels of dimerization-defective mutants (L287R and 2LR) were much lower than wild type or mutant HEXIM1s that supported dimerization in vitro.²

Our studies here have identified residues of HEXIM1 that may be involved in the mechanism of inhibition of P-TEFb. Mutation of a conserved Tyr²⁷¹ in the SETYER region of HEXIM1 greatly reduced the ability of HEXIM1 to inhibit P-TEFb in the presence of 7SK, but did not affect the recruitment of P-TEFb into the 7SK-HEXIM1 complex. Similar results were obtained with mutations of a highly conserved phenylalanine at position 208 (F208A, F208D, F208K). The biochemical phenotype of these mutations distinguishes them from mutants in the PYNT region that block recruitment of P-TEFb. Mutation of a conserved Tyr271 in the SETYER region of HEXIM1 greatly reduced the ability of HEXIM1 to inhibit P-TEFb in the presence of 7SK, but did not affect the recruitment of P-TEFb into the 7SK-HEXIM1 complex. Similar results were obtained with mutations of a highly conserved phenylalanine at position 208 (F208A, F208D, F208K). The biochemical phenotype of these mutations distinguishes them from mutants in the PYNT region that block recruitment of P-TEFb. Although separated in the linear sequence, Tyr²⁷¹ and Phe²⁰⁸ may be part of a P-TEFb inhibitory domain that is discussed below.

With all the information available now, it is possible to construct a more detailed model of how P-TEFb is controlled by association with 7SK and HEXIM proteins (Fig. 7B). From our experiments expressing P-TEFb with Cdk9 mutants in insect cells, assembly of a P-TEFb heterodimer can occur in the absence of T-loop phosphorylation (Fig. 6A). P-TEFb het-

² Q. Li, unpublished data.
erodimers were formed with wild type Cdk9 or with Cdk9 carrying T-loop mutations T186A, T186E, S175A, or S175D, but only wild type and S175A and S175D mutants were completely active and the T186E mutant was partially active (Fig. 6B). This and the MS/MS analysis strongly points toward Thr186, not Ser175, as the critical site of activating phosphorylation. Our results differ somewhat from those obtained in the Zhou laboratory (30) who found that Cdk9 expressed in HeLa cells containing T186A, T186E, and S175A mutations had no activity, and that a S175D mutant had full kinase activity. Using recombinant P-TEFb in electrophoretic mobility shift assays, we found that only kinases that were activated (wild type, S175A, and S175D) associated efficiently with the 7SK/HEXIM complex containing either HEXIM1 or HEXIM2 (Fig. 6, C and D). P-TEFb containing the partially activating mutation T186E had a low but detectable binding to the 7SK/HEXIM complex. HEXIM1 and, presumably, HEXIM2 are normally found as dimers that do not interact with or inhibit P-TEFb (Figs. 2 and 3). The binding to the first 172 residues of a single molecule of 7SK results in a conformational change that enables HEXIM to bind to and inhibit P-TEFb (Fig. 4). Because removal of the first 120 amino acids of HEXIM1 enable it to inhibit P-TEFb in the absence of 7SK (Fig. 1E), we propose that the amino-terminal domain is an autoinhibitory domain that masks the P-TEFb binding domain, and that binding of 7SK results in a conformational change that unmask the P-TEFb binding domain. The presence of an autoinhibitory domain was previously suggested to explain the ability of a HEXIM1 mutant lacking the first 180 amino acids to associate with Cdk9 with or without 7SK (29). During that study we were unable to demonstrate 7SK independent inhibition of P-TEFb for HEXIM1 (181–359).2 Perhaps that large deletion damaged the ability of HEXIM1 to inhibit P-TEFb without significantly reducing its ability to bind to P-TEFb. Finally, our results indicate that the large inactive form of P-TEFb contains one 7SK RNA, a HEXIM dimer, and two P-TEFb molecules. If no other proteins are present then the total mass of the large complex would be 430 kDa. This is in close agreement with the size estimates from glycerol gradients (19).

It is possible that HEXIM1 shares mechanistic details with the Cdk2/cyclin A inhibitor, p27kip. Like p27kip, HEXIM1 binds to the cyclin partner of the Cdk it inhibits (29). Binding of p27kip to cyclin A positions several aromatic residues (tyrosine and phenylalanine) in the catalytic cleft of Cdk2 and blocks the interaction of Cdk2 and ATP, thereby, inhibiting kinase activity (33). We found that mutation of Tyr271 to phenylalanine (Y271F) allowed continued function of HEXIM1, but substitution of alanine or glutamate significantly reduced the inhibitory function without significantly affecting the interaction with P-TEFb. HEXIM1 Phe208 mutants also blocked inhibition without disrupting the interaction with P-TEFb. Our results are consistent with Tyr271 and/or Phe208 blocking ATP binding to Cdk9. Another similarity between HEXIM1 and p27kip is that both bind to the phosphorylated (activated) form of the kinases they inhibit. The function of HEXIM1 may be more similar to the p27kip family than to the INK4 family that bind to Cdk2 and weaken the interaction of the cyclin and Cdk and distort that active site (34).

Although some details of the interactions of the components of the large inactive P-TEFb complex are known, it is still not clear how the activated P-TEFb stored there is released. Control of release of P-TEFb by removal of the phosphate from Thr186 is counter-intuitive because even though this might cause dissociation, the kinase would be inactive. We favor a model in which the association of HEXIM proteins with 7SK is regulated. Dissociation of 7SK might allow the inhibitory domain of HEXIM proteins to remask the P-TEFb binding domain, and allow P-TEFb to be free and active. Further studies are needed to uncover factors that are responsible for the regulated assembly and disassembly of the large inactive P-TEFb complex.

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REFERENCES
1. Harper, J. W., and Adams, P. D. (2001) Chem. Rev. 101, 2511–2526
2. Morgan, D. O. (1997) Annu. Rev. Cell Dev. Biol. 13, 261–291
3. Morgan, D. O. (1995) Nature 374, 131–134
4. Pavletich, N. P. (1999) J. Mol. Biol. 287, 821–828
5. Napolitano, G., Majolet, B., and Lania, L. (2002) Int. J. Oncol. 21, 171–177
6. Price, D. H. (2000) Mol. Cell. Biol. 20, 2629–2634
7. Garriga, J., and Grana, X. (2004) Gene (Amst.) 337, 15–23
8. Zhu, Y., Civelli, T., Peng, J., Ramanathan, V., Marshall, N., Marshall, T., Amendt, B., Mathews, M. B., and Price, D. H. (1997) Genes Dev. 11, 2622–2632
9. Shorer, S. M., Byers, S. A., Maury, W., and Price, D. H. (2003) Gene (Amst.) 307, 175–182
10. Peng, J., Zhu, Y., Milton, J. T., and Price, D. H. (1998) Genes Dev. 12, 755–762
11. Fu, J., Peng, J., Lee, G., Price, D. H., and Flores, O. (1999) J. Biol. Chem. 274, 34527–34530
12. Marshall, N. F., Peng, J., Xie, Z., and Price, D. H. (1996) J. Biol. Chem. 271, 27176–27183
13. Marshall, N. F., and Price, D. H. (1995) J. Biol. Chem. 270, 12335–12338
14. Mancebo, H. S., Lee, G., Frygare, T., Tomassini, J., Luo, L., Zhu, Y., Peng, J., Blau, C., Hazuda, D., Price, D., and Flores, O. (1997) Genes Dev. 11, 2633–2644
15. Zhou, Q., Chen, D., Piersoster, E., and Luo, K. (1998) EMBO J. 17, 3681–3691
16. Garber, M. E., Wei, P., and Jones, K. A. (1998) Cold Spring Harbor Symp. Quant. Biol. 63, 371–380
17. Wei, P., Garber, M. E., Pang, S. M., Fischer, W. H., and Jones, K. A. (1998) Cell 92, 451–462
18. Barboric, M., Nissen, R. M., Kanazawa, S., Jabrane-Ferrat, N., and Peterlin, B. M. (2001) Mol. Cell 8, 327–337
19. Nguyen, V. T., Kiss, T., Michels, A. A., and Bensaude, O. (2001) Nature 414, 322–325
20. Yang, Z., Zhu, Y., Luo, K., and Zhou, Q. (2001) Nature 414, 317–322
21. Michels, A. A., Nguyen, V. T., Fraldi, A., Labas, V., Edwards, M., Bonnet, F., Lania, L., and Bensaude, O. (2003) Mol. Cell. Biol. 23, 4859–4869
22. Yik, J. H., Chen, R., Nishimura, R., Jennings, J. L., Link, A. J., and Zhou, Q. (2003) Mol. Cell 12, 971–982
23. Byers, S. A., Price, J. P., Cooper, J. J., Li, Q., and Price, D. H. (2005) J. Biol. Chem. 280, 8329–8344
24. Yik, J. H., Chen, R., Pezda, A. C., and Zhou, Q. (2005) J. Biol. Chem. 280, 16368–16376
25. Sano, M., Abdellatif, M., Oh, H., Xie, M., Bagella, Li, Giordano, A., Michael, L. H., DeMayo, F. J., and Schneider, M. D. (2002) Nat. Med. 8, 1310–1317
26. Sano, M., and Schneider, M. D. (2003) Cell Cycle 2, 99–104
27. Kulkarni, P. A., Sano, M., and Schneider, M. D. (2004) Recent Prog. Horm. Res. 59, 125–139
28. Yik, J. H., Chen, R., Pezda, A. C., Samford, C. S., and Zhou, Q. (2004) Mol. Cell. Biol. 24, 5094–5105
29. Michels, A. A., Fraldi, A., Li, Q., Adamson, T. E., Bonnet, F., Nguyen, V. T., Sedore, S. C., Price, J. P., Price, D. H., Lania, L., and Bensaude, O. (2004) EMBO J. 23, 2608–2619
30. Chen, R., Yang, Z., and Zhou, Q. (2004) J. Biol. Chem. 279, 4153–4160
31. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Anal Chem. 68, 850–858
32. Peng, J., and Gygi, S. P. (2001) J. Mass Spectrom. 36, 1083–1091
33. Russo, A. A., Jeffrey, P. D., Patten, A. K., Massague, J., and Pavletich, N. P. (1996) Nature 382, 325–331
34. Jeffrey, P. D., Tong, L., and Pavletich, N. P. (2000) Genes Dev. 14, 3115–3125
Analysis of the Large Inactive P-TEFb Complex Indicates That It Contains One 7SK Molecule, a Dimer of HEXIM1 or HEXIM2, and Two P-TEFb Molecules Containing Cdk9 Phosphorylated at Threonine 186

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