Positive Transcription Elongation Factor b Phosphorylates hSPT5 and RNA Polymerase II Carboxyl-terminal Domain Independently of Cyclin-dependent Kinase-activating Kinase*

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The CDK9-cyclin T kinase complex, positive transcription elongation factor b (P-TEFb), stimulates the process of elongation of RNA polymerase (Pol) II during transcription of human immunodeficiency virus. P-TEFb associates with the human immunodeficiency virus Tat protein and with the transactivation response element to form a specific complex, thereby mediating efficient elongation. Here, we show that P-TEFb preferentially phosphorylates hSPT5 as compared with the carboxyl-terminal domain of RNA Pol II in vitro. Phosphorylation of hSPT5 by P-TEFb occurred on threonine and serine residues in its carboxyl-terminal repeat domains. In addition, we provide several lines of evidence that P-TEFb is a CDK-activating kinase (CAK)-independent kinase. For example, CDK9 was not phosphorylated by CAK, whereas CDK2-cyclin A kinase activity was dramatically enhanced by CAK. Therefore, it is likely that P-TEFb participates in regulation of elongation by RNA Pol II by phosphorylation of its substrates, hSPT5 and the CTD of RNA Pol II, in a CAK-independent manner.

Efficient transcription of full-length proviral DNA of human immunodeficiency virus (HIV) is controlled by the viral protein Tat. Tat is expressed early in the viral life cycle and is essential for viral replication and gene expression (1–3). Tat recognizes the bulge region of TAR (transactivation response element), an RNA stem-loop structure located at the 5′ end of HIV transcripts (4, 5). Although the precise molecular mechanism by which Tat exerts its transcriptional activation is not completely understood, it has been suggested that Tat stimulates transcription by RNA polymerase (Pol) II predominately at the level of elongation rather than initiation (6, 7).

Recent studies indicate that P-TEFb (positive transcription elongation factor b) is a key cellular factor supporting Tat-dependent elongation (8–12). P-TEFb, originally identified from Drosophila melanogaster, was purified as a suppressor of an inhibitor of elongation by RNA Pol II (8, 9) and is composed of kinase subunit CDK9 and its cyclin partner cyclin T (11–15). P-TEFb efficiently phosphorylates the CTD of RNA Pol II and in fact is associated with elongating RNA Pol II in vitro (16–18). The kinase activity of P-TEFb is sensitive to DRB, an inhibitor of elongation by RNA Pol II. Furthermore, Tat binds specifically to human P-TEFb via a cyclin T subunit (13, 14, 19). A specific cysteine residue, 261 of human cyclin T, is critical for the interaction of Tat with P-TEFb, and rodent cells that encode a cyclin T lacking this cysteine residue are defective for Tat activation (13, 14, 19–23). Finally, depletion of P-TEF from HeLa nuclear extract decreased not only basal transcription but also Tat-dependent transcription elongation (11, 24).

In addition to P-TEFb, Tat-dependent activation of transcription is also regulated by other cellular factors including TAT-SF1 and DSIF (hSPT4 and 5). TAT-SF1 was identified using a partially purified reconstituted reaction that supports Tat-dependent TAR-specific stimulation of elongation (25, 26). TAT-SF1, a phosphoprotein, contains two RNA recognition motifs and a highly acidic domain at its carboxyl terminus (26). TAT-SF1 binds to Tat, and its overexpression can stimulate Tat-dependent activation in vivo (26, 27). In addition, TAT-SF1 forms a protein complex including TFIIH (RAP30), P-TEFb, hSPT5, and RNA Pol II that is thought to mediate Tat-dependent activation in vitro (27–30).

DSIF (hSPT4 and 5) was originally isolated as a factor that renders transcription in vitro inhibitable by DRB (35). DSIF forms a protein complex with the negative elongation factor to inhibit promoter proximal elongation by RNA Pol II (31–34). Release from this inhibition is mediated by P-TEFb, specifically through phosphorylation by its DRB-sensitive kinase CDK9 (32, 34). DSIF is also important for Tat activation, because nuclear extracts depleted of hSPT5 do not support Tat-dependent elongation in vitro (27, 29, 30). Additionally, over-expression of hSPT5 stimulates Tat-specific activation in vivo (27). In yeast, SPT5 increases the efficiency of elongation of RNA Pol II complexes (35).

Several studies have also implicated TFIIH in Tat activation (36–39). TFIIH is a general transcription factor that forms a preinitiation complex with RNA Pol II (40–43). TFIIH is composed of nine polypeptides (p34, p44, p54, p62, CDK7, cyclin H, MAT1, ERCC2, and ERCC3). TFIIH is not only essential for RNA Pol II-dependent transcription but is also important for DNA repair and cell cycle regulation (41, 42). An important component of TFIIH is the CDK7 subunit, which interacts with...
Phosphoamino Acid Analysis and Phosphopeptide Mapping—Phosphoamino acid analyses of purified proteins were performed by high voltage paper electrophoresis at pH 2.7 (35). Phosphopeptide maps were generated essentially as described (50, 51), except that the silver staining was replaced by autoradiography. Phosphopeptides were excised from the phosphopeptide maps, eluted with 1% trifluoroacetic acid, and subjected to amino acid analysis as described (50, 51). The peptide maps were calibrated with synthetic peptides of known sequence. Phosphopeptides were identified by amino acid analysis as described above.

RESULTS

**P-TEFb Phosphorylates hSPT5 and the CTD of RNA Pol II**—DRB inhibits transcription at the stage of elongation in vivo (52, 53). During transcription in vitro, DRB also selectively inhibits elongation as compared with initiation, probably by suppressing kinase activities such as those responsible for CTD hyperphosphorylation (54, 55). Among many CTD kinases, CAK and P-TEFb have relatively well-characterized functions in RNA Pol II transcription in vitro. Both kinases are DRB-sensitive (10, 11, 38, 39). As part of the characterization of P-TEFb, the DRB sensitivity of both kinases was examined using baculovirus-expressed P-TEFb (CDK9-cyclin T) and CAK (CDK7-cyclin H). When GST-CDT was used as substrate, the IC_{50} of DRB was estimated as 2.5 and 20 μM for P-TEFb and CAK, respectively (Fig. 1A). This result suggests that P-TEFb is significantly more sensitive to inhibition by DRB than CAK, which is consistent with previous results (9–11). This observation is also consistent with the inhibition of P-TEFb controlling the DRB-sensitive elongation process both in vivo and in vitro. Approximately 2–5 μM DRB yields a 50% decrease in transcription activity (10, 11).

To date, the CTD of RNA Pol II is the best known substrate of P-TEFb. To test whether P-TEFb can phosphorylate other molecules involved in Pol II-dependent transcription, P-TEFb kinase assays were performed with several other substrates: hSPT5, CAK, CDK2-cyclin A, TAT-SF1, and histone H1. GST-CTD was included as a positive control. As shown in Fig. 1B, P-TEFb phosphorylated both GST-CTD and hSPT5 more efficiently than the other proteins tested. In fact, P-TEFb phosphorylated hSPT5 more effectively than GST-CTD at the same substrate concentration (Fig. 1B). We therefore further investigated the enzyme-substrate specificity of P-TEFb. Both hSPT5 and GST-CTD proteins were serially diluted to measure the KM values for P-TEFb (Fig. 1C). The calculated values for hSPT5 and GST-CTD were 18 and 55 nM, respectively. Moreover, because the GST-CTD contains 52 YSPTPSP repeats, each of which is a potential site of phosphorylation, whereas hSPT5 probably contains fewer potential sites, these KM values probably underestimate the preference of hSPT5 as a substrate.

**P-TEFb Phosphorylates the Carboxyl Terminus of hSPT5**—To determine the preferential phosphorylation domains of hSPT5 by P-TEFb, hSPT5 was divided into the following three domains: the amino-terminal domain, which contains acidic amino acid residues; the middle domain, which contains KOW motifs similar to those of E. coli NusG proteins; and the carboxy-terminal domain, which contains CTR heptapeptide repeats (29) (Fig. 2A). Each of the recombinant hSPT5 proteins was histidine-tagged and expressed in E. coli (Fig. 2B). As shown in Fig. 2C, P-TEFb efficiently phosphorylated the carboxy-terminal domain of hSPT5 rather than the amino-terminal or middle domains, although a low level of phosphorylation of the middle domain was detected. The CTR domain contains multiple serine and threonine residues that could be potential phosphorylation sites by the serine/threonine kinase P-TEFb. To test which amino acids of hSPT5 are phosphorylated by P-TEFb, phosphoamino acid analysis was performed. As shown in Fig. 2D, 75 and 25% of hSPT5 phosphorylations occurred at the threonine and serine residues, respectively, whereas tyrosine phosphorylation was not observed. To confirm that the CTR is the major phosphorylation domain of hSPT5 by P-TEFb, two-dimensional phosphopeptide mapping was performed with recombinant FL and CTR proteins. FL and CTR phosphopeptides produced by digestion with trypsin were resolved on TLC plates. Most of the separated phosphopeptides of the CTR proteins comigrated with those of the FL protein, suggesting...
that the high affinity phosphorylation sites of hSPT5 are in the CTR domain.

Phosphorylation of Threonine 186 of CDK9 and Kinase Activity—The kinase activities of CDKs can be regulated by several mechanisms. The binding of a cyclin partner is one of the activation processes. Some CDKs are activated by phosphorylation of a specific threonine residue in their T-loop. CAK (CDK7-cyclin H) is responsible for phosphorylation in the T-loop of several CDKs including CDK2, 4, and 6 (48, 49). CDK9 shows 39% identity with CDK2, the best characterized kinase whose activity is controlled by CAK. More importantly, CDK9 has a threonine residue at position 186 in the T-loop region that is conserved in the corresponding region of CDK2. Two different point mutants of CDK9 were made to study the functional role of phosphorylation of this site (Fig. 3A). First, threonine 186 of CDK9 was mutated to alanine (CDK9TA), which does not undergo phosphorylation. Second, aspartic acid 167 of CDK9 was mutated to asparagine (CDK9DN), which abrogates its kinase activity. Although catalytically inactive, the latter mutant protein probably folds into a native conformation because it functions as a dominant negative in vivo (10). These CDK9 mutants were expressed in insect cells by use of the baculovirus system and purified. The kinase activity of CDK9 was enhanced 4–5-fold when bound by cyclin T (Fig. 3B, lanes 1 and 2).

FIG. 1. DRB sensitivity of P-TEFb kinase and identification of P-TEFb substrates. A, DRB sensitivities of P-TEFb (CDK9-cyclin T) and CDK7-cyclin H kinase complexes were examined with GST-CTD as substrate. 10 ng of P-TEFb or CDK7-cyclin H complex were used to phosphorylate GST-CTD (100 ng) with indicated amounts of DRB. B, in vitro P-TEFb kinase assay with various substrates. Recombinant P-TEFb (CDK9-cyclin T) complex was mixed with indicated proteins (50 ng) during the in vitro kinase assay. Positions of heavily phosphorylated GST-CTD and SPT5 are marked with arrowheads. Positions of TAT-SF1, cyclin T, CDK9, and histone H1 are marked with arrows on the right. C, titration of recombinant hSPT5 and GST-CTD proteins during the in vitro P-TEFb kinase assay. Indicated amounts of substrates of either hSPT5 or GST-CTD were used with 20 ng of P-TEFb. To get a similar intensity, the film of phosphorylated GST-CTD was exposed two times longer than that of phosphorylated SPT5.

FIG. 2. Analysis of hSPT5 phosphorylation by P-TEFb kinase. A, schematic of recombinant hSPT5 proteins. The amino-terminal domain contains aa 1–271 of the hSPT5 protein; the middle domain contains aa 272–756 of the hSPT5 protein; the carboxyl-terminal domain contains aa 757–1087 of the hSPT5 protein; and CTR contains aa 757–920 of the hSPT5 protein. NT, amino-terminal domain; MD, middle domain; CT, carboxyl-terminal domain. B, immunoblotting (IB) of recombinant hSPT5 proteins with anti-His antibody. 30–50 ng of histidine-tagged recombinant hSPT5 proteins were resolved via a 4–20% SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-His antibody. C, in vitro P-TEFb (CDK9-cyclin T) assay with various hSPT5 recombinant proteins. 10 ng of P-TEFb kinase were mixed with indicated hSPT5 proteins during the in vitro kinase assay. Positions of phosphorylated CDK9 and cyclin T are marked with arrowheads. D, phosphoamino acid analysis of wild type hSPT5. Phosphorylated full-length hSPT5 proteins were obtained by an in vitro kinase assay with P-TEFb and then analyzed by phosphoamino acid analysis. Positions of phosphoserine (P-S), phosphothreonine (P-T), and free phosphate (Pi) are marked. E, two-dimensional phosphopeptide mapping. Recombinant FL and CTR of hSPT5 proteins were phosphorylated by P-TEFb as in C. Phosphorylated proteins were digested with trypsin (20 units), eluted, and then loaded for TLC.
1, 2, 5, and 6). As expected, CDK9DN had no kinase activity, whereas CDK9TA had approximately the same activity as CDK9WT. (Note that in Fig. 3A a lower amount of CDK9TA than CDK9WT was used in the reactions shown in lanes 3 and 7.) In addition, phosphorylation of CDK9 was detected in CDK9WT and CDK9-cyclin T assays (Fig. 3C, lanes 1, 2, 5, and 6), but it was not detected when CDK9TA was tested. This shows that 1) the CDK9 kinase can undergo autophosphorylation and 2) the primary site of autophosphorylation of CDK9 under these conditions is at Thr-186. In addition, these data indicate that the kinase activity of CDK9 is not strongly dependent upon phosphorylation of the threonine residue in its T-loop.

**CDK9 Kinase Activity and Phosphorylation**—Although threonine 186 of CDK9 is dispensable for its kinase activity, it is important to determine whether this conserved threonine of CDK9 could be a substrate for phosphorylation by CAK, which could modulate its kinase activity. Two different approaches were used to test the possibility that CAK could phosphorylate and thus activate CDK9-cyclin T. First, CDK9WT and mutants including CDK9TA and CDK9DN were tested as substrates for CAK. As shown in Fig. 4A, phosphorylation of CDK9WT but not CDK9TA or CDK9DN was observed in the presence of CAK (CDK7-cyclin H). The lack of phosphorylation of CDK9DN or CDK9TA was not due to the loss of CAK activity because CAK could actively phosphorylate histone H1 (Fig. 4A, lane 2). Therefore, threonine 186 of CDK9 is apparently not a substrate site of CAK, because CDK9DN, which contains this threonine, was not phosphorylated by CAK. Second, the CDK9-cyclin T complex was tested as a substrate instead of a CDK9 monomer in a CAK assay because the associated cyclin might induce a structural change in the T-loop. In parallel, CDK2-cyclin A was used as a positive control for the CAK assay. Confirming previous results, CDK2-cyclin A has negligible ability to autophosphorylate, whereas the level of phosphorylation of CDK2-cyclin A was increased about 68-fold in the presence of CAK (Fig. 4, B and C). Unlike CDK2-cyclin A, phosphorylation of the CDK9 subunit of the CDK9-cyclin T complex was not increased by CAK (Fig. 4, B and C). These observations strongly suggest that CD9 is not phosphorylated by CAK at either threonine
The phosphorylation of CDK9 appears to be due to its autokinase activity. We also tested whether P-TEFb kinase activity might be cooperatively regulated by CAK. In this case, P-TEFb kinase activities were examined using two substrates, hSPT5 and GST-CTD, in the absence or presence of CAK. As shown in Fig. 5, A and B, CDK2-cyclin A kinase activity was greatly enhanced by CAK (105-fold or more), whereas the P-TEFb kinase activity for either substrate hSPT5 or GST-CTD was barely changed by CAK. When different CDK substrates (GST-CTD (a common substrate of P-TEFb, CAK, and CDK2-cyclin A) and hSPT5 (a P-TEFb-specific substrate)) were compared, each of these kinase combinations showed a similar pattern of substrate phosphorylation. We conclude that the CDK9 kinase activity of P-TEFb is independent of CAK.

Phosphorylation of CDK9 and Kinase Activity—CDK9 is autophosphorylated on Thr-186 in the T-loop. To test whether this autophosphorylation further activated the kinase, CDK9 was preincubated with ATP to form phosphorylated CDK9. Then the kinase activities of CDK9 with or without preincubation were compared. As shown in Fig. 6, the degree of phosphorylation of CDK9 was increased in an ATP preincubation-dependent manner (Fig. 6, lanes 2, 4, 6, and 8). However, the phosphorylation of hSPT5 was not increased by preincubation of CDK9. This observation is consistent with Fig. 3, which showed that CDK9TA had kinase activity. Thus,
these data suggest that the phosphorylation of CDK9 is not a critical post-translational modification needed for kinase activity.

**DISCUSSION**

The results presented indicate that P-TEFb, composed of CDK9 and cyclin T, preferentially phosphorylates hSPT5 as compared with the CTD of RNA Pol II in vitro. Furthermore, the CDK9 kinase is not regulated by CAK even though it contains a conserved threonine common to many CAK-activated CDKs. These results suggest that P-TEFb in a CAK-independent manner could activate Tat-dependent elongation by phosphorylating hSPT5 as well as the CTD of RNA Pol II.

The CDK7 kinase of TFIIH probably has at least two distinct functions in cells. Several studies indicate that CDK7 regulates RNA Pol II activity by phosphorylation of its CTD. In addition, because CDK7 phosphorylates the conserved threonine of CDK2 and other CDKs, it is also thought to be a regulator of the cell cycle machinery (48). A functional role for CDK7 in Tat-stimulated elongation is still controversial. Several studies with CAK inhibitors support the idea that CDK7-cyclin H is also important for Tat activation (38, 39). However, there is accumulating evidence that P-TEFb is the major kinase involved in Tat-activated transcription (12, 16). Recently, the physical association of elongating RNA Pol II with P-TEFb, but not with CAK, provided strong evidence for the primacy of P-TEFb in Tat-dependent elongation (17).

We have tested whether CAK phosphorylation of the CDK9 component of P-TEFb would modulate the activity of this kinase. This does not appear to be the case, because CDK9 phosphorylation did not increase by incubation with CAK, and the kinase activity of CDK9 was not enhanced by CAK. The finding that a catalytically inactive mutant of CKD9, which is defective for autophosphorylation, was not detectably phosphorylated by CAK is the most direct evidence for the former. CDK9 autophosphorylation was observed with the WT kinase and was inactivated by mutation of threonine 186 to alanine. This change did not alter the kinase activity of P-TEFb for either CTD or SPT5. The observation that the threonine 186 to alanine mutant protein is not autophosphorylated appears to conflict with results reporting that CDK9 will autophosphorylate its carboxyl terminus and that this modification is important for binding to TAR (57, 58). The explanation may be that the reactions in this study contained lower concentrations of the CDK9 kinase and were incubated for much briefer periods of time than the previous work. We tested whether CAK-partially phosphorylated CTD of RNA Pol II was a preferred substrate for P-TEFb. However, this was not the case, because phosphorylation of GST-CTD by P-TEFb alone was not significantly different from P-TEFb plus CAK. Taken together, it is likely that CAK and P-TEFb work in an independent manner in RNA Pol II-dependent transcription.

The kinase activity of P-TEFb is important for CTD phosphorylation of RNA Pol II, which correlates with efficient elongation (9, 11). DSIF, composed of hSPT4 and hSPT5, was originally purified on the basis of rendering transcription in vitro dependent upon P-TEFb and thus inhibitable by DRB (31–33). Furthermore, a newly identified negative elongation factor complex works in conjunction with DSIF to stall elongation of RNA Pol II shortly after initiation (34). Extensive studies by Handa and coworkers (32–34) suggest that P-TEFb stimulates RNA Pol II elongation from this position by CTD phosphorylation. Thus, the kinase activity of P-TEFb alleviates the repression activity of DSIF at an early stage of elongation (32).

Many studies have also shown that P-TEFb is critical for HIV-1 Tat-activated transcription elongation (12). For example, the sensitivity to inhibition by the drug DB8 and other compounds of Tat activation of transcription in vitro corresponds to the sensitivity of the kinase activity of P-TEFb but not of other known CDKs (10). DSIF is also important for Tat activation of elongation, as shown by depleting reactions in vitro of this complex. Other evidence that Spt5 functions in a positive mode in elongation is the phenotype of mutations in this gene in yeast. Loss of function mutants can have a decreased efficiency of elongation (35).

Phosphorylation of SPT5 by P-TEFb could be important in Tat activation of elongation. This kinase preferentially phosphorylates the repetitive carboxyl domain of SPT5, which is necessary to observe Tat activation in vitro (56). The opposing roles of SPT5 in elongation, inhibition, and stimulation could reflect roles in two processes. In the promoter proximal process, SPT4 and 5 (DSIF) stalls polymerase elongation, which is relieved by phosphorylation by P-TEFb probably of the CTD of Pol II (32). This process has been well documented in vitro for many promoters. A second process, which for HIV is Tat-mediated and dependent upon TAR, probably forms after polymerase has elongated beyond the TAR element. This process would depend upon P-TEFb, bound to TAR in the presence of Tat, interacting with SPT5, probably by phosphorylation, to stimulate elongation over long segments of the template. The existence of two P-TEFb-dependent processes would be consistent with the results of Fong and Zhou (58) and would explain why cyclin T from sources other than humans can function in general transcription from the HIV promoter, but only human cyclin T can function in Tat-dependent stimulation of transcription (14). Finally, recent evidence showing that SPT5 and SPT6 are associated with elongating polymerase, whereas SPT4 and SPT5 (as DSIF) are adequate for promoter proximal events, further supports two roles for SPT5 (59, 60).

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