【SIII-3】

Regulation of HIV-1 Tat-dependent Transcription Elongation by Tat-SF1, DSIF and P-TEFb

Jiyoung Park, Kang Ho Kim and Jae Bum Kim
School of Biological Sciences, Seoul National University, Seoul, Korea

Introduction

The efficient transcription of the full length proviral 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 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 which 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 &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 C-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 TFIIF (RAP30), P-TEFb, hSPT5 and RNA Pol II which is thought to mediate Tat-dependent activation *in vitro* (27-30).

DSIF (hSPT4 & 5) is also an essential factor for Tat-activation of elongation (27, 29, 30). DSIF forms a protein complex with NELF (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). Nuclear extracts depleted of hSPT5 did not support Tat-dependent elongation *in vitro* (27, 29, 30). Additionally, overexpression of hSPT5 stimulates Tat-specific activation *in vivo* (27). In yeast, SPT5 probably directly associates with elongating RNA Pol II complex and increases the efficiency of elongation (35).

Several studies have also implicated TFIIH in Tat activation (36-39). TFIIH is a general transcription factor (GTF) which 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 cyclin H and MAT1 to form CAK (CDK-activating kinase) and phosphorylates the CTD of RNA Pol II (41-44). Recent genetic and biochemical evidence strongly suggests that this phosphorylation of the CTD is important for CTD's interaction with capping enzymes that modify the nascent transcript (45-47). Surprisingly, these results also showed that the phosphorylation state of the CTD is significantly altered as the polymerase continues to elongate. The CAK, CDK7/cyclin H, is also known to specifically phosphorylate a threonine residue in the T-loop of other CDKs (48, 49). This phosphorylation stimulates the activity of these CDK kinases.

In this study, we have shown that P-TEFb prefers hSPT5 as a substrate as compared to the CTD of RNA Pol II. Unlike other CDKs, CDK9 is not phosphorylated by CAK but undergoes autophosphorylation. These results suggest that P-TEFb mediates RNA Pol II CTD phosphorylation independent of CAK activity during stimulation of elongation.

Results

1.P-TEFb Phosphorylates hSPT5 and the CTD of RNA Pol II.

DRB inhibits transcription at the stage of elongation *in vivo*. During transcription *in vitro* , DRB also selectively inhibits elongation as compared to initiation probably by suppressing kinase activities such as those responsible for CTD hyperphosphorylation. 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. 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-CTD was used as substrate, the IC_{50} of DRB was estimated as 2.5 mM and 20 mM 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. This observation is

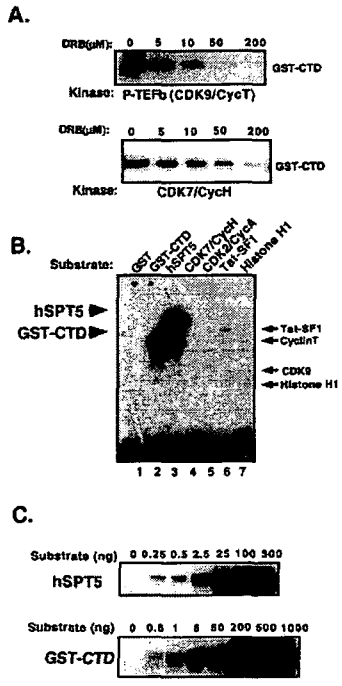

그림 1

also consistent with the inhibition of P-TEFb controlling the DRB-sensitive elongation process both *in vivo* and *in vitro*. Approximately 2-5 mM of DRB yields a 50% decreased in transcription activity.

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 value for P-TEFb (Fig. 1C). The calculated values for hSPT5 and GST-CTD were 18 nM and 55 nM, respectively. Moreover, since the GST-CTD contains 52 YSPTPSP repeats, each of which is a potential site of phosphorylation, while hSPT5 probably contains fewer potential sites, these Km values probably underestimate the preference of hSPT5 as a substrate.

2. P-TEFb Phosphorylates the C-terminus of hSPT5

In order to determine the preferential phosphorylation domains of hSPT5 by P-TEFb, hSPT5 was divided into the following three domains: N-terminus domain (NT), which is rich in acidic amino acid; middle domain (MD), which contains KOW motifs similar to those of *E. coli* NusG proteins; C-terminus domain (CT), which contains CTR heptapeptide repeats (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 CT of hSPT5 rather than the NT or MD domains although a low level of phosphorylation of the MD was detected. The CTR domain contains multiple serine and threonine residues which could be potential phosphorylation

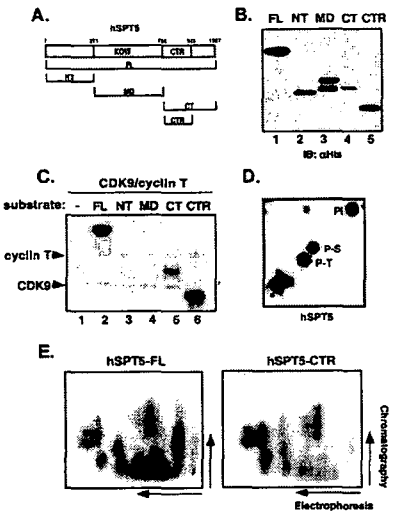

그림 2

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, while 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 full length (FL) and CTR proteins. FL and CTR phosphopeptides produced by digestion with trypsin, were resolved on TLC plates. Most of the separated phosphopeptides comigrated with those of the CTR proteins of the FL protein suggesting that the high affinity phosphorylation sites of hSPT5 are in the CTR domain.

3. 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. 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 which 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, the aspartic acid 167 of CDK9 was mutated to asparagine (CDK9DN) which abrogates its kinase activity. These CDK9 mutants were expressed in insect cells by use of the baculovirus system and purified. The kinase activity of CDK9 was enhanced 4 to 5 fold when bound by cyclin T (Fig. 3B lanes 1,2,5 and 6). As expected, CDK9DN had no kinase activity while CDK9TA had approximately the same activity as CDK9WT. 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: first the CDK9 kinase can undergo autophosphorylation, second, the sole site of autophosphorylation of CDK9 is at the Thr186, and third, these data indicate that the kinase activity of CDK9 is not strongly dependent upon phosphorylation of the threonine residue in its T-loop.

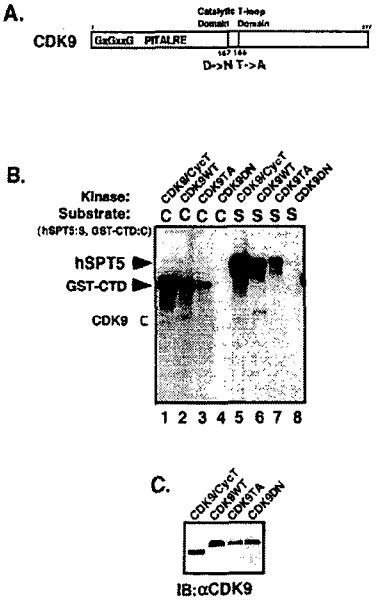

그림 3

4. 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 CDK9 mutants, including CDK9TA and CDK9DN, were tested as substrates for the CAK kinase. 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 kinase activity since CAK could actively phosphorylate histone H1 (Fig. 4A, lane 2). Therefore, threonine 186 of CDK9 is apparently not a substrate site of CAK since 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 kinase assay since 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 kinase 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. 4B 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 CDK9 is not phosphorylated by CAK at either threonine 186 or any other residue(s). The phosphorylation of CDK9 appears to be due to its autokinase activity.

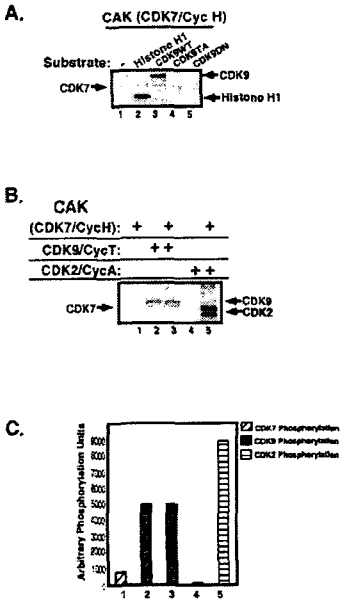

그림 4

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 5A and B, CDK2/cyclin A kinase activity was greatly enhanced by CAK (105 fold or more) while the P-TEFb kinase activity for either substrate hSPT5 or GST-CTD was barely changed by CAK. When different CDK kinase substrates (GST-CTD-a common substrate of

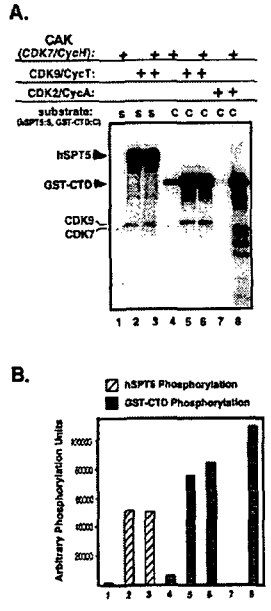

그림 5

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.

5. Phosphorylation of CDK9 and Kinase Activity

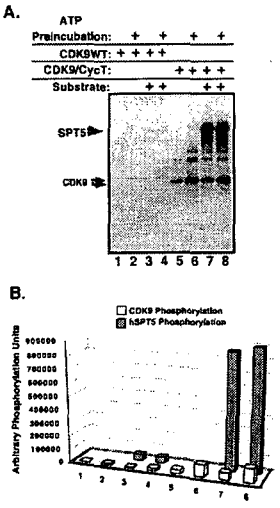

그림 6

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. 6A lanes 2,4,6 and 8). However, the phosphorylation of hSPT5 was not increased by preincubation of CDK9 (Fig. 6A and B). 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.

References

1. Cullen, B. R. (1993) *Cell* **93,** 685-692
2. Emerman, M., and Malim, M. H. (1998) *Science* **280,** 1880-1884
3. Jones, K. A. (1997) *Genes Dev* **11,** 2593-2599
4. Feng, S., and Holland, E. C. (1988) *Nature* **334,** 165-167
5. Ratnasabapathy, R., Sheldon, M., Johal, L., and Hernandez, N. (1990) *Genes Dev.* **4,** 2061-2074
6. Marciniak, R. A., Calnan, B. J., Frankel, A. D., and Sharp, P. A. (1990) *Cell* **63,** 791-802
7. Marciniak, R. A., and Sharp, P. A. (1991) *Embo J.* **10,** 4189-4196
8. Marshall, N. F., Peng, J., Xie, Z., and Price, D. H. (1996) *J. Biol. Chem.* **271,** 27176-27183
9. Marshall, N. F., and Price, D. H. (1995) *J. Biol. Chem.* **270,** 12335-12338
10. Mancebo, H. S., Lee, G., Flygare, J., Tomassini, J., Luu, P., Zhu, Y., Peng, J., Blau, C., Hazuda, D., Price, D., and Flores, O. (1997) *Genes Dev.* **11,** 2633-2644
11. Zhu, Y., Pe'ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M. B., and Price, D. H. (1997) *Genes Dev.* **11,** 2622-2632
12. Price, D. H. (2000) *Mol. Cell. Biol.* **20,** 2629-2634
13. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998) *Cell* **92,** 451-462
14. Peng, J., Zhu, Y., Milton, J. T., and Price, D. H. (1998) *Genes Dev.* **12,** 755-762
15. Peng, J., Marshall, N. F., and Price, D. H. (1998) *J. Biol. Chem.* **273,** 13855-13860
16. Chen, D., and Zhou, Q. (1999) *Mol. Cell. Biol.* **19,** 2863-2871
17. Ping, Y. H., and Rana, T. M. (1999) *J. Biol. Chem.* **274,** 7399-7404
18. Isel, C., and Karn, J. (1999) *J. Mol. Biol.* **290,** 929-941
19. Garber, M. E., Wei, P., KewalRamani, V. N., Mayall, T. P., Hermann, C. H., Rice, A. P., Littman, D.

R., and Jones, K. A. (1998) *Genes Dev.* **12,** 3512-3527
20. Bieniasz, P. D., Grdina, T. A., Bogerd, H. P., and Cullen, B. R. (1998) *Embo J.* **17,** 7056-7065
21. Fujinaga, K., Taube, R., Wimmer, J., Cujec, T. P., and Peterlin, B. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1285-1290
22. Chen, D., Fong, Y., and Zhou, Q. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2728-2733
23. Kwak, Y. T., Ivanov, D., Guo, J., Nee, E., and Gaynor, R. B. (1999) *J. Mol. Biol.* **288,** 57-69
24. Zhou, Q., Chen, D., Pierstorff, E., and Luo, K. (1998) *Embo J.* **17,** 3681-3691
25. Zhou, Q., and Sharp, P. A. (1995) *Embo J.* **14,** 321-328
26. Zhou, Q., and Sharp, P. A. (1996) *Science* **274,** 605-610
27. Kim, J. B., Yamaguchi, Y., Wada, T., Handa, H., and Sharp, P. A. (1999) *Mol. Cell. Biol.* **19,** 5960-5968
28. Li, X. Y., and Green, M. R. (1998) *Genes Dev.* **12,** 2992-2996
29. Wu-Baer, F., Lane, W. S., and Gaynor, R. B. (1998) *J. Mol. Biol.* **277,** 179-197
30. Parada, C. A., and Roeder, R. G. (1999) *Embo J.* **18,** 3688-3701
31. Wada, T., Takagi, T., Yamaguchi, Y., Ferdous, A., Imai, T., Hirose, S., Sugimoto, S., Yano, K., Hartzog, G. A., Winston, F., Buratowski, S., and Handa, H. (1998) *Genes Dev.* **12,** 343-356
32. Wada, T., Takagi, T., Yamaguchi, Y., Watanabe, D., and Handa, H. (1998) *Embo J.* **17,** 7395-7403
33. Yamaguchi, Y., Wada, T., Watanabe, D., Takagi, T., Hasegawa, J., and Handa, H. (1999) *J. Biol. Chem.* **274,** 8085-8092
34. Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999) *Cell* **97,** 41-51
35. Hartzog, G. A., Wada, T., Handa, H., and Winston, F. (1998) *Genes Dev.* **12,** 357-369
36. Garcia-Martinez, L. F., Mavankal, G., Neveu, J. M., Lane, W. S., Ivanov, D., and Gaynor, R. B. (1997) *Embo J.* **16,** 2836-2850
37. Parada, C. A., and Roeder, R. G. (1996) *Nature* **384,** 375-378
38. Yankulov, K., Yamashita, K., Roy, R., Egly, J. M., and Bently, D. L. (1995) *J. Biol. Chem.* **270,** 23922-23925
39. Cujec, T. P., Okamoto, H., Fujinaga, K., Meyer, J., Chamberlin, H., Morgan, D. O., and Peterlin, B. M. (1997) *Genes Dev.* **11,** 2645-2657
40. Holstege, F. C. P., and Young, R. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2-4
41. Svejstrup, J. Q., Vichi, P., and Egly, J. M. (1996) *Trends Biochem. Sci.* **20,** 346-350
42. Hoeijmakers, J. H. J., Egly, J. M., and Vermeulen, W. (1996) *Curr. Opin. Genet. Dev.* **6,** 26-33
43. Hampsey, M. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 465-503
44. Tirode, F., Busso, D., Coin, F., and Egly, J.-M. (1999) *Mol. Cell* **3,** 87-95
45. Cho, E. J., Takagi, T., Moore, C. R., and Buratowski, S. (1997) *Genes Dev.* **11,** 3319-3126
46. Rodriguez, C. R., Cho, E. J., Keogh, M. C., Moore, C. L., Greenleaf, A. L., and Buratowski, S. (2000) *Mol. Cell. Biol.* **20,** 104-112
47. Hirose, Y. and Manley, J. L. (2000) *Genes Dev.* **14,** 1415-1429
48. Morgan, D. O. (1995) *Nature* **374,** 131-134
49. Dynlacht, B. D. (1997) *Nature* **389,** 149-152
50. Kamps, M. P., and Sefton, B. M. (1989) *Anal. Biochem.* **176,** 22-27
51. van der Geer, P., and Hunter, T. (1994) *Electrophoresis* **15,** 544-554
52. Sehgal, P. B., Darnell, J. E., and Tamm, I. (1976) *Cell* **9,** 473-480
53. Tamm, I., Hand, R., and Caliguiri, L. A. (1976) *J. Cell. Biol.* **69,** 229-240
54. Egyhazi, E. (1974) *J. Mol. Biol.* **84,** 173-183
55. Chodosh, L. A., Fire, A., Samuels, M., and Sharp, P. A. (1989) *J. Biol. Chem.* **264,** 2250-2257