Regulation of Carboxyl-terminal Domain Phosphatase by HIV-1 Tat Protein*

(Received for publication, September 11, 1998, and in revised form, September 28, 1998)

Nicholas F. Marshall, Grace K. Dahmus, and Michael E. Dahmus‡

From the Section of Molecular and Cellular Biology, Division of Biological Sciences, University of California, Davis, California 95616

The phosphorylation state of the carboxyl-terminal domain (CTD) of RNA polymerase (RNAP) II is directly linked to the phase of transcription being carried out by the polymerase. Enzymes that affect CTD phosphorylation can thus play a major role in the regulation of transcription. A previously characterized HeLa CTD phosphatase has been shown to processively dephosphorylate RNAP II and to be stimulated by the 74-kDa subunit of TFIIF. This phosphatase is shown to be comprised of a single 150-kDa subunit by the reconstitution of catalytic activity from a SDS-polyacrylamide gel electrophoresis purified protein. This subunit has been previously cloned and shown to interact with the HIV Tat protein. To determine whether this interaction has functional consequences, the effect of Tat on CTD phosphatase was investigated. Full-length Tat-1 protein (Tat 86R) strongly inhibits the activity of CTD phosphatase. Point mutations in the activation domain of Tat 86R, which reduce the ability of Tat to transactivate in vivo, diminish its ability to inhibit CTD phosphatase. Furthermore, a deletion mutant missing most of the activation domain is unable to inhibit CTD phosphatase activity. The ability of Tat to transactivate in vitro also correlates with the strength of inhibition of CTD phosphatase. These results are consistent with the hypothesis that Tat-dependent suppression of CTD phosphatase is part of the transactivation function of Tat.

The largest subunit of RNA polymerase (RNAP) II from all eukaryotes contains at its carboxyl terminus varying numbers of repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Each cycle of transcription involves the reversible phosphorylation of this carboxyl-terminal domain (CTD) (1). RNAP II with an unphosphorylated CTD is designated RNAP IIA, and polymerase with a highly phosphorylated CTD is designated RNAP IIO. The activity of many key regulatory factors appears to be mediated by the CTD although its role in transcription is not clearly defined. An understanding of transcriptional regulation is dependent on elucidating the function of the CTD and the mechanisms and regulation of the enzymes that modify it.

Because RNAPs IIA and IIO have distinct roles in the transcription cycle, the activities of enzymes that phosphorylate and dephosphorylate the CTD must be tightly regulated. RNAP IIA is efficiently recruited to promoters during the assembly of a preinitiation complex (2–6) whereas RNAP IIO is associated with the elongation complex (7, 8). Therefore, at some point subsequent to the binding of RNAP II to the promoter and likely before RNAP II has cleared the promoter, the CTD is phosphorylated by CTD kinase(s) (2). After completion of a nascent transcript, RNAP IIA must be regenerated by CTD phosphatase. An enzyme capable of selectively dephosphorylating the CTD has been described (9–12).

CTD phosphatases and kinases can be inhibitory or stimulatory to transcription depending on the phase of the transcription cycle during which they act. According to present models, the phosphorylation of RNAP II in a preinitiation complex stimulates transcription whereas the phosphorylation of free RNAP IIA inhibits transcription by diminishing the pool of RNAP II capable of being recruited to the promoter. Conversely, CTD phosphatase acting on free RNAP IIO increases the pool of RNAP IIA available for initiation thereby acting as an activator of transcription, whereas CTD phosphatase acting on RNAP IIO in an elongation complex would likely result in a paused or stalled polymerase (13).

CTD phosphatase is specific for RNAP IIO and processively removes phosphates from the CTD to generate RNAP IIA. Furthermore, CTD phosphatase appears to interact directly with a site on RNAP II that is distinct from the CTD and is regulated by several of the general transcription factors (10). Specifically, the RAP74 subunit of TFIIF stimulates CTD phosphatase as much as 20-fold while TFIIB inhibits the IIF-dephosphorytase as much as 20-fold while TFIIB inhibits the IIF-dependent stimulation but not the basal activity of the enzyme. The analysis of deletion constructs of RAP74 indicates that the carboxyl terminus and part of the conserved amino-terminal domain are required for maximal stimulation of CTD phosphatase (10).

The HIV-1 transcriptional activator, Tat, increases gene expression by increasing the processivity of RNAP II (14–17). The increase in processivity correlates with an increased level of CTD phosphorylation (18). Accordingly, both the CTD and CTD kinases have been implicated in Tat function (18–24). Because an increased level of CTD phosphorylation can result from either an activation of CTD kinase(s) or an inhibition of CTD phosphatase(s), CTD phosphatase is a potential Tat target. Recently, a two-hybrid screen using RAP74 as bait resulted in the isolation of the mammalian FCP1 cDNA (25). FCP1 was shown to be a subunit of the previously described CTD phosphatase and was found to interact with Tat (9, 25). Therefore, Tat has the ability to interact with proteins that can either phosphorylate or dephosphorylate the CTD.

This report establishes that the 150-kDa polypeptide (FCP1)
corresponds to the catalytic subunit of CTD phosphatase. Tat directly inhibits the activity of CTD phosphatase, and mutants of Tat that are defective in transactivation in vivo are defective in their ability to inhibit CTD phosphatase in vitro. The ability of Tat to regulate both basal and RAP74-stimulated CTD phosphatase activities suggests that the control of transcription from the HIV long terminal repeat is in part mediated by CTD phosphatase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mono Q, HiTrap columns (heparin, NHS-activated, SP, Q), and radiolabeled [γ-32P]ATP (800 Ci/mmol) were purchased from Amersham Pharmacia Biotech. The DEAE-15HR column is from Waters. [γ-32P]ATP (3000 Ci/mmol) was obtained from NEN Life Science Products. Human recombinant casein kinase II is from UBI. Glutathione-agarose and human thrombin were purchased from Sigma. SYPRO Red Lysysin was purchased from Promega. RNase T1 was purchased from U. S. Biochemical Corp. Transcription templates TAR-G400 and ΔTAR-G100 were provided by P. A. Sharp (Massachusetts Institute of Technology). Expression vectors for GST-Tat fusion proteins were obtained from the NIH AIDS Research and Reference Reagent Program.

**Purification of CTD Phosphatase**—Human CTD phosphatase was purified from HeLa cells as described by Chambers and Dahmus (9) with several modifications. The purification was begun with 2 × 10^11 cells. HiTrap heparin columns (5 ml, three in series) were used in place of heparin-Sepharose resin, and a DEAE-15HR (20 × 100 mm) column was used in place of DE52 resin. The Resource Q column was replaced by three 5-ml HiTrap Q columns run in series. The phenyl-Superose and Superose 12 steps were replaced by a 5-ml HiTrap NHS-activated column that had been coupled with a 1-ml Mono Q column. Renaturation of Polypeptides from SDS-PAGE—Proteins purified by SDS-PAGE were renatured as described by Conaway et al. (26).

**Expression and Purification of GST-Tat Fusion Proteins**—GST-Tat and mutant proteins were expressed and purified as described previously (27). Tat proteins were recovered from glutathione-agarose by thrombin cleavage of the bound GST-fusion protein. Tat was further purified and renatured by adjusting to 400 mM KCl, loading onto 1-ml HiTrap heparin columns in TGED buffer (50 mM Tris-HCl, pH 7.9, 20% glycerol, 0.1 mM EDTA, 5 mM DTT) containing 400 mM KCl followed by step elution with TGED containing 1× KCl. For Tat and Tat mutants, samples of the peak HiTrap fractions were dialyzed against TGED containing 10× KCl until the KCl concentration was less than 25 mM as determined by conductivity. Tat proteins were quantitated by silver staining of bands in SDS-PAGE with SYPRO Red stain. RNase T1 was from FMC BioProducts. RNase T1 was purchased from Promega. RNase T1 was labeled with [32P]ATP (3000 Ci/mol) and lies outside the consensus repeat, dephosphorylated by CTD phosphatase activity (20 µl) and for protein (60 µl) by SDS-PAGE on a 5–17.5% polyacrylamide gel. The gel was sliced into 3-mm sections. Proteins were eluted and renatured as described under “Experimental Procedures.” Aliquots of each fraction were assayed for CTD phosphatase activity (20 µl) and for protein (60 µl) by SDS-PAGE on a 5–17.5% gel. A, CTD phosphatase activity. Lanes 1 and 11 contain marker RNAP IIA. The positions of subunits Ila and Ilo are as indicated. B, silver-stained SDS-PAGE. Lane 1 contains 30 units of input CTD phosphatase; lanes 2–6 contain aliquots of fractions 8–12, respectively. The position of M\(_{r}\) markers (× 10^3) are indicated on the left.

**RESULTS**

The 150-kDa Polypeptide Is Necessary and Sufficient for CTD Phosphatase Activity—Initial purification of human CTD phosphatase yielded an active fraction with several polypeptides as putative subunits (9). The 150-kDa polypeptide has recently been shown to be equivalent to the RAP74 interacting protein FCP1 (28). The finding that a polyclonal antibody directed against FCP1 precipitates CTD phosphatase activity suggests that the 150-kDa protein is an essential subunit. However, the 150-kDa subunit contains no recognizable phosphatase motif (12, 25). To determine which polypeptides present in the CTD phosphatase-containing fraction are required for activity, highly purified CTD phosphatase was denatured and fractionated by SDS-PAGE. Each of 34 gel slices was extracted for protein and renatured as described by Conaway et al. (26). Fractions were assayed for CTD phosphatase activity in the presence of RAP74. CTD phosphatase activity was recovered only in fractions 10 and 11 (Fig. 1A, lanes 7 and 8). The processive dephosphorylation of RNAP IIO is characteristic of CTD phosphatase and is preserved in the renatured enzyme. Based on PhosphorImager quantitation of the input and renatured activity, approximately 0.1% of the input CTD phosphatase activity was recovered.

The polypeptides recovered from gel slices were re-run on SDS-PAGE, silver-stained, and are shown in Fig. 1B. Fractions containing CTD phosphatase activity correspond to those fractions containing the 150-kDa subunit. A sample of the input CTD phosphatase is shown in Fig. 1B, lane 1. Accordingly, the
Tat regulates CTD Phosphatase Activity—The basal transcription factors TFIIF and TFIIIB have previously been shown to regulate the activity of CTD phosphatase in vitro (10). The finding that the 150-kDa subunit (FCP1) interacts with the HIV-1 transcriptional regulator, Tat, suggests that Tat might also regulate CTD phosphatase activity (25). To test this hypothesis, purified recombinant Tat 86R (wt) was examined for its ability to influence CTD phosphatase activity in the standard RAP74-dependent CTD phosphatase assay. For increased sensitivity, CTD phosphatase was titrated to the point where approximately 50% of the input RNAP IIO was dephosphorylated during the course of the reaction (Fig. 2A, lane 2). The addition of increasing amounts of Tat 86R resulted in inhibition of CTD phosphatase activity.

To determine the specificity of this inhibition and to define domains of Tat required for the regulation of CTD phosphatase activity, three mutant Tat proteins were assayed (Fig. 2, B–D). C22G, P18IS, and D2/36 are, respectively, a substitution of one critical cysteine, an insertion of a single amino acid in the activation domain, and a nearly complete deletion of the activation domain. All mutations are in the wild type (86R) background. Both C22G and P18IS are partially effective in inhibiting the activity of CTD phosphatase. Relative to wild type Tat, an approximately three times higher concentration of C22G and P18IS is required for 50% inhibition (Fig. 2B). The presence of D2/36, even at very high concentrations, does not result in significant inhibition of CTD phosphatase. These results suggest that the activation domain of Tat is required for CTD phosphatase inhibition, but a completely wild type domain is not strictly required.

Tat Inhibition of CTD Phosphatase Correlates with Transcriptional Transactivation—The three mutants tested in Fig. 2 have been defined as transactivation-deficient in vivo, but at least two of them retain some ability to inhibit CTD phosphatase in vitro. Wild type Tat and the mutants described above were assayed in parallel for their ability to transactivate in vitro and inhibit CTD phosphatase activity (Fig. 3, A and B). Results were quantified and are presented in Fig. 3C. Tat 86R supports a 5-fold transactivation and, as reported above, is also the most effective in inhibiting CTD phosphatase activity. P18IS shows a reduced but parallel effect on both transactivation and inhibition of CTD phosphatase. C22G is modestly inhibitory but does not transactivate, whereas D2/36 is ineffective in either assay. These results show a parallel between the ability to support transactivation and the inhibition of CTD phosphatase for Tat 86R and mutant Tat proteins.

All CTD phosphatase reactions described to this point were carried out in the presence of RAP74. To determine whether the effect of Tat is in part mediated by RAP74, the effect of Tat on CTD phosphatase activity was determined in the absence of RAP74. Tat 86R and the mutants were titrated as in Fig. 2, A–D. The pattern of inhibition in the absence of RAP74 is similar to that in its presence (data not shown). A reproducible stimulation of CTD phosphatase activity was observed at low concentrations of 86R and P18IS (data not shown). Approximately 5-fold more Tat is required to inhibit CTD phosphatase to a comparable extent in the absence of RAP74. This is expected in that RAP74-independent reactions contained about 40-fold more CTD phosphatase than those in Fig. 2.

DISCUSSION

These studies establish that human CTD phosphatase is comprised of a single subunit of molecular size 150 kDa. The recovery of CTD phosphatase activity from renatured SDS-PAGE-purified 150-kDa (FCP1) polypeptide, in the presence of recombinant RAP74 and highly purified RNAP IIO, supports the idea that CTD phosphatase is comprised of a single subunit. The fact that FCP1 contains no recognizable phosphatase motifs suggests that CTD phosphatase is a novel protein phosphatase. Alternatively, the 150-kDa protein might be a regulatory subunit that is essential for activation of a catalytic
subunit that resides elsewhere. Because RAP74 is recombinant and not essential for CTD phosphatase activity, the only fraction that might contribute required protein(s) is the substrate, RNAP IIO. The observation that normal CTD phosphatase activity can be demonstrated with labeled RNAP IIO as the substrate, RNAP IIO. The observation that RNAP II catalyzes transcript elongation. Indeed, increasing evidence supports the idea that the phosphorylated CTD plays an essential role in processive elongation (18, 20, 22, 39). Accordingly, dephosphorylation of RNAP IIO in an elongation complex will likely result in pausing and subsequent termination. Tat is known to increase gene expression by increasing the processivity of RNAP II (14–17). Furthermore, Tat leads to an increase in the ratio of RNAP IIO to IIA, an effect due in part to the activation of TFIIF kinase (18). Both TFIIF and P-TEFb have recently been directly implicated in mediating Tat transactivation (18, 22, 23, 40–42).

The results presented in this manuscript indicate that transactivation by Tat might also be mediated by the direct inhibition of CTD phosphatase activity. According to this hypothesis, initiation of transcription from the HIV long terminal repeat leads to early elongation complexes that are sensitive to CTD phosphatase. In the absence of Tat, CTD phosphatase dephosphorylates RNAP IIO, which leads to pausing and subsequent termination. In the presence of Tat, CTD phosphatase is inhibited, and the level of RNAP II phosphorylation is maintained resulting in processive elongation. Because Tat recruits P-TEFb, through interaction with cyclin T, the activation of CTD phosphorylation and suppression of CTD dephosphorylation may be coordinately regulated by Tat. Accordingly, during the phase of transcript elongation when RNAP II is sensitive to dephosphorylation, Tat shifts the equilibrium in favor of CTD phosphorylation by both inhibiting dephosphorylation and promoting phosphorylation. An understanding of the biochemical mechanisms that underlie this complex regulation is dependent on understanding the molecular interactions between the various components and whether or not the interaction of Tat with cyclin T and CTD phosphatase are mutually exclusive.

A corollary of this proposal is that RNAP II initiating transcription from different promoters may result in elongation complexes that are differentially sensitive to CTD phosphatase. Accordingly, it is important to understand the mechanisms by which CTD phosphatase is recruited to elongation complexes and how this interaction is regulated.

**Acknowledgments**—We thank Nandu Chatterjee and Alan Lehman for assistance in the purification of CTD phosphatase. We also thank Jack Greenblatt for helpful discussions early in the course of this work and Alan Lehman, Julia Munsch, and Patrick Lin for critical reading of this manuscript. In addition, we thank David Price for recombinant P-TEFb.

**REFERENCES**

1. Dahmus, M. E. (1996) *J. Biol. Chem.* 271, 19009–19012
2. Dahmus, M. E. (1994) *Prog. Nucleic Acids Res. Mol. Biol.* 48, 143–179
3. Lu, H., Flores, O., Weinmann, R., and Reinberg, D. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 950–954

—N. F. Marshall and M. E. Dahmus, unpublished observations.
