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A Human Primary T-Lymphocyte-Derived Human Immunodeficiency Virus Type 1 Tat-Associated Kinase Phosphorylates the C-Terminal Domain of RNA Polymerase II and Induces CAK Activity

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Tat protein mediates transactivation of human immunodeficiency virus type 1 (HIV-1), which results in more-efficient transcript elongation. Since phosphorylation of C-terminal domain (CTD) of RNA polymerase II correlates with its enhanced processivity, we studied the properties of a Tat-associated CTD kinase derived from mitogenically stimulated human primary T lymphocytes (TTK). TTK binds to full-length Tat and specifically phosphorylates CTD and CDK2. This dual kinase activity is characteristic of CDK-activating kinase (CAK). The CTD kinase activity is induced upon mitogenic stimulation of primary T lymphocytes. Fractionation of T-cell lysate demonstrates that Tat-associated CTD kinase activity elutes in two peaks. About 60% of Tat-associated CTD kinase copurifies with CDK2 kinase activity and contains the CAK components CDK7 and cyclin H. The rest of Tat-associated kinase is free of CDK2 kinase activity and the CAK components and thus may represent a novel CTD kinase. The kinase activities of TTK are blocked by the adenosine analog 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) as well as by the kinase inhibitor H8 at concentrations known to block transcript elongation. Importantly, the Tat-associated kinase markedly induced CAK. We suggest that the mechanism of Tat-mediated processive transcription of the HIV-1 promoter includes a Tat-associated CAK activator.

Transcriptional latency in human immunodeficiency virus type 1 (HIV-1) infected cells in the absence of Tat is characterized by short promoter-proximal transcripts. Late in infection or in response to Tat in transient transfection assays, there is a marked stimulation of full-length promoter-distal transcripts (1, 2, 4, 19). Highly processive transcription in response to Tat involving efficient initiation and elongation has been noted in a number of studies (11, 22, 25, 28). The proposed mechanisms envision Tat being able to tether to its *cis*-acting TAR RNA target site host cell factors that are required to promote efficient processive transcription (23, 42, 44–46). However, the nature of such a Tat cofactor and its mechanism of action remain unclear.

The carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II (Pol II) consists of heptapeptide repeats (Tyr-Ser-Pro-Tyr-Ser-Pro-Ser) which are known to be phosphorylated during transcriptional elongation (8, 9, 30, 46). Involvement of Pol II CTD phosphorylation in transcript elongation is supported by the fact that inhibitors of elongation also block CTD phosphorylation (6, 26, 27, 31, 42). A likely candidate for CTD kinase in vivo is the general transcription factor TFIIH-associated CDK-activating kinase (CAK [10, 36–39]). However, it is not clear whether the CAK activity associated with TFIIH is sufficient for regulating processive transcription of specific promoters in vivo or whether the TFIIH-associated kinase or a unique CTD kinase is induced in response to promoter-specific activators, such as Tat. Furthermore, under specific conditions, CAK activity is known to be regulated either by the phosphorylation of CDK7, the kinase subunit of CAK, or by the formation of a CDK7-cyclin H complex in response to the assembly factor MAT 1 (12, 13, 40). We suggest that the processive transcription of viral promoter in response to Tat is mediated in part by induction of CAK with a Tat-associated kinase.

With respect to the mechanism of Tat-mediated transcriptional elongation of the HIV-1 promoter, recent reports have identified the putative Tat cofactors which include transcription factors (17, 18, 21, 29, 32, 41, 45, 46). A CTD kinase activity that was isolated from HeLa cells based on its affinity for the activation domain of Tat (TAK kinase [17]) appears to be unique in that it bears no antigenic similarity to the TFIIH-associated CAK (18). On the other hand, the activation domain of Tat appears to be required for the stimulation of TFIIH-associated CTD kinase (32). It is still unclear whether the induction is a result of the association of TFIIH with Tat or is due to a unique Tat-associated CTD kinase. Another Tat-associated kinase that has not yet been characterized has been implicated in phosphorylation of a Tat-TAR RNA-specific factor that is required for transactivation (45).

We asked if the CTD kinase involved in processive transcription functions as an activator of CAK and whether it is distinct from the TFIIH-associated kinase. The Tat-associated, primary-T-cell-derived kinase (TTK) complex contains the CAK catalytic subunit CDK7, as well as a CTD kinase activity that is free of CDK7, cyclin H, and TFIIH. Both the CAK and the CTD kinase activities associated with the Tat-bound fraction are blocked by the adenosine analog 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) and the kinase inhibitor H8 at concentrations known to inhibit transcript elongation. Importantly, the TTK fraction markedly stimulated reconstituted CAK activity. When TTK was microinjected in primary human cells, it augmented Tat-mediated transcriptional transactivation of the HIV-1 promoter. We suggest that one of the mechanisms of Tat-mediated transcriptional transactivation of the

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HIV-1 promoter includes stimulation of processive transcription by induction of CDK7 kinase activity.

MATERIALS AND METHODS

Primary T lymphocytes from healthy donors were purified (90 to 95%) by countercurrent centrifugal elutriation. The T cells were activated for 24 h by treatment with 1-μg/ml phytohemagglutinin (PHA; Murex Diagnostics, Dartford, England) and 5-ng/ml phorbol myristate acetate (Sigma Chemical Company, St. Louis, Mo.). Activation of the T cells was characterized by flow cytometry (35). Mitogenically activated and unstimulated cells were lysed in 20 mM HEPES (pH 7.9) buffer containing 0.1% Nonidet P-40 (NP-40), 5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10-μg/ml aprotinin. The lysates were precleared by successive incubation with glutathione-agarose and glutathione *S*-transferase (GST)-agarose beads to remove proteins nonspecifically associated with agarose and GST. Tat expression plasmids (GST-Tat 86, GST-Tat 86 *dl*2-36, GST-Tat 72, GST-Tat 72 cys22, GST-Tat 48, and GST-Tat 48 cys22), which were a gift from John Brady (National Cancer Institute, National Institutes of Health), were expressed in *Escherichia coli* and purified on glutathione-agarose beads as described elsewhere (20). A Tat-associated CTD kinase was fractionated from the precleared lysate of activated T cells on GST-agarose beads containing full-length Tat protein (Tat 86 or Tat 72 [product of the first exon]). Approximately 100 μg of total protein was incubated with GST-Tat beads (15-μl suspension containing 1 μg of Tat protein) for 1 h at 4°C. After incubation, the beads were pelleted and washed three times with 100 μl of lysis buffer containing 250 mM NaCl, 1% NP-40, and 0.03% sodium dodecyl sulfate (SDS) to remove nonspecific proteins. The pellet was washed twice with the kinase buffer (50 mM HEPES [pH 7.9], 10 mM $MgCl_2$, 6 mM EGTA, 2.5 mM dithiothreitol) prior to the kinase reaction in 50 μl of buffer containing 10 μM ATP, 10 μCi of [γ-^{32}P]ATP (specific activity, 6,000 Ci/mmol), and 100 ng of a substrate. After 30 min of incubation at 30°C, unless otherwise indicated, the reactions were resolved on an SDS-4 to 20% gradient polyacrylamide gel electrophoresis (PAGE) gel and the phosphorylated substrates were visualized by autoradiography. Proteins present in the TTK fraction were analyzed by Western blot analysis by using polyclonal antibodies to CDK7, cyclin H, the p89 and p62 subunits of TFIIH, and cdc2 (Santa Cruz Biochemical, Santa Cruz, Calif.).

Partial purification of TTK. About 15 mg of total protein (combined T-cell lysates from 10 donors) was subjected to ammonium sulfate fractionation. The 25 to 40% ammonium sulfate cut was applied to a DEAE-Sepharose column. The column was eluted with a linear gradient of from 100 to 500 mM KCl. Fractions were collected and analyzed by Western blotting CTD and CDK2 kinase activities were assayed as described above.

CAK assay. Baculovirus constructs expressing hemagglutinin (HA) epitope-tagged CDK7, cyclin H, and CDK2 were kindly provided by David Morgan (University of California, San Francisco). Fractionation of CAK components and the CAK assay were as described elsewhere (12, 13). Immunoprecipitations of CDK7, CDK2, and cyclin H were carried out with monoclonal antibody 12CA5 against the HA epitope (Boehringer Mannheim, Indianapolis, Ind.). Purification of CDK2 was carried out as described elsewhere (34); GST-CTD (obtained from Richard Gaynor, University of Texas Southwestern Medical Center) and GST-Rb (a gift from John Brady [National Cancer Institute]), were purified on glutathione-agarose beads as described elsewhere (33). Immunoprecipitated CDK7, CDK2, and cyclin H were added when indicated to GST-Tat-fractionated T-cell lysate, and the incubated mixtures were incubated under the conditions of the kinase reaction described above. The autoradiograms were quantitated on a Storm 860 PhosphorImager (Molecular Dynamics).

RESULTS

Human primary-T-lymphocyte-derived Tat-associated CTD kinase. We used GST-Tat beads to fractionate CTD kinase from human primary T lymphocytes obtained from healthy donors. Mitogenically stimulated primary T lymphocytes have been shown in a variety of studies to support viral replication (3, 5, 14, 43). Additionally, even though the N-terminal activation domain of Tat seems to be sufficient for transactivation, full-length Tat has been shown to be required for optimal activity (37). Therefore, we fractionated the primary-T-cell lysate on full-length Tat (GST-Tat) columns. T cells derived from Tat 86-associated kinase (TTK) phosphorylated GST-CTD fusion protein (Fig. 1, lane 2), but there was little or no phosphorylation of either GST-Rb or histone H1 (Fig. 1, lane 4 and 6, respectively), suggesting a specific CTD kinase activity of the TTK fraction. In contrast to the other CTD kinases reported (17), we did not observe major shifting in the mobility of the phosphorylated CTD.

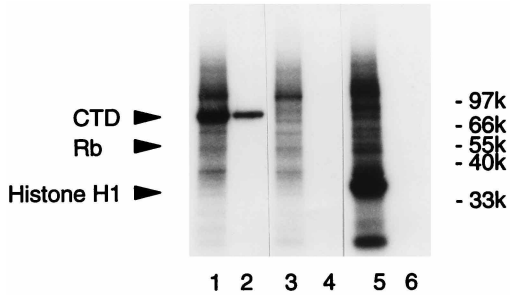

FIG. 1. TTK phosphorylates CTD. Primary-T-cell lysates were fractionated on GST-Tat 86 fusion protein coupled to agarose beads as described in Materials and Methods. TTK on beads was incubated with different substrates in reactions containing [γ-^{32} P]ATP. Lanes 2, 4, and 6, TTK reactions with GST-CTD, GST-Rb, and histone H1, respectively; lanes 1, 3, and 5, kinase reactions with GST-CTD, GST-Rb, and histone H1 incubated with unfractionated T-cell lysate, respectively. Arrows, positions of phosphorylated substrates.

Full-length Tat (Tat 86 or Tat 72) is required for TTK fractionation. Domains of Tat protein that are required to bind TTK were determined with the following GST fusion constructs: GST-Tat 86, GST-Tat 72, GST-Tat 86 *dl*2-36, GST-Tat 72 cys22, GST-Tat 48, and GST-Tat 48 cys22 or GST alone. Both GST-Tat 86 and GST-Tat 72 efficiently retained CTD kinase activity (Fig. 2, lanes 2 and 3, respectively). The deletion mutant of Tat 86 lacking the N-terminal 2 to 36 residues retained only partial CTD kinase activity, suggesting an important role of the activation domain of Tat protein (Fig. 2, lane 4 [a decrease of about 50%]). Tat 72 cys22 mutant was as active as Tat 72 (Fig. 2, lane 5). However, when either GST-Tat 48, GST-Tat 48 cys22, or GST alone was used, only a background level of CTD phosphorylation was detected (Fig. 2, lanes 6 to 8). The level of CTD kinase retained on GST-Tat 48 was less than 3% of the activity bound to GST-Tat 86. Therefore, although the activation domain of Tat is an important contributor of Tat-associated kinase activity, the N-terminal 48 residues appear to be insufficient to bind the TTK fraction.

TTK activity in mitogenically stimulated T lymphocytes. We asked whether the kinase activity of TTK paralleled the mitogenically stimulated state of the T cells. Figure 3 shows phosphorylation of CTD by TTK fractionated from either activated or nonactivated primary T cells. Compared to TTK activity from activated T cells (Fig. 3, lane 2), a background level of CTD phosphorylation was observed when GST beads alone were incubated with the activated or unstimulated T-cell lysate (Fig. 3, lanes 1 and 4, respectively) or when unstimulated T-cell

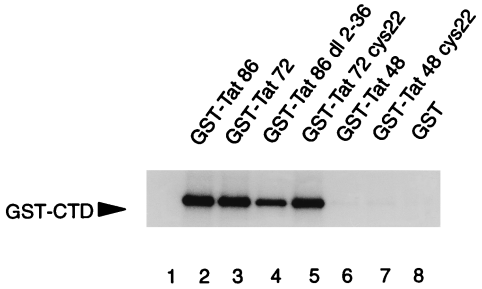

FIG. 2. Full-length Tat is required to bind CTD kinase activity. Primary-T-cell lysate was fractionated on wt or mutant Tat (GST) beads as indicated (lanes 2 to 7) or GST-agarose (lane 8). The kinase activity was determined using GST-CTD as substrate. Lane 1, control in which GST-CTD was incubated in the absence of a Tat-associated kinase. Arrow, position of the phosphorylated GST-CTD (the hypophosphorylated form [molecular mass, 90 kDa]).

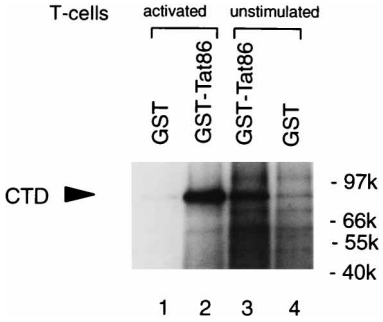

FIG. 3. A higher level of TTK activity is observed in mitogen-activated primary human T lymphocytes. TTK fractions from unstimulated and mitogen-activated T cells were prepared as described in Materials and Methods. Their kinase activities were determined with GST-CTD as the substrate in the presence of [γ-^{32}P]ATP. Lanes 1 and 4, phosphorylation of CTD by lysates from activated and unstimulated T cells, respectively, fractionated on GST-agarose beads; lanes 2 and 3, phosphorylation of CTD by TTK from activated and unstimulated T cells, respectively. Arrow, position of phosphorylated CTD.

lysate was fractionated on GST-Tat beads (Fig. 3, lane 3). These results suggest that TTK activity is induced upon mitogenic stimulation of primary T lymphocytes.

TTK phosphorylates CDK2. To ascertain whether the Tat-associated CTD kinase activity was related to CAK, we utilized CDK2 as a substrate for TTK assays. As an experimental control, CAK was reconstituted from baculovirus-expressed human CDK7 and cyclin H (12). Phosphorylation of CDK2 with the Tat-associated kinase fraction alone was similar to or better than that of the reconstituted CAK complex (Fig. 4, compare lanes 3 and 4). There was little endogenous CDK kinase associated with the baculovirus-expressed CDK7 or cyclin H (Fig. 4, lane 1). As in the case of the CTD kinase activity described above, CDK2 kinase was observed mostly associated with GST-Tat 86 or GST-Tat 72, whereas GST-Tat 48 did not show CDK2 phosphorylation (data not shown).

Partial purification of TTK. To determine whether the CDK2 and CTD kinase activities associated with the Tat-bound kinase fraction were related, we undertook partial purification of the primary T-cell lysate. A 25 to 40% ammonium sulfate cut which contained most of the CTD kinase activity was subjected to anion-exchange chromatography on DEAE-Sepharose as described elsewhere (7). Fractions were assayed for kinase activity with GST-CTD or recombinant CDK2 as substrates. As shown in Fig. 5A and as summarized in Fig. 6A, the CTD and CDK2 kinase activities were resolved into different fractions. For the sake of discussion, we have designated the fractions shown in Fig. 6 regions F1, F2, and F3, since they eluted with increasing salt concentrations. To analyze the

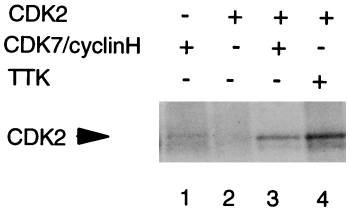

FIG. 4. Phosphorylation of CDK2 by TTK fraction. Recombinant CDK2 was incubated with the TTK fraction in an in vitro kinase reaction as described in Materials and Methods. As a control, CAK was reconstituted in vitro with recombinant CDK7 and cyclin H, and phosphorylation of CDK2 substrate was assayed as previously described. Lanes 1, CDK7-cyclin H complex; 2, CDK2 alone; 3, CDK2 incubated with reconstituted CAK; 4, CDK2 substrate incubated with TTK. Arrow, position of phosphorylated CDK2.

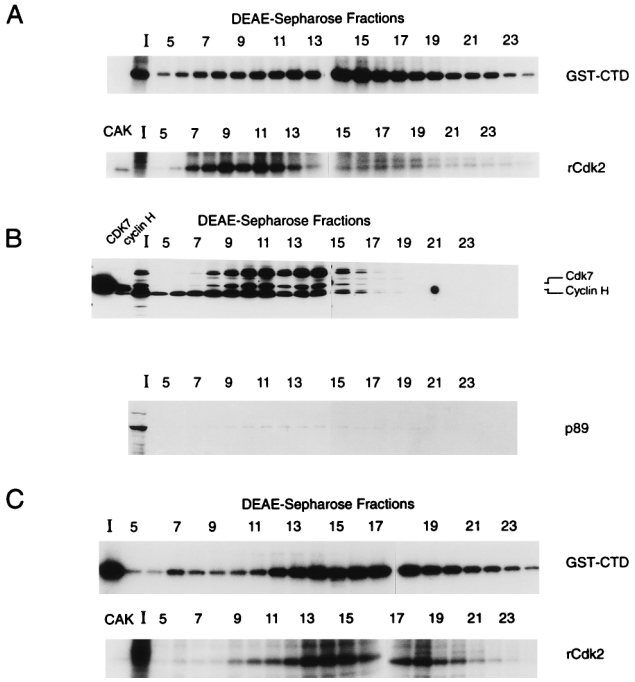

FIG. 5. Fractionation of T-cell lysate on DEAE-Sepharose. Chromatography was performed as described in Materials and Methods. Lanes I, control showing column input; lanes CAK, control showing recombinant CDK2 (rCDK2) phosphorylated by reconstituted CAK. Eluted fractions were numbered as indicated at the top. (A) DEAE-Sepharose fractions directly assayed for CTD and CDK2 kinase activity; (B) the same fractions assayed by Western blotting for CDK7, cyclin H, and the p89 subunit of TFIIH; (C) DEAE-Sepharose fractions, which were affinity purified on GST-Tat 72 beads, assayed for CTD and CDK2 kinase activities.

DEAE fraction for the CTD kinase and components of the TFIIH, the fractions were immunoblotted with antibodies against CDK7, cyclin H, the TFIIH p62 and p89 subunits, cdc2, and casein kinase II (CKII). As shown in Fig. 5B and 6B, CDK7 and cyclin H coelute with fractions in regions F1 and F2. Interestingly, the TFIIH subunits p89 (Fig. 4B and 5B) and p62 (not shown) coelute mostly with fractions in region F1. The cdc2 was not present in any of the fractions (F1 to F3) and eluted much earlier in fractions 1 to 5 (data not shown). When immunoblotted with anti-CKII antibody, the DEAE-Sepharose fractions 14 to 20 (Fig. 5A) showed coelution of CKII with the peak of CTD activity (data not shown). However, CKII was not retained on GST-Tat when the DEAE-Sepharose fractions were rechromatographed on GST-Tat (data not shown).

To analyze Tat-associated kinase activities specifically, the DEAE fractions were further affinity fractionated on Tat beads. As shown in Fig. 5C and 6C, the two kinase activities bound to Tat were clearly resolved within the F2 and F3 regions. The change in the profiles of CTD and CDK2 kinase activities in fractions before and after Tat affinity purification (compare Fig. 5A and C) may be due to the elimination of contaminating CTD kinase (such as CKII; data not shown). Furthermore, Tat-bound CTD kinase activity (in the area for F2 [Fig. 5C]), copurifies with CDK7 and cyclin H and also with TFIIH, suggesting that a fraction of the Tat-associated CTD kinase is clearly related to CAK. Finally, the fraction in region F3 (Fig. 6C), which contains 40% of the total Tat-associated CTD kinase activity, does not appear to be associated with either CAK or TFIIH components, suggesting that a significant

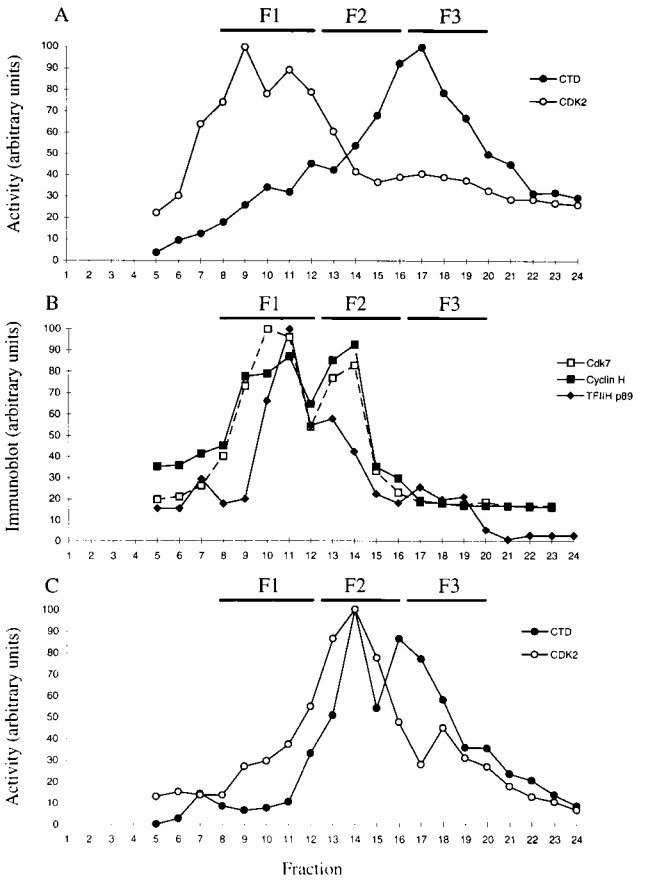

FIG. 6. Summary of the fractionation profiles shown in Fig. 5. The data presented in Fig. 5 were quantitated on a PhosphorImager (A and C) or by scanning densitometry (B). The results are expressed as arbitrary units for CTD and CDK2 phosphorylation (A and C) or for density of immunoblots (B). Region F1, fractions coeluted with the peaks of TFIIH and CAK components; F2, fractions coeluted with CAK components; F3, fractions free of TFIIH and CAK components.

fraction of CTD kinase associated with Tat is distinct from TFIIH-associated CAK (18).

CDK7 directly binds to full-length Tat. We then examined whether CDK7/MO15 associates with the domains of Tat that were required for affinity fractionation of the TTK kinase fraction. Immunoblot analysis of the GST-Tat 86- or GST-Tat 72-bound fractions and not the GST-Tat 48 or GST-agarose beads alone revealed the presence of CDK7 (Fig. 7, lanes 3 to

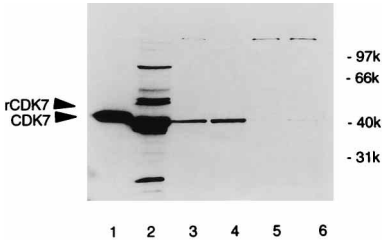

FIG. 7. CDK7 is present in the TTK fraction. TTK was affinity purified with various GST-Tat fusion proteins, resolved by SDS-PAGE, and immunoblotted with an anti-CDK7 antibody. Lanes: 1, insect cell lysate expressing epitope-tagged CDK7; 2, primary-T-cell lysate; 3 to 6, T-cell lysate fractionated on GST-Tat 86, GST-Tat 72, GST-Tat 48, and GST, respectively. Arrows, positions of HA-tagged CDK7 and human CDK7. rCDK7, recombinant CDK7.

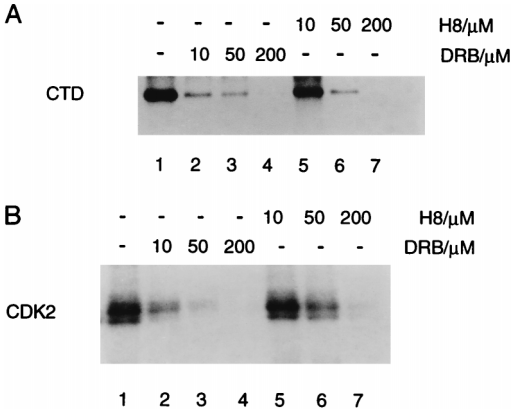

FIG. 8. Effect of DRB and H8 on TTK activity. Kinase reactions were performed in the presence of the indicated amounts of H8 or DRB with GST-CTD or purified CDK2 as substrates. Lanes: 1, control, untreated reaction; 2 to 7, phosphorylation in the presence of the indicated amounts of inhibitors. (A) Phosphorylation of GST-CTD; (B) phosphorylation of purified CDK2.

6, respectively). Thus, the affinity fractionation of Tat-associated CDK7 paralleled the CTD kinase activity with respect to its affinity for the Tat protein domain. Importantly, the binding of CDK7 to the GST-Tat 86- or GST-Tat 72-associated kinase fraction was stable following extensive washing (with the buffer containing 0.25 M NaCl, 1% NP-40, and 0.03% SDS). Furthermore, we did not detect CDK7 in immunoblots when insect cell lysate containing the recombinant CDK7 was directly incubated with GST-Tat 72 (data not shown), suggesting that the primary-T-cell-derived TTK fraction may either include a factor(s) that is required to stabilize the CDK7-Tat interaction or induce CDK7 kinase activity (see below).

DRB and H8 block CTD/CDK2 phosphorylation by TTK. The kinase inhibitors DRB and H8 have been shown to block both transcription elongation and CTD phosphorylation by TFIIH-associated CAK, implying a direct role of CTD phosphorylation in elongation (6, 42). We determined whether the TTK-associated kinase activities were blocked by DRB and H8 at concentrations known to inhibit the TFIIH-associated CAK. Addition of 10 μm DRB resulted in more than 90% inhibition of CTD (Fig. 8A, lane 2) and 60% inhibition of CDK2 phosphorylation (Fig. 8B, lane 2) mediated by TTK. Addition of 50 μM DRB resulted in more than 90% inhibition of both CTD and CDK2 phosphorylation (Fig. 8A, lane 3, and Fig. 8B, lane 3, respectively). Addition of 50 μM H8 resulted in 90% inhibition of CTD (Fig. 8A, lane 6) and 30% inhibition of CDK2 (Fig. 8B, lane 6) phosphorylation. Addition of either DRB or H8 at 200 μM completely blocked both kinase reactions (Fig. 8A, lane 7, and Fig. 8B, lane 7, respectively). Therefore, the sensitivity of the Tat-associated kinase to both of the inhibitors was similar to that of TFIIH kinase inhibition, suggesting that TTK included CTD kinase activity analogous to that of TFIIH-associated CAK.

TTK stimulates CAK activity. Phosphorylation of the conserved threonine (T160 in cdc2 and T161 in CDK2) residue of cyclin-dependent kinases by CAK is essential for their activity during cell cycle progression. CAK itself is a heterodimeric protein consisting of the catalytic subunit CDK7 and cyclin H. The kinase activity of CAK appears to be stimulated either by CDK7 phosphorylation or by MAT 1-mediated assembly of active CAK (13, 40). To determine whether the Tat-associated kinase fraction included CAK-stimulating activity, we reconstituted CAK from baculovirus-expressed human CDK7 and cyclin H and determined its kinase activity based on CDK2

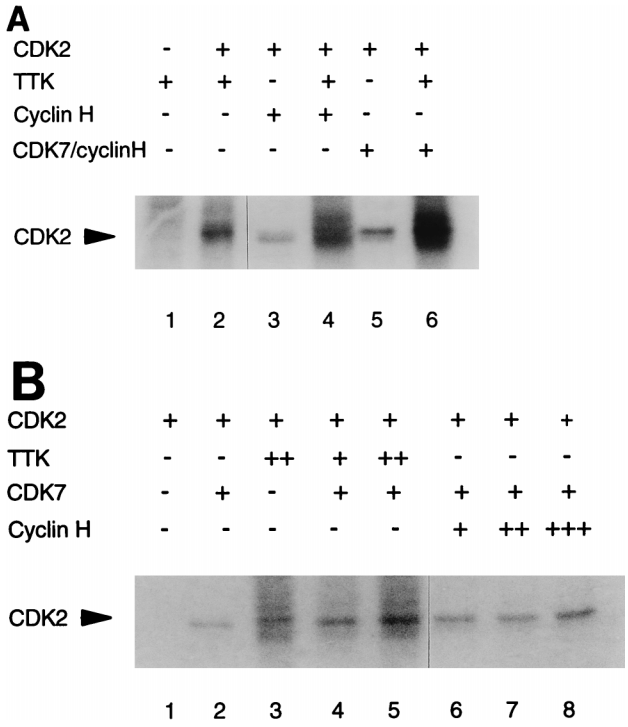

FIG. 9. TTK increases the activity of in vitro-reconstituted CAK. (A) Recombinant CDK2 (CAK substrate), cyclin H, and immunoprecipitated CDK7 were incubated with TTK fractions in kinase reaction mixtures as described previously. Lanes 1, TTK fraction alone; 2, recombinant CDK2 incubated with TTK; 3, recombinant cyclin H incubated with CDK2; 4, cyclin H incubated with CDK2 and TTK; 5, CDK7-cyclin H complex incubated with CDK2; 6, CDK7-cyclin H complex incubated with CDK2 and TTK. Arrow, position of phosphorylated CDK2. (B) Increasing amounts of TTK or cyclin H were incubated with immunoprecipitated CDK7 and CDK2 to assess the induction of CAK. Lanes: 1, CDK2 alone; 2, CDK2 incubated with CDK7; 3, CDK2 incubated with TTK (200 μg of T-cell lysate fractionated on GST-Tat 86); 4 and 5, increasing amounts of TTK (100 and 200 μg of T-cell lysates fractionated on GST-Tat 86, respectively) incubated with fixed amounts of CDK7 and CDK2; 6 to 8, increasing amounts of cyclin H (1, 2, and 3 μg, respectively) incubated with fixed amounts of CDK7 and CDK2.

phosphorylation in the presence of added TTK. Compared to the level of CDK2 phosphorylation by the TTK fraction alone (Fig. 9A, lane 2) or that of the reconstituted CDK7-cyclin H complex (Fig. 9A, lane 5), addition of the TTK fraction (along with CDK7-cyclin H) resulted in a marked increase in CDK2 phosphorylation (Fig. 9A, lane 6). There was also an activation of CDK2 phosphorylation when cyclin H alone was added along with the TTK fraction (Fig. 9A; compare lanes 3 and 4), suggesting that the Tat-associated kinase fraction contains an active CDK7 component which is complemented with added cyclin H. However, supplementing reconstituted CAK with the TTK fraction resulted in a more-pronounced phosphorylation of the CDK2 substrate, suggesting that the Tat-associated kinase fraction is capable of CDK7 activation (Fig. 9A, lane 6).

These results suggest that the Tat-associated T-cell-derived kinase contains components of CAK activation which are capable of markedly stimulating its CDK2 kinase activity. It is not clear whether the mechanism of the Tat-associated kinase in CAK stimulation involved phosphorylation of the catalytic subunit CDK7 or the accelerated assembly of active CAK. We addressed the issue of whether the T-cell-derived TTK fraction might provide a candidate CAK assembly factor similar to that of MAT 1 by utilizing increasing concentrations of cyclin H in the reconstituted CAK assay described above. An increase of as much as fivefold in CDK2 phosphorylation was observed upon addition of increasing amounts of TTK fraction along with CDK7 (Fig. 9B, lanes 4 and 5); however, such an increase in CDK2 phosphorylation was not observed when increasing amounts of cyclin H were added to the CDK7 fraction (Fig. 9B, lanes 6 to 8). The results suggest that the primary-T-cell-derived Tat-associated kinase includes a CAK activation function, principally by the induction of active CDK7.

DISCUSSION

The dual role of mammalian CAK, i.e., its pivotal role in linking cell cycle progression with basal transcription and DNA repair function (as part of the general transcription factor TFIIH), poses something of a paradox. Structurally, at least at the level of amino acid sequence, there is little similarity between the two CAK substrates, that is, the T loop of CDKs and the CTD repeats (YSPTSPS) of the large subunit of Pol II. How is it that CDK7 functions as an essential upstream kinase for CDKs and as the kinase responsible for promoter clearance during the transcription of Pol II genes? Since CAK content changes little during the cell cycle and since CAK activity is regulated both by CDK7 phosphorylation and stabilization of the CAK complex by p36 MAT 1, it is formally possible that regulation of CAK activity plays a significant role in the regulation of promoter-specific transcriptional activation. Particularly with respect to the CTD kinase involved in stimulation of processive transcription, it is likely that the components of CTD kinase regulation form part of a promoter-specific transcription complex such as the Tat-associated TTK kinase. Such a role for Tat cofactor has been suggested based on a variety of in vitro transcription assays.

Our results showing a Tat-associated kinase with CAK activation function is consistent with the mechanism of regulation of processive transcription of the HIV-1 promoter by a post-translational modification of the general transcription factor. It is likely that the absence of CAK components reported from HeLa cell-derived CTD kinase (18) reflects the differences of their upstream regulatory kinases. As we show, the TTK complex is heterogeneous. A part of it (Fig. 6B and C [fraction F2]) contains CAK and TFIIH components and possesses both CDK2 and CTD kinase activities. Interestingly, this fraction of Tat-bound kinase also promotes hyperphosphorylation of CTD (Fig. 5C, lanes 11 to 14). However, we did not observe a complete shift in hyperphosphorylated CTD, which may require factors other than the TFIIH-associated CTD kinase (15). The general transcription factor TFIIF was shown to be required for the hyperphosphorylation of Pol II CTD by TFIIH-Tat stimulated CTD kinase (32). Importantly, an appreciable fraction of Tat-bound CTD kinase is free of CAK and TFIIH components. It is likely that such a CTD kinase forms a predominant fraction of HeLa cell-derived, Tat-bound TAK kinase (17).

Our results show that fractionation of Tat-bound kinase utilizing full-length Tat reveals components of CTD kinase in addition to those seen associated with the N-terminal activation domain of Tat (17, 18, 32). Such an apparent discrepancy with the published results (17, 32) may also be due to the likely possibility that in nonprimary cells, the CTD kinase function is predominantly bound to the N-terminal activation domain of Tat protein, whereas a full complement of regulatory components that are needed to induce CAK activity in vivo require full-length Tat. The results of Tat transactivation experiments do in fact show that although the N-terminal domain of the Tat protein is sufficient for induction of the HIV-1 long terminal repeat, full-length Tat is required for optimal activity (16, 37).

By direct microinjection of the TTK fraction (eluted from Tat 72 beads) into human primary foreskin fibroblasts, we recently observed an augmentation of Tat-mediated transactivation in a CTD-dependent manner, indicating that TTK functions as an in vivo CTD kinase (24). However, it is not clear which component of TTK is responsible for the effect; clearly, further purification of the Tat-bound CTD kinase will be important to resolve this question.

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