A cofactor, TIP30, specifically enhances HIV-1 Tat-activated transcription

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Contribution by Robert G. Roeder, December 29, 1997

The HIV-1 genome encodes a regulatory protein, Tat, that functions primarily to increase the level of transcription from the single promoter in the viral long terminal repeat (LTR) during viral growth (reviewed in ref. 1). Unlike most transcriptional activators, Tat binds to an RNA element, termed the Tat-responsive (TAR) element, near the 5′ end of the nascent transcript and acts mainly to enhance transcriptional elongation (1). Tat contains a TAR-binding domain and a nuclear localization signal, which lie in the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II elongation (13) and CTD phosphorylation (reviewed in ref. 13). Further support for this model was provided by the finding that the CTD is required for Tat-mediated transactivation in vitro (14–16) and in vivo (15). DRB may exert its effect by direct inhibition of a CTD kinase activity, and two DRB-sensitive cellular kinases, the CAK component of TFIIH (14, 17–19) and Tat-associated kinase (TAK) (20), have been found to interact with the activation domain of Tat. Consistent with these observations, the CTD kinase activity of TFIIH can be stimulated by Tat (14, 18, 19), and a pseudosubstrate inhibitor of CDK7 that blocks CAK-mediated phosphorylation significantly inhibits Tat-mediated synthesis of long HIV transcripts without affecting synthesis of short (attenuated), Tat-independent HIV transcripts (19). In addition, TAK recently was identified as the previously reported elongation factor P-TEFb (21–23).

We report here the identification of a cellular factor, TIP30, that interacts with the activation domain of Tat both in vitro and in vivo. We demonstrate that TIP30 is selectively required for Tat-mediated transactivation both by anti-TIP30 immunodepletion and recombinant TIP30 complementation assays in vitro and by transient transfection assays involving ectopic TIP30 expression in vivo. Our results suggest that, like previously characterized general transcription factors, TIP30 may also serve as a direct target for Tat.

Abbreviations: GST, glutathione S-transferase; CTD, carboxyl-terminal domain; TAR, Tat-associated kinase; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat. Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF039103).

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MATERIALS AND METHODS

Plasmids. The plasmid pGEX-3X-Tat(1–48) was described previously (18). The pGEX-3X-Tat(1–48K41T) and pGEX-3X-TatF38A plasmids used for expression of mutant GST-Tat proteins were constructed by digesting the plasmids pGEX-3X-TatK41T and pGEX-3X-TatF38A with Eaql and EcoRI, filling in the ends, and recircularizing. To construct the series of mutant GST-Tat plasmids, the Tat-coding sequence amino acids 1–72 was first cloned into pBluescript (+) (Stratagene), and mutations were then introduced at positions 41 and 38 by oligonucleotide-directed mutagenesis (Amer sham). Subsequently, the Tat-coding sequences containing mutations were cloned into pGEX-3X in-frame with the glutathione S-transferase (GST) gene. For expressing TIP30 in bacteria and for generating polyclonal antibodies against TIP30, a PCR-based method was used to generate a TIP30 cDNA containing an Ndel site at the N-terminal end and a BamHI site at the C-terminal end after the stop codon. The resulting PCR product was subcloned into the plasmid Hist-pRSET (24) to generate 6His-pRSETTIP30. For production of TIP30 by in vitro transcription/translation, the plasmid pET11aTIP30 was used to produce TIP30. This plasmid was constructed by insertion of a 730-bp DNA fragment, which was isolated by digestion of Hist-pRSETTIP30 with Ndel and BamHI, into pET11a (Novagen). The plasmid (pRSETTat) used for producing Tat was constructed by inserting a BglII Tat cDNA fragment (encoding amino acids 1–72) into the BamHI site of pRSET-C (Invitrogen). Affinity Chromatography and Protein Purification. HeLa whole cell extract was prepared as described previously (25). To purify Tat-binding proteins on a large scale, 100 ml of HeLa whole cell extract (5 mg/ml protein) in ACB buffer (10 mM Hepes, pH 7.9/1 mM EDTA/1 mM DTT/10% glycerol) containing 100 mM NaCl was chromatographed on a 5-ml GST-Tat (1–48) affinity column. The bound proteins were eluted with 20 ml of 1 M NaCl in ACB and dialyzed against 0.1 M NaCl in ACB (25). Fifteen milliliters of the dialyzed bound proteins was loaded onto a 0.5-ml phosphocellulose column and step-eluted with 2.5 ml each of 0.3, 0.5, and 0.85 M NaCl in ACB. Twenty-microliter aliquots of the eluted fractions (0.5 ml) were first analyzed by SDS-PAGE followed by silver staining. The 30-kDa protein band present in the 0.3 M NaCl fraction, which were collected, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE. The 30-kDa band was visualized by staining with Coomassie blue, excised from the gel, and digested with endoproteinase C. The resulting peptides were isolated by HPLC and subjected to microsequencing. For antibody production, His-tagged TIP30 protein expressed in bacteria was purified and then used for immunization of rabbits as described previously (26). To purify TIP30 cDNA was subcloned into the mammalian expression vector pcDNA3.1 (Novagen) to create pcDNA3.1TIP30. Lipofectamine was used for transfection according to the manufacturer’s instructions (GIBCO/BRL). The same amounts of DNA were used for each dish. Cell lysates were first assayed for β-galactosidase activity (32). Normalized quantities of cell extracts were then analyzed by thin-layer chloramphenicol acetyltransferase (CAT) assays (32). Acetylated and nonacetylated forms of [14C]chloramphenicol were quantified by PhosphorImager (Molecular Dynamics Storm 840). Percent conversions of [14C]chloramphenicol to acetylated forms were determined. The fold stimulation by TIP30 was determined from an average of at least three independent experiments.

In Vitro Transcription Assays. In vitro transcription assays with Gal-VP16 and Gal-Sp1 were performed in HeLa nuclear extracts as described previously (27). Gal-VP16 and Gal-Sp1 were purified as described previously (33, 34). In vitro transcription assays with Tat were performed as described previously (11) with modifications. Supercoiled pHIV+TAR-G400 (100 ng) and pHIVD Tar-G100 (100 ng) templates were used in each reaction (25 μl). The reactions were further incubated for 30 min and then stopped by addition of 1 μl of 250 mM EDTA. Each reaction was then supplemented with 2 μl of RNase T1 (5 units/μl) and incubated at 37°C for 10 min.

RESULTS

Identification of Tat-Interacting Proteins. To identify cellular proteins that interact with the activation domain of Tat, we employed a GST fusion protein [GST-Tat(1–48)] containing the amino-terminal 48 aa of HIV-1 Tat for protein affinity chromatography. The bacterially expressed fusion protein was bound to glutathione-Sepharose and used as a ligand (17). As controls, two GST-Tat(1–48) fusion proteins containing amino acid substitutions K41T and F38A within the activation domain were also used as ligands. Both amino acid substitutions significantly weaken transcriptional activation by GAL4-Tat(1–48) (35), indicating that they might affect the interaction between Tat and its cofactor(s). To carry out affinity chromatography, a HeLa whole cell extract was loaded onto the columns at 100 mM NaCl, and the columns were then washed at 100 mM NaCl. Bound proteins were eluted with buffer containing 1M NaCl, resolved by SDS/PAGE, and stained with silver. Two polypeptides with apparent molecular masses of 30 kDa and 56 kDa selectively bound to the GST-Tat(1–48) column (Fig. 1, lane 2), but not to the GST column (lane 1). These two polypeptides bound somewhat less efficiently to the GST-Tat mutant columns (compare lane 2...
and 56-kDa proteins. Aliquots of HeLa whole cell extract were chromatographed on affinity columns containing either GST or GST-Tat(1–48) as described previously (17). Bound proteins were subjected to SDS-PAGE and stained with silver. Arrows indicate the 30- and 56-kDa proteins.

with lanes 3 and 4). Because Tat is likely to interact with multiple cellular factors that are involved in stimulation of transcription, abrogation of Tat-mediated transactivation by these mutations may result from the cumulative effect of weakening several protein–protein interactions between Tat and cellular factors. Therefore, although they were not completely abolished by the mutations, the interactions between the activation domain of Tat and these two proteins may still be biologically important. We also have observed binding of the 30- and 56-kDa proteins when HeLa nuclear extracts, rather than whole cell extracts, were subjected to Tat affinity chromatography (data not shown). The 30-kDa Tat-interacting protein is designated TIP30, and the 56-kDa Tat-interacting protein is designated TIP56. Proteins similar in size to subunits of P-TEFb (21–23) were not apparent in the high-salt GST-Tat column eluates, although it is possible that they remained bound to the column.

Identification of a Cognate cDNA and Characterization of the 30-kDa Tat-Interacting Protein (TIP30). For further characterization, a large preparation of affinity-purified TIP30 was subjected to further purification and microsequencing. Of the seven peptide sequences obtained from TIP30, five matched amino acid sequences encoded by a randomly cloned human cDNA in the EST database. Our subsequent sequence analysis of a 1.2-kb insertion revealed an ORF encoding a 242-aa protein with a calculated mass of 27.3 kDa and an estimated isoelectric point of 9.01 (Fig. 2a). All seven of the original peptide sequences were found within this ORF (Fig. 2a, underlined sequences), which appears to encode full-length TIP30 on the basis of the following. First, there is a good match to the consensus Kozak sequence (CCA/GCCATGG) at the first AUG (36); the nucleotide at the −3 position is a G and the nucleotide at position +4 is G. Second, a search of the database with the entire TIP30 sequence resulted in the identification of 15 related cDNA clones derived from several libraries. An in-frame stop codon located 30-bp upstream of the first ATG codon was found in a cDNA clone derived from a pancreatic islet cell library. Third, as shown in Fig. 2b, the recombinant protein (lane 2) expressed from the cloned cDNA

![Fig. 2. Sequence analysis and characterization of the cloned TIP30 cDNA.](image-url)
is indistinguishable in size from the endogenous TIP30 (lane 1) in HeLa nuclear extract. The smaller $^{35}$S-labeled polypeptide in lane 2 may be either a truncated TIP30 resulting from an internal translation start site or a proteolytic product. Finally, as shown below, the recombinant TIP30 has transcriptional activity.

Interaction Between TIP30 and the Activation Domain of Tat. To further ascertain that the cDNA encodes authentic TIP30, the eluates from GST and GST-Tat(1–48) columns were immunoblotted with anti-TIP30 antibody (Fig. 2c). An immunoreactive polypeptide, identical in size to TIP30 in the nuclear extract (lane 1), was detected in the eluate from the affinity column containing the GST-Tat activation domain fusion protein (lane 3) but not in the eluate from the control column containing GST alone (lane 2). Moreover, in vitro translated Tat labeled with $[^{35}$S]$\text{methionine}$ was bound to a GST-TIP30 fusion protein (Fig. 2d) but not to GST alone. Bacterially expressed TIP30 was found to bind weakly to GST-Tat(1–48) fusion protein but not to GST in an in vitro binding assay (data not shown), suggesting that TIP30 may be able to contact directly the activation domain of Tat. However, the stronger binding of natural TIP30 from nuclear extracts or recombinant TIP30 from reticulocyte lysates leaves open the possibility that other proteins may facilitate interactions between Tat and TIP30 by acting as intermediates or by covalent modification (e.g., phosphorylation) of TIP30.

To test for interactions between Tat and TIP30 in vivo, nuclear extracts from control HeLa cells and from HeLa cells that stably express an HA-tagged Tat protein were immunoprecipitated by anti-HA antibodies. In this analysis (Fig. 2e), TIP30 was detected in the immunoprecipitate from a nuclear extract containing Tat protein (lane 4) but not in that from a control extract lacking Tat protein (lane 3). Consistent with an earlier report (37) that Tat can associate with an RNA polymerase II holoenzyme complex (29, 38), RNA polymerase II (RPB1 subunit) and SRB7 (a component diagnostic of yeast and mammalian holoenzyme; ref. 29), but not RNA polymerase III (RPC53 subunit), were also coimmunoprecipitated with Tat (Fig. 2e). A protein affinity chromatography assay (Fig. 2f) also demonstrated selective binding of RNA polymerase II and SRB7 in nuclear extract to GST-TIP30 relative to GST, whereas no binding of RNA polymerase III was found. Therefore, TIP30 may enhance formation of a Tat-containing RNA polymerase II holoenzyme complex via interactions with both Tat and components of an RNA polymerase II complex.

Selective Involvement of TIP30 in Tat-Mediated Transcription in Vitro. To directly assess whether TIP30 is required for both basal and activated transcription, we used antigen-purified anti-TIP30 antibody to immunodeplete TIP30 from HeLa nuclear extracts. A quantitative Western blot analysis with anti-TIP30 showed that this treatment removed approximately 90% of TIP30 from the extract, whereas a mock-depleted extract showed little loss of TIP30 (Fig. 3A). Mock-depleted and anti-TIP30-depleted nuclear extracts were first assayed for transcription from both the adenovirus major late core promoter and a promoter containing the HIV TAR box and five Gal4-binding sites upstream of the TATA box (27). As shown in Fig. 3B, there were no significant effects of TIP30 depletion on either basal transcription or on transcription activated by the DNA-binding activators Gal-VP16 and Gal-Sp1.

We next tested whether TIP30 is required for in vitro activation of the HIV-1 promoter by Tat. As demonstrated in Fig. 3C, Tat strongly activated transcription in the mock-depleted extract (compare lanes 1 and 2). A 2-fold increase in the activity of a template with a mutant TAR site in response to Tat is consistent with previous reports by others (11, 14, 39) and may reflect the ability of a sufficiently high concentration of Tat to moderately suppress the effects of TAR mutations in vitro. An analogous DNA-binding-independent transcriptional

activation by high levels of a DNA-binding activator has also been observed in prokaryotic systems (40). No significant effect on Tat-independent transcription from the HIV-1 promoter was observed in TIP30-depleted extract (Fig. 3C, lane 1 vs. lane 3). In striking contrast, the TIP30-depleted extract failed to support Tat-dependent transcription from either the highly responsive intact template or the weakly responsive TAR-deficient template (Fig. 3C, lane 2 vs. lane 4).

When added to the TIP30-depleted nuclear extract, the endogenous TIP30 preparation isolated from HeLa nuclear extract (about 10–20% pure judged by silver stain) fully restored Tat-activated transcription (Fig. 3D, lane 6 vs. lane 5), whereas bacterially expressed recombinant TIP30 only partially restored this activity (lane 4 vs. lane 3). In contrast, neither the endogenous TIP30 nor recombinant TIP30 increased transcription from the HIV-1 promoter in the absence of Tat (lane 1 vs. lanes 3 and 5). As expected, the endogenous TIP30 and recombinant TIP30 also restored some activity of the weakly responsive TAR-deficient templates in the presence of Tat (data not shown). This result strongly suggests that, among the activators tested, TIP30 is required specifically for
transactivation by Tat. The observation that recombinant TIP30 is less active than the natural TIP30 indicates that the full activity of TIP30 may require either posttranslational modifications or an associated factor(s) that can be partially depleted by anti-TIP30 antibodies.

**Potentiation of Tat-Mediated Transactivation by TIP30 in Vivo.** To examine the role of TIP30 in transcriptional activation by Tat in vivo, HeLa cells were cotransfected with a plasmid (pcDNA3.1.TIP30) that contains the TIP30 cDNA under the control of a CMV promoter, a plasmid p167 (ref. 41) that contains the CAT gene under the control of the HIV LTR, and a plasmid (pSVtat) that expresses HIV-1 Tat (42). No significant effect on expression of the CAT gene was observed when TIP30 was expressed in the absence of Tat (Fig. 4a, lane 4 vs. lane 1). However, overexpression of TIP30 increased Tat-activated transcription by approximately 9-fold (8.9 ± 3, means ± SEM, n = 4) (lane 3 vs. lane 2).

We also tested whether TIP30 could increase activation by other regulatory factors. The pcDNA3.1.TIP30 was cotransfected with vectors expressing Gal-VP16 (ref. 35), Gal-p53 (ref. 43), or Gal-E1A (ref. 35) and an HIV-LTR CAT reporter plasmid p167 (2 µg) and other plasmids expressing Gal-VP16 (20 ng) and TIP30 (4 µg), as indicated. (b) Effects of TIP30 on transcription controlled by other activators. HeLa cells were cotransfected with CAT reporter plasmid pGl63HIVLTRDTAR (2 µg) and other plasmids (20 ng), as indicated.

**DISCUSSION**

Despite considerable evidence that the ubiquitous TFIIH and TAK serve as direct targets for Tat (Introduction), Tat is unable to activate transcription in transfected rodent cells (1) or in cell-free systems reconstituted with partially purified protein fractions containing all general transcription factors (including TFIIH) and TAK (22). These observations clearly indicate that other cellular factors are required for Tat-mediated activation. We have identified and obtained a cDNA clone for a Tat-interacting protein designated TIP30. TIP30 shares no significant homology with any of the putative Tat cofactors, suggesting that it is a novel Tat-interacting factor. In contrast to previous studies of other Tat-interacting proteins that include TBP1, Tip60, HT2A, and Tat-SF1, we have employed both recombinant protein and cognate antibodies to show that TIP30 is required specifically for Tat-activated transcription in vitro. Furthermore, unlike the Tat-SF1 that was isolated as a component of a Tat stimulator fraction in vitro (11), overexpression of TIP30 also potentiated transactivation by Tat in vivo. Therefore, it is likely that TIP30 plays a role that is distinct from that of Tat-SF1 in mediating transactivation by Tat.

Our results suggest that TIP30 interacts with Tat in nuclear extract. This conclusion is based on the observations that TIP30 in nuclear extract binds to GST-Tat, that in vitro translated Tat binds to GST-TIP30, and that Tat can be coimmunoprecipitated with TIP30 from nuclear extract (Fig. 2). However, it is not clear that TIP30 binds directly to the activation domain of Tat in vivo because bacterially expressed TIP30 bound only weakly to GST-Tat. Therefore, it remains possible that other cellular factors mediate interactions between Tat and TIP30.

Our analyses also showed that an SRB-containing RNA polymerase II complex, as well as TIP30, could be coimmunoprecipitated with Tat (Fig. 2e), and that TIP30 can interact with an SRB-containing RNA polymerase II complex in the absence of Tat (Fig. 2f). These results are consistent with an earlier report claiming that Tat is associated with an RNA polymerase II holoenzyme (37). However, in contrast to SRB proteins (29, 38) and CA150, another protein that has been reported to associate with RNA polymerase II and to be involved in Tat-mediated transcription (44), TIP30 is not tightly associated with RNA polymerase II and SRB proteins and can be separated from them by phosphocellulose chromatography. It also has been shown that the RNA polymerase II holoenzyme purified by phosphocellulose and other chromatographic steps does not support Tat-activated transcription in vitro, whereas a Tat binding fraction containing RNA polymerase II holoenzyme complex can do so (37). Hence, it is conceivable that a cellular Tat cofactor(s) separated from holoenzyme during conventional purification is necessary for Tat-mediated transactivation. Our demonstrations that TIP30 is associated with RNA polymerase II in nuclear extracts, that it can interact with Tat, and that it can be coimmunoprecipitated with Tat and RNA polymerase II raise the possibility that TIP30 is a cellular cofactor involved in Tat recruitment to an RNA polymerase II–holoenzyme complex.

The mechanisms of Tat-mediated activation may involve regulation of phosphorylation of the CTD of RNA polymerase II, because this event is correlated with the transition from initiation to productive elongation (reviewed in ref. 13) and because kinase inhibitors (DRB, H8) that block phosphorylation also block Tat-stimulated transcriptional elongation by RNA polymerase II (12, 14). Many cellular kinases have been shown to phosphorylate the CTD in vitro. Two of them, general transcription factor TFIIH (14, 18–20) and elongation factor P-TEFb/TAK (21–23), were found to interact with Tat; in the case of TFIIH, Tat was shown to enhance phosphorylation of the CTD (14, 18, 19). Thus, Tat may increase transcriptional elongation by increasing the CTD kinase activity of TFIIH, and this could be effected at initiation and/or promoter-clearance steps. Other kinases, such as TAK, might function in conjunction with Tat during elongation to keep RNA polymerase II in a hyperphosphorylated form, because P-TEFb...
TAK) can stimulate elongation by RNA polymerase II after initiation and promoter clearance are completed (45).

Another possibility is that Tat may trigger a cascade of kinase reactions to increase phosphorylation of the CTD at a particular step. Indeed, it was suggested that higher levels of CTD phosphorylation (hyperphosphorylation) may require additional factors in addition to TFIHH (14). Such a scenario obviously predicts that cyclic phosphorylation/dephosphorylation events mediated by different components regulate Tat-activated transcription. Because TIP30 interacts with both Tat and an SRB-containing RNA polymerase II complex, it is predicted that TIP30 facilitates recruitment of Tat into an RNA polymerase holoenzyme which may serve to regulate phosphorylation of the RNA polymerase II CTD or of another complex involved in elongation.

TIP30, unlike other Tat-interacting proteins such as TFIHH, TAK, and TFIID (46), does not increase the level of HIV transcription in the absence of Tat, suggesting that TIP30 is neither a general transcription factor nor a general elongation factor. Moreover, that TIP30 was not found to have any significant effect on transactivation by several other DNA-binding activators suggests that Tat might be specifically required for Tat-mediated transcription and, presumably, for certain other cellular activators. Whether there are such presumptive activators, whether they reflect human counterparts of Tat, or whether they might, like Tat, affect the elongation potential of RNA polymerase II are interesting questions that remain to be explored.

We thank R. Kobayashi (Cold Spring Harbor Laboratory) for peptide sequencing, and M. Green, Q. Zhou, and P. A. Sharp for providing plasmids. We are also grateful to our colleagues for discussions. This work was supported by grants to R.G.R. from the National Institutes of Health and the Medical Research Council of Canada and the National Cancer Institute of Canada. J.G. is an International Research Scholar to J.G. from the Medical Research Council of Canada and the National Institutes of Health (AI37327) and the Tebil Foundation and by grants from the National Science Foundation. This work was supported by grants to R.G.R. from the National Science Foundation and the National Institute of General Medical Sciences (GM31520) and the National Cancer Institute (CA54125).

References