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Specific Repression of Tax *trans*-Activation by TAR RNA-Binding Protein TRBP

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The human T-cell leukemia virus type 1 (HTLV-1)-encoded Tax protein activates transcription from the long terminal repetition via association with host cellular factors. In this study, we searched for cellular proteins that interact with Tax and modulate its activity by using the yeast two-hybrid system. One of the strongest interactors was found to be identical with TRBP, which was previously shown to bind to the RNA encoded by the Tat response element of human immunodeficiency virus type 1. Interactions are demonstrated with *Escherichia coli*-expressed proteins in vitro and in mammalian cells, using one- and two-hybrid systems, and with antibodies that coprecipitate Tax and TRBP at physiological TRBP concentrations. Moreover, TRBP, when directed into the cytoplasm, is capable of preventing transport of Tax into the nucleus. A 60-amino-acid polypeptide suffices for binding to Tax. TRBP inhibits activation of transcription by both Tax and GAL4-Tax fusion proteins. Inhibition is specific for Tax and is not seen with the other activators tested. Our data are consistent with the interpretation that TRBP inhibits the interplay of Tax with the transcription machinery or accessory factors.

Tax protein from human T-cell leukemia virus type 1 (HTLV-1) strongly enhances transcription from the viral long terminal repetition (LTR) through three 21-bp repeats which contain the cyclic AMP-responsive elements (CREs) (7, 16, 19, 43, 50, 53). Tax also activates the expression of several cellular genes, for example, the cellular immediate-early genes (c-*fos*, *egr*-1, and *egr-2*), through the serum response element (so-called CArG boxes), and the interleukin-2 (IL-2), IL-2 receptor α chain, and c-*myc* genes, through the NF-κB recognition sites (2, 3, 10, 14, 34, 35). Occasionally it also inhibits expression, i.e., of the human β-polymerase gene (29).

Tax alters gene expression through direct interactions with cellular transcription factors which bind to the Tax-responsive elements (20, 38, 56). Association between Tax and SRF (serum response factor), a cellular factor mediating activation of c-*fos* gene through CArG boxes, is correlated with activation of the c-*fos* promoter by Tax (17, 18).

Interaction of Tax with the ubiquitous ATF/CREB family of transcription factors is critical for regulation of gene expression from the 21-bp repeats (1, 4, 39, 56, 60, 61). Two mechanisms have been suggested to explain transcriptional activation of the HTLV-1 promoter by Tax. First, Tax enhances the affinity of CREB for DNA. Tax may support both dimerization and DNA binding of the ubiquitous transcription factor CREB and possibly other b-ZIP factors (5, 42, 56, 59, 62). Second, Tax enhances transcription directly, following binding to CREB/ATF proteins, via an intrinsic activation domain located in the carboxy-terminal half of Tax (1, 8). As one possible molecular mechanism, it has been reported that Tax stimulates transcription by recruiting the TATA box-binding protein TBP to the promoter (8). In other reports, it was shown that Tax interacts with the gamma subunit of the general transcription factor TFIIA (9, 13). In addition, direct interactions with the transcriptional coactivator CBP (CREB-binding protein), leading to recruitment of CBP to the HTLV-1 LTR, have been described. This process is dependent on the nature of the specific CRE and requires modification of CREB at certain cellular CREs (33).

In the context of other genes, Tax may act as a second messenger. Induction of the IL-2 and IL-2 receptor α-chain genes by Tax involves the nuclear translocation of the NF-κB/Rel factors (3, 6, 26, 37, 44). NF-κB/Rel is a family of dimeric transcription factor complexes, including p50, p52, and p65 (RelA), which are retained in the cytoplasm by a family of ankyrin-rich inhibitory proteins, such as IκB, p105 (p50 precursor), and p100 (p52 precursor). Tax supports indirectly the phosphorylation of IκB, which leads to its rapid proteolytic degradation (55) and interrupts the IκB function of p105 upon p50 and p65 (57).

In an attempt to better understand the function of Tax in the mammalian cell and to further investigate the molecular interplay of Tax with host factors, we used the yeast interaction trap to isolate genes that encode Tax-interacting proteins (25). Among the genes isolated, one encoded the protein TRBP (*trans*-activation response [TAR] RNA-binding protein) (21). TRBP is a double-stranded RNA (dsRNA)-binding protein which has been reported to bind the TAR element of human immunodeficiency virus type 1 (HIV-1). It has been suggested that TRBP functions as a potent activator at the HIV-1 LTR in a manner synergistically with the HIV-1 *trans*-activator Tat (21, 22). We demonstrate physical interaction of TRBP with Tax in vitro and in mammalian cells. TRBP specifically inhibits Tax but not *trans*-activation by other cellular activators.

MATERIALS AND METHODS

Strain. *Saccharomyces cerevisiae* EGY48 *MATα trp1 ura3 his3 LEU2::plexAop6-LEU2* was used as the host for the interaction trap (30). In this strain, the upstream activating sequences of the chromosomal *LEU2* gene are replaced by *lexA* operators (15) and plasmid pJK103, which directs the expression of a *GAL1-lacZ* gene from one ColE1 *lexA* operator (31).

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Plasmids. All cloning procedures were as described by Sambrook et al. (48), and all clones were verified by sequence analysis. Tax cDNA and deletion constructs of TRBP were generated by amplification PCR from pHISLESXd SPH (24) and TRBP(211-345), respectively. Full-length TRBP was isolated from a Jurkat cDNA library by PCR. To express proteins from *Escherichia coli*, the cDNAs encoding Tax and TRBP were inserted into the expression vectors pGEX and pET11d, respectively. For interaction experiments with yeast, the plasmids used to express LexA-Tax fusion proteins are based on pL202 PL (45), which carries the HIS3$^+$ marker and the 2μm replicator. Tax cDNA was ligated as an *Nde*I-*Hin*dIII blunt-ended fragment into the *Eco*RI site of pL202 PL to give pLexA-Tax, containing the entire 353 residues of Tax and the LexA DNA-binding domain. An oligo(dT)-primed Jurkat cDNA library cloned in the vector pJG4-5 was used to direct the synthesis of library-encoded proteins from the inducible yeast *GAL1* promoter in yeast (31). Library proteins carry, at their amino termini, the influenza virus HA1 epitope tag, the B42 acidic activation domain, and the simian virus 40 (SV40) nuclear localization signal (NLS). The cDNA encoding full-length TRBP was introduced as a *Nde*I-*Hin*dIII blunt end fragment into the *Eco*RI-*Xho*I sites from the pJG4-5 yeast vector.

The Tax expression vector pHISLESXdSPH has been described previously (24). The vector pGAL4-Tax, expressing the GAL4 DNA-binding domain (amino acids 1 to 147) fused to Tax, was constructed by introducing the Tax *Nde*I-*Eco*RI fragment into pSG424 (47). Expression vectors pGAL4-VP16 and pCMV-CREB have been described elsewhere (23, 46); expression vector pM-53 was from Clontech. The vector pTRBP-VP16, expressing the VP16 activation domain fused to TRBP(211-345), was constructed by introducing the TRBP *Eco*RI-*Xba*I fragment into pVP16 (Clontech).

Plasmid Igpoly7 was used to express TRBP derivatives that were cloned into the *Eco*RI and *Xho*I sites. Igpoly7 expresses the fusion proteins of the immunoglobulin heavy-chain (Ig) domain and TRBP derivatives via the cytomegalovirus promoter (31). A related vector lacking the Ig domain, termed pTRBP, was used to clone TRBP. The vector Igpoly7nls was generated by inserting a double-stranded oligonucleotide encoding the NLS of SV40 with the sequence PKKKRKV into the *Eco*RI site of Igpoly7, which directs the Ig fusion proteins into the nucleus independently of an inherent NLS.

Tax activity was monitored by luciferase reporter constructs (pGL2-basic; Promega). pHTLV-I LUC was generated by inserting the HTLV-1 3′ LTR *Hin*dIII-*Xho*I fragment from pU3R-I (53) into pGL2-basic. pHIVLUC was generated by inserting the *Hin*dIII-*Xho*I promoter fragment (positions +167 to −80) (41) into pGL2-basic. The GAL4 reporter pGLMRG5 was constructed by inserting a fragment carrying five GAL4 recognition sites and the HIV core promoter from pMRG5 (32) into the *Hin*dIII-*Xho*I sites of pGL2-basic.

In vitro binding analysis. Expression of a glutathione *S*-transferase (GST)-Tax and His-TRBP in *E. coli* and purification of the proteins were performed by using standard protocols. Purified His-TRBP was incubated either with GST-Tax or GST bound to the glutathione-Sepharose beads (Pharmacia) in a buffer containing 20 mM Tris (pH 7.3 at room temperature), 20% glycerol, 100 mM KCl, 5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, and incubated for 2 h at 4°C. The beads were washed with 100 volumes of the incubation buffer. The bound proteins were released by boiling in sodium dodecyl sulfate (SDS) and analyzed on Western blots.

Screening for proteins that interact with Tax. For interaction trap selection (25), strain EGY48 containing pJK103 and pLexA-Tax was transformed with the Jurkat interaction library by the lithium acetate method (28). A total of 7×10^5 primary transformants were selected on eight 24- by 24-cm Ura-, Trp-, and His-deficient glucose plates, scraped, and pooled. The transformed yeast cells were plated on 10 standard Ura-, Trp-, His-, and Leu-deficient 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) galactose plates. Library plasmids from yeast colonies that grew on Leu-deficient Gal plates and turned blue on X-Gal medium were isolated as described by Hoffman and Winston (27) and introduced into *E. coli* KC8 by electroporation. As a specificity control, isolated library plasmids were introduced into strain EGY48 expressing either LexA-Tax fusion protein or an unrelated fusion protein, LexA-CD8. cDNAs were analyzed by restriction digestion with *Eco*RI and *Xho*I and DNA sequencing.

Transfection, immunostaining, and immunoprecipitation. Human Jurkat T lymphocytes (J6), 293T human kidney, and COS cells were grown in RPMI medium supplemented with 10% fetal calf serum. DNA was transfected into Jurkat cells by electroporation (Gene Pulser; Bio-Rad). A total of 1.5×10^7 Jurkat cells were transfected with 10 μg of the luciferase reporter plasmid and amounts of expression plasmids indicated in the figures or corresponding empty vectors as a control. Luciferase assays were performed 42 h after transfection as instructed by the manufacturer (Promega). Relative light units were quantified on a Top-counter (Canberra-Packard). Luciferase values were normalized to protein concentrations as determined by the protein assay (Bio-Rad) or β-galactosidase activity from cotransfected pCMVβGal. Values in the figures represent the means and standard error of at least three experiments.

The immunofluorescence assay was performed 42 h after transfection of DNA in COS cells. The DEAE-dextran method was used for transfection of expression plasmids (5 μg) in COS cells. Cells were fixed and immunostained with fluorescein isothiocyanate-labeled anti-Ig or with polyclonal anti-Tax and fluorescein isothiocyanate-labeled anti-rabbit antibodies. Antibodies against Tax were obtained through the AIDS Research Program. Polyclonal antibodies against TRBP were generated in rabbits with *E. coli*-expressed TRBP(211-345).

TRBP antibodies allowed efficient precipitation of both internal and overexpressed TRBP, while they did not function satisfactorily in immunostaining of cells. With these antibodies, internal TRBP was detected in nuclear extracts (data not shown). The coimmunoprecipitation of Tax and TRBP was performed 42 h after transfection of DNA in 293T cells by the calcium phosphate method, using 1 to 5 μg of expression vectors. Cells were lysed in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0 at room temperature), 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (20 μg of Na-*p*-tosyl-L-phenylalanine chloromethyl ketone and 20 μg of trypsin inhibitor per ml) for 30 min on ice. After centrifugation at $10{,}000 \times g$ for 5 min, the total cell extract was incubated directly with protein A-Sepharose if cells were transfected with Ig-TRBP expression vectors. Lysates of cells transfected with Tax expression vector were incubated with preimmune serum or rabbit polyclonal antibody against TRBP for 1 h at 4°C. Protein A-Sepharose beads were subsequently added to collect the antibody-TRBP complexes. After a 1-h incubation period, the beads were washed in 150 volumes of lysis buffer, and bound proteins were released by boiling in SDS and analyzed on Western blots.

RESULTS

Isolation of Tax-interacting proteins. To identify proteins interacting with Tax, we used the yeast two-hybrid system (25). A cDNA library from Jurkat T cells (31) was conditionally expressed in a yeast strain which contained a plasmid directing the synthesis of a LexA-Tax fusion protein. Among the 50 positive clones isolated, 7 encoded TRBP. TRBP was previously identified as a factor that binds to the TAR RNA of HIV-1 and activates viral transcription (21). The TRBP gene proved to encode one of the strongest Tax interactors in our screen, as judged from the levels of β-galactosidase expressed in yeast cells mediated by Tax-TRBP interactions (data not shown). TRBP specifically interacted with LexA-Tax but did not interact with control proteins such as a LexA-CD8 fusion protein (reference 31 and data not shown).

TRBP inhibits activation of transcription by Tax. To test whether TRBP controls Tax function in mammalian cells, we analyzed transcriptional activation of the HTLV-1 LTR by Tax in transfection experiments. Preceding these investigations, a full-length TRBP cDNA encoding a 345-amino-acid protein and a full-length TRBP2 protein which carries 21 additional amino-terminal amino acids were cloned. Subsequently, the HTLV-1 promoter containing the three Tax-responsive elements was cotransfected with Tax and TRBP expression vectors. Tax activated transcription of the HTLV-1 reporter in a dosage-dependent manner up to 700-fold. Full-length TRBP repressed activation of the HTLV-1 promoter by Tax 5- to 10-fold (Fig. 1A). TRBP2 exerted indistinguishable effects on Tax activation. TRBP had no effect on activation of transcription from the HTLV-1 promoter in the absence of Tax. Thus, the different activators (e.g., CREB) that bind and activate the HTLV-1 LTR are not targeted by TRBP. This observation also implies that TRBP does not inhibit basal transcription. Moreover, TRBP did not affect *trans*-activation of a defined heterologous activator, the GAL4-VP16 protein (Fig. 1A). Levels of the expressed Tax protein in cells were not changed in the presence of TRBP (Fig. 1B). Thus, TRBP specifically represses activation of transcription by the Tax protein.

TRBP directly interacts with Tax in mammalian cells. Having demonstrated that TRBP interacts with Tax in yeast and strongly represses Tax *trans*-activation, we next asked whether TRBP and Tax contacts also occur in mammalian cells. Interactions between the two proteins could be demonstrated in colocalization experiments. Both Tax and TRBP are localized predominantly in the nucleus, as was demonstrated previously (21, 52) and is shown for Tax in Fig. 2A. The original isolate in the yeast interaction assay, a deletion clone of TRBP comprising the carboxy-terminal amino acids 211 to 345, termed TRBP(211-345), apparently lacked an NLS. To be able to

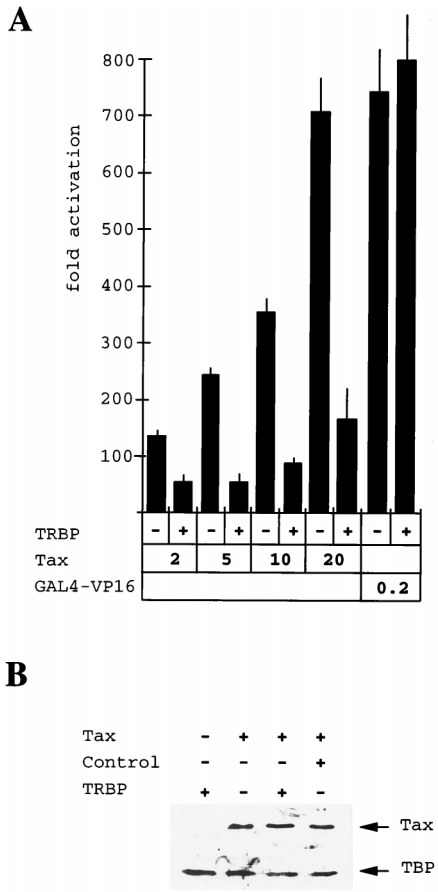

FIG. 1. (A) Overexpression of TRBP specifically inhibits Tax *trans*-activation. Relative transcriptional activation by transfected Tax and the effects of TRBP were analyzed in T-cell cotransfection assays. The HTLV-1 reporter was cotransfected with 10 μg of TRBP expression plasmid and increasing amounts (indicated in micrograms) of Tax expression vector. TRBP had no effect on activation of GAL4 reporter (pGLMRG5) by the GAL4-VP16 *trans*-activator. (B) TRBP does not affect the expression of Tax. Tax levels were monitored by Western blot analysis using antibodies directed against Tax. Nuclear extracts of cells, transfected with Tax, TRBP, or control expression vectors lacking the TRBP open reading frame (control) as indicated, were analyzed. Antibodies against TBP were used as a control (arrow labeled TBP).

distinguish between expressed and internal TRBP, TRBP(211-345) was expressed as a fusion protein with cytoplasmic Ig. The Ig domain can be detected with a secondary antibody or directly with protein A. Ig-TRBP(211-345) fusion protein was predominantly localized in the cytoplasm (data not shown). When Ig-TRBP(211-345) and Tax were coexpressed, both TRBP and Tax were found in the cytoplasm (Fig. 2B). Hence, TRBP is able to prevent import of Tax into the nucleus. Since Tax contains an NLS, colocalization of Ig-TRBP(211-345) and Tax in the cytoplasm strongly argues for interactions between Tax and TRBP in mammalian cells.

Notably, repression of Tax function by TRBP does not necessarily require aberrant sequestration of Tax protein by TRBP(211-345) in the cytoplasm. Full-length TRBP inhibits Tax *trans*-activation although it is localized in the nucleus (21). Moreover, introduction of an NLS into Ig-TRBP(211-345) generated a protein that is localized in the nucleus (Fig. 2C) and which nonetheless reduced activation of the HTLV-1 promoter by Tax fivefold. A protein consisting of the carboxy-terminal amino acids (211 to 345) and the SV40 NLS but lacking the Ig part (termed TRBP-NLS [see Fig. 4B]) inhibited Tax activity as well as did cytoplasmic TRBP(211-345).

To further demonstrate interaction of Tax with TRBP, we performed coimmunoprecipitation experiments. Tax and TRBP(211-345) fused to the Ig domain were transiently expressed in 293T cells. When Ig-TRBP(211-345) fusion protein was purified on protein A-Sepharose, Tax was retained in the presence of Ig-TRBP(211-345) but not with the Ig domain alone (Fig. 3A). More importantly, we could demonstrate that Tax associates with native TRBP at physiological concentrations of TRBP. Proteins from cells transiently expressing Tax were immunoprecipitated with a polyclonal TRBP antiserum or a nonspecific rabbit preimmune serum. Bound proteins were analyzed in Western blots (Fig. 3B). Substantial amounts of Tax protein were found in the precipitate that contained less than 1% of the total protein. This was not seen with preimmune serum (Fig. 3B) or with a control antibody (data not shown). As an additional control, TBP was not retained on the columns but was solely contained in the supernatant (Fig. 3B).

While these experiments provided clear evidence for interactions between Tax and TRBP in mammalian cells, it remained unclear whether interactions are direct or require additional components. To address this question, we analyzed the interaction of *E. coli*-expressed proteins in vitro. Purified TRBP bound specifically to GST-Tax (Fig. 3C) but not to GST alone, whereas a similar amount of expressed and purified Ets1 was not retained on GST-Tax (data not shown). Thus, Tax and TRBP interact directly with each other.

The RNA-binding domain of TRBP is dispensable for interaction with Tax. To determine the regions in TRBP involved in interaction with Tax, we analyzed TRBP deletion mutants in cotransfection experiments (Fig. 4A). Interaction was directly tested through the use of Ig-TRBP fusion proteins, which are localized in the cytoplasm. The TRBP derivatives TRBP(211-269) and TRBP(211-289) reduced Tax effects at limiting concentrations up to 15-fold, thus exceeding levels of full-length TRBP (Fig. 1A). Levels of repression increased moderately at saturating Tax concentrations (10 to 20 μg of expression vector). Under these conditions a 700-fold Tax-mediated increase of luciferase levels was reduced approximately 20-fold by TRBP(211-345) (data not shown). Proteins containing amino-terminal regions of TRBP, TRBP(1-211), and TRBP(1-170) as well as carboxy-terminal regions comprising amino acids 238 to 345 were essentially inactive (Fig. 4B). By comparing various deletion mutants as depicted in Fig. 4A, the minimal Tax interaction domain in TRBP was found to reside in between residues 211 and 269. Amino acids 211 to 238 were not sufficient for interaction with Tax. TRBP is a dsRNA-binding protein. It contains three dsRNA-binding motifs located between amino acid 17 to 89, 135 to 208, and 269 to 342 (54), respectively (Fig. 4A). Thus, the Tax interaction domain does not overlap with the dsRNA-binding motifs.

TRBP inhibits Tax independently of cellular activators. Tax mediates its effects on transcription via interaction with cellular regulatory proteins such as CREB (56, 61). To test a possible involvement of CREB, we tested GAL4-Tax fusion proteins on a reporter carrying five GAL4 recognition sites upstream of the HIV-1 core promoter. GAL4-Tax activated the promoter 30- to 70-fold. Coexpression of TRBP-NLS reduced activation by Tax approximately fivefold (Fig. 5A). This result is consistent with the hypothesis that TRBP can inhibit Tax in the apparent absence of CREB.

We reasoned that if TRBP binds to and inhibits Tax, it may itself not be able to activate transcription. To further analyze this issue, TRBP(211-345) was fused to the activation domain of VP16, establishing a tool for use in mammalian interaction

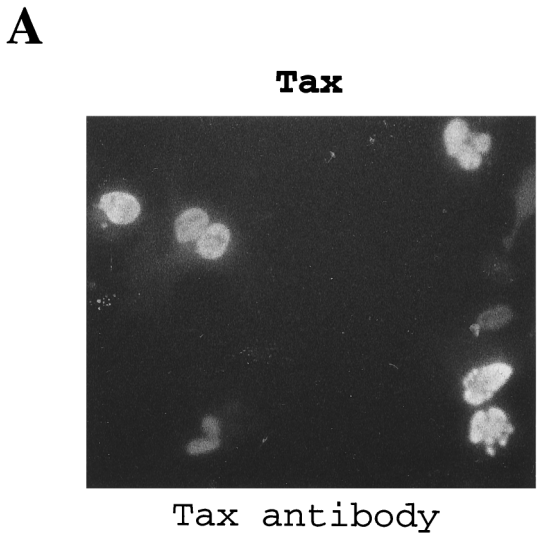

FIG. 2. Tax is sequestered by cytoplasmic TRBP in the cytoplasm. Immunostainings of cells transfected with Tax and TRBP expression vectors show nuclear localization of Tax in the absence of TRBP (A) and predominantly cytoplasmic localization of Tax in the presence of the TRBP derivative Ig-TRBP(211-345) (B). This fusion protein, consisting of the Ig domain and a TRBP deletion protein (comprising amino acids 211 to 345), lacks an NLS. Ig-TRBP(211-345) is directed into the nucleus through introduction of the NLS of SV40 in between Ig and TRBP regions (C).

assays. Cotransfection of TRBP-VP16- and GAL4-Tax-expressing vectors enhanced the activation potential of GAL4-Tax up to 15-fold on a GAL4 reporter plasmid (Fig. 5B), while VP16 alone or control proteins (p53) had no effect (Fig. 5B). A mammalian one-hybrid approach led to very similar conclusions. TRBP-VP16 was cotransfected with limiting concentrations of Tax (1 μg of expression vector) and the HTLV-1 reporter, which resulted in fivefold potentiation of Tax activity (Fig. 5C). TRBP-VP16 had no effect on activation of transcription from the HTLV-1 promoter in the absence of Tax, arguing that TRBP(211-345) cannot bind to the promoter without Tax. These experiments provide independent evidence for direct Tax-TRBP interactions in mammalian cells. Complex formation takes place at the promoter, based on the observation that repression by TRBP is reversed through addition of an activation domain to TRBP. They further suggest that TRBP does not compete with CREB but rather blocks the communication of Tax with the basal machinery or accessory factors. Consistent with this conclusion, overexpression of CREB could not relieve the negative effect of TRBP on Tax *trans*-activation when cotransfected with TRBP and Tax expression vectors (Fig. 5D). Under comparable conditions, expression of CREB stimulated the human 8.1 Vβ promoter (12) approximately fivefold in human T-cell Jurkat cells (Fig. 5E). Activation through the NF-κB pathway by Tax, monitored at the HIV LTR (36), was reproducibly not repressed by the TRBP derivative TRBP-NLS (Fig. 5F), which lacks the region that has been shown to bind the TAR region of HIV. Hence, TRBP has no effect on the release of NF-κB from IκB or on the activation of transcription by the NF-κB complex.

DISCUSSION

In this study, we identified TRBP as a specific interactor and repressor of the HTLV-1-encoded Tax protein. TRBP inhibited exclusively Tax and had no effect on the HTLV promoter or on the acidic activators VP16 and NF-κB. To our knowledge, TRBP provides the first example of a cellular protein that specifically represses activation of transcription by the viral coactivator Tax as a consequence of direct protein-protein interactions. Our findings are consistent with the interpretation that TRBP could function as a cellular inhibitor of Tax, which limits Tax activity unless Tax levels exceed TRBP concentrations. Interactions have been demonstrated at limiting concentrations of transiently transfected Tax at physiological concentrations of cellular TRBP. Two other Tax interactors have been isolated in two hybrid screens by others and were shown to interact with transiently transfected Tax (11, 44). Interaction of Tax with Int-6, a component of promyelocytic leukemia nuclear bodies, caused its delocalization into the cytoplasm (11). The other Tax interactors were components of the proteasome, HsN3 and HC9 (44). For unknown reasons, these interactors were not found in our *lexA*-based two hybrid screen.

Several possible modes of action have previously been suggested for TRBP, though the precise role in the cell is still unclear. TRBP has been shown to activate transcription from

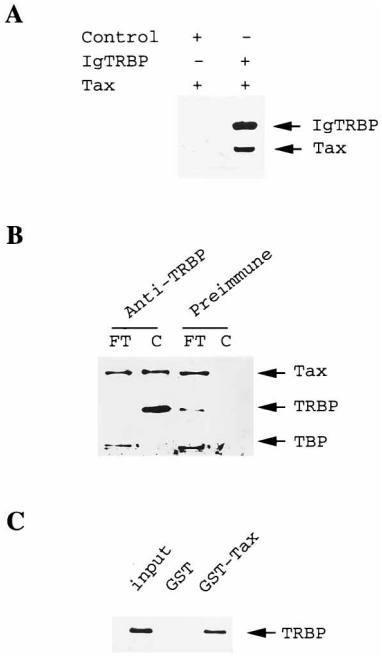

FIG. 3. Tax binds specifically to TRBP. The Tax-TRBP coimmunoprecipitation experiments were performed with 293T human kidney cells. (A) Coprecipitation of Tax and TRBP with protein A-Sepharose from cells transiently transfected with Ig (control) or Ig-TRBP fusion protein and Tax. SDS eluates of beads were analyzed with anti-Tax and anti-TRBP antibodies. (B) Immunoprecipitation of Tax with cellular TRBP. Total-cell extract from 293T cells transfected with a Tax-expressing vector (1 μg) was immunoprecipitated with rabbit preimmune serum or a polyclonal antibody directed against TRBP. Bound proteins (lanes C) and flowthrough (lanes FT) were analyzed by immunoblotting with polyclonal anti-Tax, anti-TRBP, and anti-TBP antibodies. (C) TRBP and Tax directly interact with each other. Purified TRBP from *E. coli* (lane 1) is retained on GST-Tax (lane 3) but not on GST (lane 2) columns, as demonstrated by Western blot analysis of bound and eluted fractions with antibodies against TRBP.

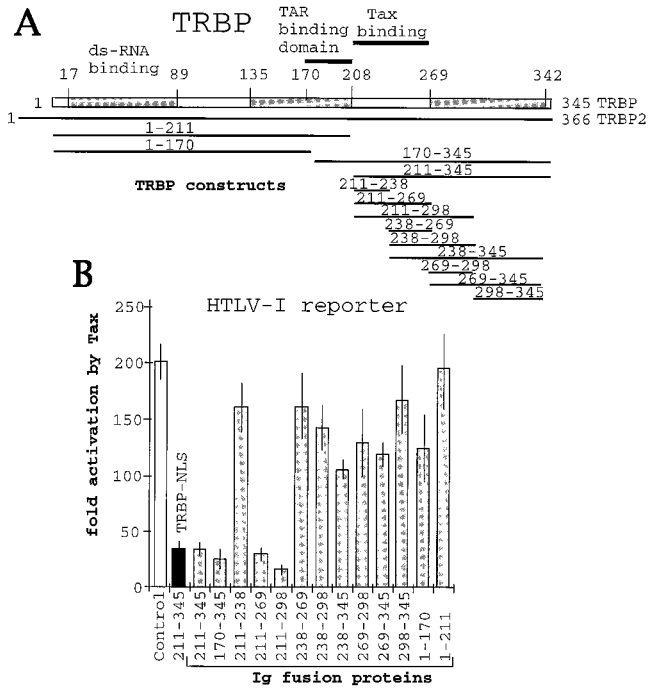

FIG. 4. (A) Schematic representation of TRBP derivatives. TRBP2 (accession number U08998) carries 21 additional amino acids amino terminally of the TRBP open reading frame. Deletion constructs of TRBP used in transfection are shown below with numbers indicating amino acid positions (numbering refers to the TRBP open reading frame). The TAR RNA-binding domain as defined previously (21) and dsRNA-binding motifs are indicated by grey boxes (54). (B) Characterization of the minimal Tax-binding domain of TRBP. Effects of TRBP derivatives on Tax were analyzed in cotransfection experiments through inhibition of Tax *trans*-activation (5 μg of Tax expression vector) of the HTLV-1 LTR with cytoplasmic Ig-TRBP fusion proteins (10 μg of expression vector) in Jurkat cells. Note that the minimal Tax-binding domain does not overlap the dsRNA-binding motifs. Similar levels of inhibition (compare with the control that contained 10 μg of an empty expression vector [white bar]) were detected with nuclear TRBP derivatives, as exemplified here with TRBP-NLS, comprising amino acids 211 to 345 of TRBP (black bar).

the HIV-1 LTR. In this investigation, activation of transcription was correlated to binding of TRBP to the stem-loop of the TAR RNA. Optimal *trans*-activation of the HIV-1 LTR by TRBP requires an intact TAR RNA structure (21). TRBP activated transcription synergistically with the *trans*-activator of HIV-1, the Tat protein, which is thought to enhance the processivity of RNA polymerase II upon binding to a bulge of the TAR RNA (reviewed in reference 21).

In other investigations, TRBP has been reported to act as a negative regulator of the dsRNA-dependent protein kinase DAI/PKR (40). DAI/PKR is induced by interferon and participates in the cellular defense against viral infections. The kinase is activated upon binding to dsRNA. TRBP prevents phosphorylation of DAI/PKR and of target proteins of DAI/PKR. It is likely but not yet proven that inhibition of the kinase is directly or indirectly related to the capacity of TRBP to interact with dsRNA. TRBP not only interacts with the TAR RNA but also binds to RNA complementary to the Rev-responsive element of HIV-1 (40). Furthermore, it has been reported that TRBP binds preferentially to GC-rich stem-loop structures (22).

While these investigations indicated specificity of TRBP for defined elements, it is also possible that TRBP binds nonspecifically to dsRNA. TRBP contains three conserved dsRNA-binding domains which have extensive homology with several other dsRNA-binding proteins such as the Staufen protein of *Drosophila melanogaster*, which is required for the localization of maternal mRNAs. Notably, the kinase DAI/PKR also contains one copy of this dsRNA-binding motif (54). In summary, all previously defined activities of TRBP involve binding of TRBP to RNA. In our investigations, we could not demonstrate a correlation of RNA-binding and repression activities. First, the highly purified *E. coli*-expressed proteins bind to each other directly in the apparent absence of RNA in vitro. Second, binding of TRBP to Tax requires a region of TRBP which does not overlap with the RNA-binding motif (amino acids 211 to 269). An isolated RNA-binding domain alone (amino acids 1 to 170) or two copies of it (amino acids 1 to 211) had no effect on activation of the HTLV-1 LTR by Tax. Moreover, the carboxy-terminal amino acids 211 to 269 of TRBP alone, i.e., in the absence of an intact RNA-binding motif, were sufficient to mediate binding of TRBP to Tax. Repression of Tax activity was also seen on HTLV-1 reporters in which the region downstream of the start site was either removed or exchanged, arguing at least against the requirement of a specific RNA-binding site in the transcribed region of HTLV-1. However, since the Tax interaction domain was mapped with TRBP deletion proteins that were located in the cytoplasm, we cannot formally rule out the possibility that repression in the nucleus is dependent on additional regions such as the carboxy-terminal RNA-binding motif of TRBP (amino acids 269 to 342).

In our investigations, we demonstrated that the inhibition of

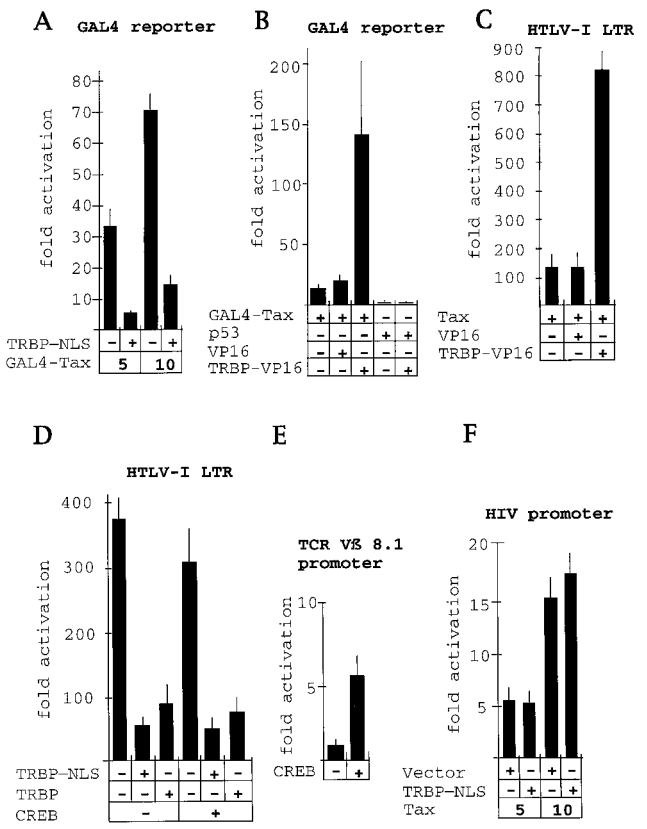

FIG. 5. Luciferase activities of transiently transfected Jurkat cells. (A) TRBP inhibits activation of transcription by GAL4-Tax fusion proteins. A GAL4 reporter plasmid (pGLMRG5; 10 μg) was cotransfected with 10 μg of TRBP-NLS and amounts of GAL4-Tax expression vector as indicated. (B) TRBP-VP16 fusion proteins activate transcription depending on the presence of GAL4-Tax. pGLMRG5 was cotransfected with 2 μg of GAL4-Tax or p53 (Clontech) expression vector and 10 μg of either TRBP-VP16 or VP16 expression vector as indicated. (C) TRBP-VP16 but not VP16 (5 μg of vector) enhances the activation of the HTLV-1 promoter by Tax (1 μg of vector). (D) Overexpression of CREB does not alleviate inhibition of Tax by TRBP. The HTLV-1 reporter was cotransfected with 6 μg of Tax expression vector, 10 μg of a CREB expression plasmid, and 10 μg of vectors expressing TRBP(211-345) or TRBP. (E) Although CREB did not affect transcriptional activity of the HTLV-1 reporter, identical amounts of CREB expression vector (10 μg) activate the Vβ promoter (10 μg) that carries a partially conserved CRE approximately 60 bp upstream of the transcription start site (12). (F) TRBP-NLS does not affect the activation of NF-κB by Tax. The HIV reporter plasmid was transfected with TRBP-NLS expression plasmid (10 μg; compare with Fig. 4B) and different amounts of Tax as indicated.

Tax activity by TRBP correlated with direct protein-protein contacts between TRBP and Tax. Direct physical interactions seem to occur, as concluded from interaction studies with recombinant proteins and the yeast two-hybrid and mammalian one- and two-hybrid systems, as well as by colocalization and immunoprecipitation experiments in mammalian cells. Our experiments do not yet address a possible role of TRBP on viral growth. However, interactions were seen at physiological concentrations of TRBP in immunoprecipitations under conditions which were frequently used to elucidate and dissect the functional properties of the viral Tax protein in previous investigations. At least under these conditions, cellular TRBP may limit Tax activity, unless Tax is produced in excess. Importantly, TRBP potentiated the activity of both wild-type Tax and GAL4-Tax proteins when fused to an activation domain on HTLV-1 and GAL4 reporters, respectively. In the absence of the VP16 activation domain, GAL4-Tax fusion proteins are efficiently inhibited by TRBP. This finding provides functional evidence that TRBP binds to Tax at the promoter and inhibits an event following promoter recognition by Tax.

It seems interesting that GAL4-Tax fusion proteins were far (at least 10-fold) less active than the natural Tax protein, although the corresponding reporter construct contained five proximal GAL4 recognition sites. One possible explanation for the moderate activity of GAL4-Tax proteins might be a limited accessibility of the bona fide activation domain due to steric hindrance. However, it is also possible that native Tax utilizes in part alternative mechanisms to stimulate transcription. These mechanisms could also account for the inability of TRBP to fully repress Tax activation. Indeed, when we titrated TRBP to saturating levels, we always retained 10- to 40-fold activation by Tax, even if we used TRBP derivatives that apparently quantitatively retained Tax in the cytoplasm.

It has been reported that Tax acts as a second messenger on genes that carry NF-κB-binding sites through release of the activator from IκB in the cytoplasm and its subsequent transport into the nucleus (11, 36, 49). We could not detect inhibition of this pathway by TRBP. This finding was not unexpected, as cellular TRBP is located in the nucleus, while activation is thought to take place in the cytoplasm and Tax is not directly involved in the activation of the transcription machinery at the HIV LTR.

We have not yet precisely mapped the interaction domain on the Tax protein. However, a preliminary characterization indicated that Tax interacts with TRBP through its amino-terminal 202 amino acids (data not shown). The amino terminus of Tax contains the CREB interaction domain and the putative NLS but lacks the activation domain (51). While it is possible that cytoplasmic TRBP prevents the transport of Tax to the nucleus by masking the localization signal, we have no evidence that inhibition by TRBP results of interference with CREB-Tax interaction. TRBP inhibits activation by Tax and by GAL4-Tax proteins, respectively, to similar extents. Inhibition of Tax does not require the transcriptional activation domain of Tax. The latter is leucine rich and is contained within the carboxy-terminal amino acids 289 and 322 of Tax. Earlier characterizations of the Tax activation domain as an acidic α-helix were not confirmed in more recent studies in which this motif was dispensable for transcriptional activation (50, 51).

Several mechanisms of *trans*-activation have been assigned to Tax. For example, it has been suggested that Tax alters the activity of the coactivator CBP, which could account for enhanced activity of native Tax compared with GAL4-Tax fusion proteins (33). However, since TRBP inhibits Tax independently of CREB, we have as yet no evidence for interference with CREB-CBP complex formation. We propose a model in which binding of TRBP to the amino-terminal region of Tax indirectly inhibits the function of the transcriptional activation domain. For example, binding of a cofactor or components of the basal transcription machinery might be sterically inhibited by TRBP. Candidates are TBP and TFIIA, which have been reported to functionally interact with Tax (8, 9). In future studies, the TRBP could be a useful tool to specifically target Tax and to investigate the molecular mechanism by which Tax interacts with cofactors and stimulates the activity of RNA polymerase II.

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