Human Immunodeficiency Virus Type 1 Tat Binding Protein-1 Is a Transcriptional Coactivator Specific for TR

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The DNA-binding domain of nuclear hormone receptors functions as an interaction interface for other transcription factors. Using the DNA-binding domain of TRβ1 as bait in the yeast two-hybrid system, we cloned the Tat binding protein-1 that was originally isolated as a protein binding to the human immunodeficiency virus type 1 Tat transactivator. Tat binding protein-1 has subsequently been identified as a member of the ATPase family and a component of the 26S proteasome. Tat binding protein-1 interacted with the DNA-binding domain but not with the ligand binding domain of TR in vivo and in vitro. TR bound to the amino-terminal portion of Tat binding protein-1 that contains a leucine zipper-like structure. In mammalian cells, Tat binding protein-1 potentiated the ligand-dependent transactivation by TRβ1 and TRα1 via thyroid hormone response elements. Both the intact DNA-binding domain and activation function-2 of the TR were required for the transcriptional enhancement in the presence of Tat binding protein-1. Tat binding protein-1 did not augment the transactivation function of the RAR, RXR, PPARγ, or ER. The intrinsic activation domain in Tat binding protein-1 resided within the carboxyl-terminal conserved ATPase domain, and a mutation of a putative ATP binding motif but not a helicase motif in the carboxyl-terminal conserved ATPase domain abolished the activation function. Tat binding protein-1 synergistically activated the TR-mediated transcription with the steroid receptor coactivator 1, p120, and cAMP response element-binding protein, although Tat binding protein-1 did not directly interact with these coactivators in vitro. In contrast, the N-terminal portion of Tat binding protein-1 directly interacted in vitro and in vivo with the TR-interacting protein 1 possessing an ATPase activity that interacts with the activation function-2 of liganded TR. Collectively, Tat binding protein-1 might function as a novel DNA-binding domain-binding transcriptional coactivator specific for the TR probably in cooperation with other activation function-2-interacting cofactors such as TR-interacting protein 1. (Molecular Endocrinology 15: 1329–1343, 2001)
binding factors independent of DNA binding (19, 22–24). Direct evidence that the DBD-binding factor of NRs is involved in ligand-dependent transactivation has been shown by Cheng et al. (27) who reported that the hematopoietic bZIP protein, p45/NF-E2 interacts with the DBD of TR and RAR and potentiates ligand-dependent gene activation in cooperation with the cAMP response element-binding protein-binding protein (CBP). A novel protein termed SNURF (small nuclear ring finger protein), which was cloned using the DBD of AR as bait, can potentiate gene activation mediated by AR, GR, and PR in a ligand-dependent manner, possibly by functioning as a bridging factor between these NRs and the TATA-binding protein, a basal transcription factor (28). A cold-inducible coactivator of NRs, PGC-1, binds to the DBD and a part of the hinge region of PPAR γ (29). The p300/CBP associated factor has a histone acetylase activity and directly binds to the DBD of NRs to function as a coactivator (30). Paradoxically, histone deacetylases, HDAC1 and HDAC2, have also been found to bind the DBD of TR and might participate in the negative regulation of the TSH β subunit gene by thyroid hormone (31). These findings suggest that the DBD-interacting proteins might be classified as a novel class of coregulators involved in transcriptional control by NRs.

To gain additional insights into the role of DBD-binding factors of NRs for ligand-dependent transactivation, we cloned a protein that binds to the DBD of human TRβ1 using the yeast two-hybrid system. The nucleotide sequence of the cloned cDNA was found to be identical to the human immunodeficiency virus type 1 (HIV-1) Tat binding protein-1 (TBP-1) that was originally isolated as a protein interacting with the HIV-1 Tat transactivator (32). TBP-1 potentiated the TR-mediated, but not other NR-mediated, transactivations in mammalian cells. Although TBP-1 itself possessed an intrinsic transactivation function, augmentation of TR-mediated transactivation by TBP-1 required the intact AF-2 of TR. TBP-1 directly interacted with the TR-interacting protein 1 (Trip1), a putative transcriptional mediator for TR that binds to the AF-2 of TR in a ligand-dependent manner (33, 34). These findings demonstrated that TBP-1 interacts not only with Tat but also with TR, and TBP-1 might function as a novel DBD-binding coactivator specifically involved in the TR-mediated gene activation.

RESULTS

Cloning of a Protein That Interacts with the DBD of TRβ1

To isolate the proteins that interact with the DBD of TR, we performed a yeast two-hybrid screen of a Hela cell cDNA library using the DBD plus a part of the hinge region of human TRβ1 as bait (Fig. 1A). Sixteen positive clones were obtained, and their partial nucleotide sequences were determined. A clone containing the identical partial cDNA sequence was also isolated from a human lung cDNA library. A computer search for data resources suggested that the partial cDNA sequence isolated from two different libraries was apparently identical to that of the human TBP-1 that was previously cloned as a protein interacting with the HIV-1 Tat transactivator (32). TBP-1 has been reported to be predominantly localized in the nucleus (32). The reported TBP-1 was encoded by 404 amino acids and was shown to possess a putative nucleotide binding motif and an RNA/DNA helicase motif in the C-terminal conserved ATPase domain (CAD), a characteristic structure of an ATPase family (Fig. 1B) (32, 35). It was also noted that a heptad repeat of hydrophobic amino acids, reminiscent of a leucine zipper, was located in the heptad-repeat of hydrophobic amino acids. The conserved ATPase domain (CAD) is shown as a shaded box. Comparison of the amino acid sequences of TBP-1, Trip1, and MIP224. The amino acid sequences of TBP-1, Trip1, and MIP224 were aligned and compared with TBP-1 and are shown as percent sequence identity. GenBank accession numbers of TBP-1, Trip1, and MIP224 are M34079, L38810, and U27515, respectively.

Fig. 1. Cloning of a Protein Interacting with the DBD of TRβ1 Using the Yeast Two-Hybrid System

A, Schematic representation of the structure of hTRβ1. The portion of TRβ1 used for the yeast two-hybrid screen is shown as bait. B, Schematic representation of the structure of human TBP-1. The positions and amino acid alignments of the putative leucine zipper-like structure, nucleotide binding motif, and helicase motif are indicated. Underlines indicate the heptad-repeat of hydrophobic amino acids. The conserved ATPase domain (CAD) is shown as a shaded box. C, Comparison of the amino acid sequences of TBP-1, Trip1, and MIP224. The amino acid sequences of Trip1 and MIP224 that were cloned as proteins interacting with NRs (9, 31) were compared with TBP-1 and are shown as percent sequence identity.

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the ATPase family that have been isolated as the factors interacting with the LBD of TR/RAR and an orphan NR, MB67, respectively (Fig. 1C) (34, 37, 38). Further sequence analyses of the cDNA clone isolated in our yeast dual-hybrid screen revealed that the cDNA contained the full-open reading frame of the originally isolated TBP-1 (32). Ohana et al. reported the cloning of a longer version of TBP-1 cDNA that has two additional in-frame ATG codons 35 and 19 amino acids upstream of the first ATG in the originally isolated TBP-1 (39). The present clone lacked the sequence corresponding to the first in-frame 16 amino acids of the longer version of TBP-1. The third ATG codon was previously established to be used as an initiation codon (32). Therefore, the third and probably the second ATG codon might be used in our cloned TBP-1 cDNA when translated in vitro. For convenience, numbering of the amino acids of TBP-1 in this manuscript followed that of the longer TBP-1 cDNA version (39).

**TBP-1 Interacts with the DBD but Not with the LBD of TR**

To confirm the interaction between TBP-1 and TR, TRDBD plus a part of the hinge region (TRD-H) ligated to GAL4DBD (pGBT9TRD-H) and the full-length TBP-1 ligated to GAL4AD (pGAD10TBP-1) were cotransformed into yeast containing the lacZ reporter gene under control of the GAL4-binding site, and the protein-protein interaction was analyzed by a β-galactosidase assay. To determine whether TBP-1 also binds to a domain other than TRDBD, the interaction of TBP-1 with GAL4DBD separately fused to the DBD alone (pGBT9TRDBD), and the remainder of the hinge region plus LBD (pGBT9TRLBD) of TRβ1 was tested. As shown in Fig. 2, TBP-1 interacted with TRDBD and TRD-H, but not with TRLBD. GAL4DBD itself (pGBT9) did not interact with TBP-1 (Fig. 2A), and GAL4TRDBD did not show any β-galactosidase activity in the presence of GAL4AD alone (data not shown), indicating specificity of the interaction. TRLBD did not interact with TBP-1 even in the presence of T3 (Fig. 2A), whereas the interaction of TRLBD with Trip1 fused to GAL4AD was T3-dependent in our yeast system in agreement with a previous study (34) (data not shown). To next examine whether TBP-1 physically associates with TR in mammalian cells, CV-1 cells were cotransfected with TR and FLAG-tagged TBP-1 expression vectors. Protein complexes associated with TBP-1 in mammalian cells, CV-1 cells were cotransfected with TR and FLAG-tagged TBP-1 expression vectors. Protein complexes associated with TBP-1 were first immunoprecipitated with the anti-FLAG monoclonal antibody, and the bound proteins were subsequently analyzed by immunoblotting with an anti-TR-specific antibody. As shown in Fig. 2B, TR protein was detected in immunoprecipitates from cells transfected with both TBP-1 and TR expression vectors, but not with the empty FLAG vector and TR expression plasmid, confirming that TR and TBP-1 are found as complexes in vivo.

**Glutathione-S-Transferase (GST) Interaction Assay**

We next assessed the in vitro interaction of TBP-1 with TR using a GST pull-down assay. [35S]-labeled TRβ1 proteins encoding the A/B domain (TRα/B), A/B plus DBD (TRα/B-DBD), A/B to a part of the hinge region (TRα/B-hinge), the remainder of the hinge region plus LBD (TRLBD), and the full-length TRβ1 were synthesized using in vitro transcription/translation and were incubated with a GST-fused full-length TBP-1 (GST-TBP-1) immobilized to glutathione beads. After extensive washing, TRα/B-DBD, TRα/B-hinge, and the full-length TR were retained by GST-TBP-1, but not by GST itself (Fig. 3A). In contrast, TRα/B and TRLBD did not bind to GST-
TBP-1. The interaction of the full-length TR with TBP-1 was not T₃ dependent as expected from the findings obtained in the yeast experiment. The binding of TR to GST-TBP-1 was comparable to that of a GST-fused receptor interaction domain of the nuclear receptor corepressor (N-CoR) (Fig. 3B). These findings confirmed the interaction of TBP-1 with the DBD of TR in vitro.

Mapping the TR-Interacting Domain in TBP-1

To localize the domain necessary for interaction with TR in TBP-1, GST-fusion proteins of the N-terminal (residues 17–176) and C-terminal portions (residues 176–439) of TBP-1 were synthesized, and their interaction with [³⁵S]-labeled full-length TR was examined using a pull-down assay. The polypeptides encoding the A/B domain (A/B), A/B plus DBD (A/B-DBD), and A/B to a part of the hinge region (A/B-hinge) of TR were synthesized as fusion proteins with GAL4DBD for better resolution in a 15% polyacrylamide gel. Interaction of TR LBD and the full-length TR was analyzed in a 10% gel. The positions of the molecular size markers are indicated. “Input” represents 10% of input protein. The percent bound protein is shown below each lane. B, Binding of [³⁵S]-labeled full-length TR to equivalent amounts of GST TBP-1 and the GST-fused receptor interaction domain of N-CoR was compared using a pull-down assay in the presence or absence of 100 nM T₃ (left panel). SDS-PAGE of GSTTBP-1 and GST-fused receptor interaction domain of N-CoR is shown in the right panel.

TBP-1 Potentiated the Ligand-Dependent Transactivation by TRβ1 in Mammalian Cells

We next analyzed whether TBP-1 affects the ligand-dependent transactivation function of the TR in mammalian cells using a transient transfection assay. In TR-deficient CV-1 cells, the activity of promoter carrying the palindromic thyroid hormone response element (TRE) was stimulated by the cotransfected hTRβ1 in the pres-
The cotransfection of TBP-1 augmented the ligand-dependent stimulation of the promoter activity by TR in a dose-dependent manner (Fig. 5A). A similar ligand-dependent augmentation by TBP-1 was observed with a reporter construct carrying another TRE, DR4, that is direct repeat-type TRE with a spacer of four nucleotides (Fig. 5B). In contrast, TBP-1 did not potentiate the activities of thymidine kinase (TK), Simian virus 40 early and Rous sarcoma virus (RSV) long terminal repeat (LTR) promoters that lack the positive TRE in the presence of liganded TR (data not shown). The transcriptional enhancement by TBP-1 of the palindromic TRE was not observed when a TR with a mutation in the first zinc finger motif in DBD (C127S) was cotransfected (Fig. 5C). Moreover, TBP-1 lacking the N-terminal leucine zipper-like structure did not potentiate transcription in the presence of wild-type TR in transient transfection assays (Fig. 5D). These findings collectively suggest that TBP-1 potentiated the ligand-dependent transactivation of TR in mammalian cells, and that TBP-1 functioned in concert with TR that bound to DNA.

TR is known to be encoded by two different genes, c-erb A α and β (40, 41). To clarify whether TBP-1 functions in a TR isoform-specific manner, hTRα1 was cotransfected with TBP-1 into CV-1 cells in the presence of the palindromic TRE reporter gene. TBP-1 could potentiate transactivation by TRα1 in the presence of T3 similar to TRβ1 (Fig. 6A). Identical findings were obtained using the DR4 reporter gene (data not shown). We next examined whether TBP-1 can potentiate transactivation by other members of NRs in CV-1 cells. As shown in Fig. 6B, the cotransfected TBP-1 did not significantly enhance activation of the promoters containing cognate hormone response elements by RAR, RXR, PPARγ, and ER in the presence of individual specific ligands. The direct interaction of TBP-1 with RXR and RAR, known heterodimer partners for TR (5, 42), was also evaluated using the yeast two-hybrid system. As shown in Fig. 6C, TBP-1 interacted neither with the full-length RXR in the absence and presence of 9-cis-retinoic acid (9-cis-RA) nor the DBD plus the hinge region of RAR. Moreover, [35S]-labeled full-length RAR, RXR, PPAR, and ER did not bind to GST-TBP-1 in in vitro pull-down assays (Fig. 6D). These findings indicated that TBP-1 selectively potentiates the transactivation function of TR.

To investigate whether TBP-1 possesses an intrinsic activation function, the N-terminal and C-terminal portions of TBP-1 were ligated to GAL4DBD and cotransfected with the upstream activating sequence (UAS)-TK reporter gene into CV-1 cells. As shown in Fig. 7, GAL4VP16 significantly activated the UAS-TK promoter activity. GAL4TBP-1N did not stimulate the promoter activity over that of GAL4DBD itself. In contrast, GAL4TBP-1C significantly stimulated the promoter activity. Identical findings were obtained using HeLa cells (data not shown). These findings indicated that TBP-1 might possess an intrinsic activation function in the C-terminal CAD region.

Ohana et al. (39) reported that mutation or deletion of the putative ATP-binding motif and helicase motif in
the CAD region abolished the intrinsic activation potential of TBP-1. We, therefore, evaluated whether these motifs in the CAD are necessary for enhancement of the TR-mediated transactivation by TBP-1 using a transient transfection assay. As shown in Fig. 8A, a mutation introduced into the putative ATP-binding site (K233H) abolished augmentation of the palindromic reporter activity. In contrast, TBP-1 with a mutation within the putative helicase motif (D286A) showed significant enhancement similar to the wild-type TBP-1. These mutant TBP-1s were similarly bound to TR in a GST pull-down assay (Fig. 8B). These findings clearly revealed that the potential nucleotide binding motif, but not the helicase motif in CAD, was required for augmentation by TBP-1 of the TR-mediated gene activation.

Fig. 5. TBP-1 Potentiates Ligand-Dependent Transcriptional Activation by TR

A, pKCR2TRβ1 (83 ng/well) was cotransfected into CV-1 cells with the palindromic TRE reporter gene (PAL) (1.7 μg/well) in the absence or presence of increasing amounts of pKCR2TBP-1 (170, 500, and 830 ng/well). Luciferase activity was measured after 48 h incubation with or without 10 nM T3. Values represent the mean ± SE from triplicate determinants. B, pKCR2TRβ1 (83 ng/μl) was cotransfected with the DR4 TRE reporter gene (1.7 μg/well) in the presence of pKCR2 or pKCR2TBP-1 (830 ng/well). C, The wild-type or a mutant TRβ1 possessing a substitutive mutation in DBD (C127S) (83 ng/well) was cotransfected with PAL (1.7 μg/well) in the absence or presence of pKCR2TBP-1 (830 ng/well). D, The wild-type or mutant TBP-1 lacking the N-terminal leucine zipper-like structure (830 ng/well) was cotransfected with PAL (1.7 μg/well) in the presence of pKCR2TRβ1 (83 ng/well).
Fig. 6. TBP-1 Specifically Potentiates TR-Mediated Transactivation

A, The TRα1 or TRβ1 expression vector (83 ng/well) was cotransfected with PAL (1.7 μg/well) in the absence or presence of pKCR2TBP-1 (830 ng/well). B, Expression vectors for hRARα, RXRα, PPARγ, or ER (83 ng/well) was transfected with the reporter vector containing retinoic acid response element (DR5), retinoid X response element (DR1), peroxisome proliferator-activated response element (DR1) or estrogen response element (1.7 μg/well), respectively, in the absence or presence of pKCR2TBP-1 (830 ng/well). One micromolar all-trans-RA (atRA), 1 μM 9-cis-RA, 10 μM troglitazone (Tz), or 10 nM E2 was used as a specific ligand for RAR, RXR, PPAR, or ER, respectively. C, Two hybrid interactions of pGBT9RAR (DBD plus a part of the hinge region) and pGBT9RXR with pGAD10TBP-1 in yeast were measured using the β-gal assay. One micromolar 9-cis-RA was used as a ligand for RXR. pGBT9TRDBD was used as a positive control. D, Interactions between [35S]-labeled full-length RAR, RXR, PPAR, and ER vis-à-vis GST alone or full-length TBP (GST TBP-1) were analyzed using a pull-down assay in the presence or absence of cognate ligands.
TBP-1 Requires the AF-2 Domain of TR for Transcriptional Enhancement

In addition to its intrinsic activation potential, it was unclear whether TBP-1 requires interaction with other transcriptional regulators to augment ligand-dependent transactivation by TR. To assess this possibility, we first examined whether TBP-1 could enhance the transactivation function of an AF-2 mutant TR (E457A). E457A has been shown to bind to TRE and possesses an affinity for T3 similar to the wild-type receptor, but lacks the ligand-dependent transactivation function because of its impaired ability for interacting with co-activators (43). As shown in Fig. 9, TBP-1 did not potentiate the promoter activity carrying the palindromic TRE in the presence of liganded E457A, although TBP-1 could directly bind to TRE and possesses an affinity for T3 similar to the wild-type receptor, but lacks the ligand-dependent transactivation function because of its impaired ability for interacting with co-activators (43). As shown in Fig. 9, TBP-1 did not potentiate the promoter activity carrying the palindromic TRE in the presence of liganded E457A, although TBP-1 could directly bind to E457A in a pull-down assay. This indicated that the potentiation by TBP-1 of transactivation by TR might be dependent on the AF-2 domain of TR.

TBP-1 and Coactivators Synergistically Stimulate TR-Mediated Transactivation

The AF-2 of TR has recently been demonstrated to directly interact with multiple protein complexes containing the coactivators and corepressors (6–9). We, therefore, examined whether TBP-1 influences the enhancement of TR-mediated transactivation by coactivators using a transient transfection assay. As shown in Fig. 10A, cotransfection of three coactivators of TR, steroid receptor coactivator 1 (SRC-1), p120, and CBP enhanced the ligand-dependent stimulation by TR of the palindromic TRE in a magnitude similar to the cotransfected TBP-1. When TBP-1 was simultaneously transfected with these coactivators, the promoter activity in the presence of liganded TR was strongly augmented (Fig. 10A). We next examined whether TBP-1 can directly interact with these coactivators using the in vitro pull-down assay. As shown in Fig. 10B, GST-TBP-1 did not interact with in vitro translated SRC-1, p120, and CBP. These findings suggest that TBP-1 could synergistically function with the AF-2-interacting coactivators in the absence of direct interaction.

TBP-1 Directly Interacts with Trip1 in Vivo and in Vitro

TBP-1 has been shown to be able to form a homodimer with TBP-1 itself and to weakly form a heterodimer with TBP7, a putative ATPase highly homologous to MIP224 (38), possibly through its N-terminal leucine zipper-like motif (39). These findings suggested that the homo- or heterodimer formation of TBP-1 might be important for its function and led us to evaluate the interaction between TBP-1 and Trip1, a...
member of the ATPase family that binds to the AF-2 of TR in a ligand-dependent manner (34). As shown in Fig. 11A, [35S]-labeled Trip1 bound to GST-TBP-1, but not to GST itself. Moreover, Trip1 interacted with the N-terminal, but not the C-terminal, region of TBP-1 (Fig. 11A). Synthesis of TBP-1 proteins of appropriate molecular weights were confirmed by SDS-PAGE analyses (Fig. 11B). In agreement with the previous findings (39), [35S]-labeled TBP-1, in addition to Trip1, bound to GST-TBP-1 in a pull-down assay (data not shown). The interaction of the N-terminal, but not the C-terminal, region of TBP-1 with Trip1 was also confirmed using the yeast two-hybrid system (Fig. 11C).

We next examined the effect of transfection of Trip1 on TR-mediated transcription in the presence of cotransfected TBP-1 using a transient transfection into CV-1 cells. Transfection of Trip1 did not enhance the palindromic TRE-driven promoter activity in the presence of cotransfected TBP-1 (E457A) and GST-fused TBP-1 was analyzed using a GST pull-down assay (right panel). These in vitro and in vivo findings suggest that TBP-1 binds to the DBD of TR might cooperate with Trip1, which interacts with the AF-2 via a protein-protein interaction.

**DISCUSSION**

TBP-1 was originally isolated as a protein interacting with the HIV-1 Tat transactivator and was reported to inhibit the TAT-mediated activation of the HIV-1 LTR promoter (32). Proteins highly homologous to the human TBP-1 have subsequently been cloned from different species indicating that TBP-1 might have some evolutionary conserved functions (49–52). The TBP-1 gene is mapped to human chromosome 11p (53, 54) and is ubiquitously expressed in a variety of human cell lines and in rodent tissues (32, 55, 56). However, the physiological importance of TBP-1 in the regulation of the transcription of specific cellular genes remains to be elucidated. The present study, therefore, provides the first evidence that TBP-1 is involved in transcriptional activation of the thyroid hormone-responsive gene.
TBP-1 belongs to a protein family possessing the CAD named AAA family (ATPases associated with a variety of cellular activities) (35), and the CAD region in TBP-1 shares significant homology with those of Trip1, mSUG1, and other members of the ATPase family (34, 35, 37). When compared with Trip1/mSUG1, several unique features of TBP-1 as a transcriptional cofactor for TR were observed in the present study. First, TBP-1 constitutively bound to TR in the presence and absence of T3, whereas Trip1/mSUG1 binds to the receptors in a ligand-dependent manner (34, 37). Second, TBP-1 interacted with the DBD of TR, whereas Trip1/mSUG1 interacts with the LBD of TR (34, 37). Third, overexpression of TBP-1 strongly potentiated the ligand-dependent transactivation by TR in mammalian cells. In contrast, cotransfected Trip1 did not enhance the TR-mediated transactivation in transient transfection assays (Fig. 11D and Ref. 34). Lastly, TBP-1 appeared to selectively function with TR, whereas Trip1/mSUG1 has been shown to interact not only with TR, but also with RAR, RXR, VDR, and ER (34, 37, 57).

In addition, TBP-1 has a characteristic function that requires the AF-2 domain of TR to potentiate the TR-mediated transcription. The AF-2 of NRs was recently shown to interact with multiprotein complexes containing coactivators and cointegrators in the presence of ligand (6–9). In the present study, we showed that TBP-1 was able to directly interact and synergistically function with Trip1, one of the AF-2 interacting cofactors that bind to TR in a ligand-dependent manner. Moreover, the present transfection study revealed that TBP-1 could synergistically enhance the TR-mediated transactivation with a subset of coactivators such as SRC-1, p120, and CBP that interact with the AF-2 of TR, although TBP-1 did not interact with these coactivators in vitro. These findings collectively suggest that TBP-1 might function in cooperation with other transcriptional cofactors interacting with the AF-2 of TR, such as Trip1, to augment the ligand-dependent gene activation.

In agreement with a previous finding reported by Ohana et al. (39), we could detect an intrinsic activation function of TBP-1 using a mammalian one-hybrid...
assay and showed that the activation domain resided within the C-terminal CAD region. TBP-1 lacked the consensus L-x-x-L-L sequence, a characteristic NR-binding site in many coactivators that possess intrinsic histone acetyltransferase activity (36). Mutation of the potential ATP-binding motif, but not the helicase motif, abolished the enhancement of TR-mediated transactivation by TBP-1 in the present study. The recombinant rat SUG1 has been established to possess an RNA-dependent ATPase activity (58). Moreover, mSUG1 has been shown to have a bona fide helicase activity (59) and interacts with the p89/XPB (xeroderma pigmentosum B) subunit of TFIIH (60). The DEAD (the predicted ATPase B motif)-box proteins such as the eukaryotic translation initiation factor 4A and p68 exhibit an RNA-unwinding activity as well as RNA-stimulated ATPase activity (60, 61). Endoh et al. (62) recently reported that p68 stimulates the ER-mediated gene activation via a protein-protein interaction with the AF-1 of ER and showed that the intrinsic helicase activity was dispensable for the coactivator function of p68. In conjunction with these findings, the present study indicates that the putative ATPase activity of TBP-1 is responsible for enhancement of the ligand-dependent gene activation by TR.

TBP-1 and Trip1/mSUG1 are the integral components of the 19S regulatory subunit of the 26S proteasome that degrades the ubiquitinated and nonubiquitinated proteins contributing to a variety of cellular regulatory processes (35, 44, 45). It was, therefore, anticipated that ectopic expression of TBP-1 might alter the levels of TRβ1 protein, thereby affecting the

Fig. 11. TBP-1 Interacts with Trip1 in Vivo and in Vitro
A, Interactions of the GST-fused full-length, N-terminal, and C-terminal portions of TBP-1 with [35S]-labeled full-length Trip1 were examined using a pull-down assay. B, SDS-PAGE analysis of the GST-fused TBP-1 proteins used in panel A. C, Interactions of the N-terminal and C-terminal portions of TBP-1 in pGBT9 with pGAD10Trip1 and pGAD10 were examined using the yeast two-hybrid system. β-gal assay was performed as describe above. D, Increasing amounts of pKCR2TBP-1 (170, 500, and 830 ng/well) were transfected with pKCR2TRβ1 (17 ng/well) into CV-1 cells in the absence or presence of pKCR2Trip1 (170 ng/well). Luciferase activities of PAL in the absence or presence of T3 were measured and were expressed as light units/µg protein.
MATERIALS AND METHODS

TR-mediated transactivation. However, TBP-1 selectively enhanced the TR-mediated, but not other NR-mediated, activation in the present transfection studies. Moreover, TBP-1 overexpressed in a condition identical to that used in transfection studies did not alter the steady-state levels of TRβ1 protein. Makino et al. recently reported that the complex of proteasomal ATPases could directly interact with the TATA-binding protein, and that TBP-1 was present in the complex as well as mSUG1, SUG2, TBPF7, MSS1, S4, and a novel transcriptional regulator, TIP (TBP-interacting protein)120 (63). These findings taken together suggest that the proteasomal function of TBP-1 was likely to be dispensable for augmentation of the TR-mediated gene transcription. TBP-1, like other proteasomal ATPases, might be a multifunctional protein directly involved in the regulation of gene transcription as well as in protein degradation.

The HIV-1 Tat is a powerful activator of viral gene expression from the integrated LTR (64). TR has previously been demonstrated to directly interact with Tat through its DBD and has been shown to stimulate activation of the HIV-1 LTR promoter in the presence of Tat (22). HIV-1 Tat has recently been shown to directly recruit the host transcriptional coregulators to activate the viral gene expression from the integrated LTR promoter (64). TR has previously been shown to stimulate activation of the Tat-mediated HIV-1 LTR promoter in the presence of T3 (22). Further studies are required to ascertain this possibility.

Fig. 12. Overexpression of TBP-1 Did Not Alter the Levels of TR Proteins in CV-1

Cells CV-1 cells in 100-mm² dishes were transfected with pKCR2TRβ1 (5 μg/dish) and TBP-1 (10 μg/dish) using a calcium phosphate precipitation method, and nuclear extracts were prepared after incubation for 48 h with 10 nM T3 or vehicle alone. Forty micrograms of nuclear proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with an anti-TRβ1 monoclonal antibody. Levels of TRβ1 protein were visualized as described in Materials and Methods.

MATERIALS AND METHODS

Plasmids

Expression vectors for human (h)TRβ1, TRα1, RARα, RXRα, PPARγ, and ER in a pKCR2 expression vector were de-
media (Trp+, Leu). Isolation of yeast DNA was performed as described previously (43). The plasmids isolated from positive colonies were amplified on a large scale, and the nucleotide sequences of both strands were determined using an autosequencer (PRISMSTM 310, PE Applied Biosystems, Tokyo, Japan). All the PCR-amplified cDNA fragments used for plasmid construction were verified by direct sequencing.

Sequence Analysis

The plasmids isolated from positive colonies were amplified on a large scale, and the nucleotide sequences of both strands were determined using an autosequencer (PRISMSTM 310, PE Applied Biosystems, Tokyo, Japan). All the PCR-amplified cDNA fragments used for plasmid construction were verified by direct sequencing.

GST Pull-Down Assay

[35S] methionine-labeled full-length and partial proteins of hTRß1 were synthesized by in vitro transcription/translation from pKCR2TRA/B, TRA/B-DBD, TRA/B-hinge, TRLBD, and the full-length TR using T7 RNA polymerase and a TNT-coupled reticulocyte lysate system (Promega Corp.). [35S]-labeled full-length RAR, RXR, PPARγ, and ER proteins were synthesized in vitro from the pKCR2 expression vectors. Synthesis of proteins of expected molecular weights was confirmed by SDS-PAGE analyses. The full-length and PCR-amplified partial fragments of TBP-1 cDNA were ligated in-frame into pGEX4T1 (Pharmacia Biotech, Piscataway, NJ) to yield GST fusion proteins in E. coli DH5α. A cDNA fragment encoding the receptor interaction domain of N-CoR was amplified partial fragments of TBP-1 cDNA were ligated in-frame into pGEX4T1 (Pharmacia Biotech, Piscataway, NJ) to yield GST fusion proteins in E. coli DH5α. A cDNA fragment encoding the receptor interaction domain of N-CoR was amplified by PCR using pKCR2N-Cori (43) as a template and subcloned in frame into pGEX4T1. The GST fusion proteins were purified on glutathione-agarose beads (Sigma, St. Louis, MO) and analyzed by SDS-PAGE. Interaction assays and autoradiographies were performed as described previously (43). Bound protein was quantified by Molecular Imager FX (Bio-Rad Laboratories, Inc., Hercules, CA).

Cell Culture, Transfection, and Luciferase Assay

CV-1 and Hela cells were grown in DMEM supplemented with 10% FBS, as described previously (67, 68). Twenty-four hours before transfection, cells were split into six-well plates in subconfluency. Transient transfection was performed using a calcium phosphate precipitation method, as described previously (67, 68). The total amounts of transfected plasmids were adjusted by adding an empty expression vector in all experiments. Luciferase assay was performed and the luciferase activity was normalized by the protein concentration, as described previously (67, 68). All the transfection experiments were repeated at least twice with triplicate determinants. T3, all-trans-RA, 9-cis-RA, and E2 were purchased from Sigma. Trogilitazone was from Sankyo Co., Ltd. (Tokyo, Japan).

Immunoprecipitation and Immunoblotting

The full-length TBP-1 cDNA was ligated in frame into a pFLAG-CMV-2 expression vector (Sigma) and transfected into CV-1 cells cultured in 100 mm² dishes in the presence of pKCR2TRA/B using a calcium phosphate precipitation method. Forty-eight hours after transfection, nuclear extracts were prepared as previously described (70). After immunoprecipitation with an anti-FLAG M2 monoclonal antibody (Sigma), the samples were resolved on 10% polyacrylamide gels under denaturing conditions and transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Arlington Heights, IL). After incubation in a blocking buffer [20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 5% skimmed milk] for 1 h at room temperature, the membranes were incubated overnight at 4 C with an anti-TR antibody (J52), which recognizes the human TRß1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After extensive washing, the membranes were incubated with a sheep antimouse Ig antibody, and TR proteins were visualized with the ECL Plus Western blotting detection kit (Amersham Pharmacia Biotech).

Mammalian One-Hybrid Assay

The cDNAs corresponding to the N-terminal (residues 17–176) and C-terminal portions (residues 176–439) of TBP-1 were amplified by PCR, ligated in frame into the EcoRI site of pMGAL4DBD (CLONTECH Laboratories, Inc.), and transfected with UAS-TK luciferase reporter (43) into CV-1 and Hela cells as described above. The herpes simplex virus VP16 ligated to GAL4DBD (GAL4VP16) (CLONTECH Laboratories, Inc.) was used as a positive control. Luciferase activities were measured as described above.

Statistical Analysis

Statistical analyses were performed using Duncan’s multiple range test among multiple groups. The level of significance was set at P < 0.05.

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