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Molecular Cloning and Characterization of a Novel TBP-1 Interacting Protein (TBPIP):Enhancement of TBP-1 Action on Tat by TBPIP

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The human immunodeficiency virus-1 (HIV-1) protein Tat, encoded by one of the HIV regulatory genes, tat, is reported to be essential for HIV gene expression and replication in infected cells. Observations suggest that several cellular factors cooperate with Tat in this process. Tat binding protein-1 (TBP-1) is reported to be one such cellular factor that specifically suppresses Tat-mediated transactivation of HIV replication in vitro. Here we have cloned a novel factor, TBP-1 interacting protein (TBPIP) from the mouse, which interacts with mouse TBP-1. TBPIP contains several kinase phosphorylation sites and co-localizes with TBP-1 in vivo. The fact that Tat activity is altered synergistically by the TBP-1 and an additional TBP-1 binding protein has not been reported before. We provide evidence that expression of TBPIP enhances the inhibitory action of TBP-1 on Tat-mediated transactivation in vitro. Our results suggest that TBPIP may have a key role in suppressing the Tat-mediated transactivation.

Human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, is a retrovirus that encodes two classes of genes; structural genes such as gag, pol and env that identify viral capsid and core protein and regulatory genes that modulate the replication of viral genes. HIV-1 Tat protein, encoded by one of the viral regulatory genes, tat, is a powerful transactivator of viral gene expression and is essential for virus replication (1-3). Tat activates the viral gene expression from the HIV-1 long terminal repeat (LTR) promoter through the transactivation responsive RNA element, TAR, which is a nascent mRNA located immediately downstream of the transcription initiation site (4-8). It has been reported that several candidate cellular factors (9-15) which interact with Tat may play a role in Tat-mediated transactivation. The function of these cellular factors in Tat-mediated transactivation remains to be elucidated. TBP-1 was identified as one such cellular factor several years ago. It was suggested that TBP-1 would specifically suppress Tat-mediated transactivation of HIV-1 replication in vitro (12). Ever since TBP-1 was first reported, it has been controversial as to whether TBP-1 is an inhibitory factor on Tat activity or not (16, 17). In addition, the several molecules which have the similar *c*onserved *A*TPase containing *d*omain (CAD) of TBP-1 have been reported (conserved ATPase family), and then one such molecule enhances Tat activity (e.g., MSS-1) (18). The N-terminal of TBP-1 is critical to the uniqueness of TBP-1 among the conserved ATPase family molecules (19), but the original assayed human TBP-1 cDNA lacked its N-terminal region (12). Hence, we have shown (i) the inhibitory action of TBP-1 on Tat activity with the full-length mouse TBP-1 (mTBP-1) cDNA that we have obtained and (ii) its heterogenous localization in vivo, especially robust expression in the cells in seminiferous tubules of testis (Nakamura, T. et al. submitted.).

Here, using the mouse testis cDNA library, we have cloned TBP-1 interacting protein (TBPIP), a novel factor that interacts with mTBP-1. TBPIP is co-localized with TBP-1 in vivo and enhances the inhibitory action of TBP-1 on Tat activity in vitro.

MATERIALS AND METHODS

Preparation of mammalian expression plasmids (pCI-mTBP1, pCI-TBPIP). The mTBP-1 cDNA was amplified with Taq DNA polymerase using two primers: a forward primer which includes the initiation site of mTBP-1 with EcoR I restriction site in the 5′ end: 5′-TTCGGCGAATTCATGCAGGAAATGAATCTGCTG-3′, a reverse primer

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Abbreviations: PCR, polymerase chain reaction; PVDF, poly vinylidene difluoride; SSC, saline sodium citrate; SDS, sodium dodecyl sulfate; kb, kilobase pair(s); KDa, kilodalton.

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which includes the termination site of mTBP-1 with Sal I restriction site in the 3′ end: 5′-TAGAGGCAGCTGCTAGGCATAGTATTGTAG-GTT-3′. Condition for PCR were 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min, and then a final extension at 72°C for 10 min. The amplified fragments were digested with EcoR I and Sal I restriction enzymes, then were subcloned into the EcoR I/ Sal I site of pCI mammalian expression vector (Promega) to make an mTBP-1 expression plasmid (pCI-mTBP1). TBPIP cDNA, obtained in pGAD10-TBPIP which we had identified by the two-hybrid method (see two-hybrid library screening section), was amplified with two primers. Sequence of the primers used are as follows: a forward primer including the initiation site of TBPIP with Xho I restriction site in the 5′ end: 5′-CCGCTCGAGATGAGTAAAAGC-CGGGCCGAG-3′, a reverse primer including terminal site of TBPIP with EcoR I restriction site in the 3′ end: 5′-CCGGAATTCTTACCA-GCCTCTATCTAACAC-3′. PCR was performed under the same condition as mentioned above. This Xho I/ EcoR I fragments were cloned into the pCI mammalian expression vector to make a TBPIP expression plasmid (pCI-TBPIP).

Cotransfections and CAT assay. The pCI-mTBP1, pCI-TBPIP, or both plasmids were cotransfected into CHO cells with the Tat-expression plasmid (pCV1-Tat) (20) and the reporter plasmid (pBennCAT) (21) using the Tfx-50 reagent (Promega). pBennCAT contains HIV LTR and a chloramphenicol acetyltransferase (CAT) reporter gene. Tat is reported to increase the number of steady-state mRNA transcribed from HIV LTR, that is, to increase CAT activity. Cells were harvested 48hr after transfection, and CAT activities were measured by Quan-T-CAT assay system according to the manufacturer's protocol (Amersham). For quantification of CAT activity, the activity of [^{3}H] acetylated biotinylated chloramphenicol was measured.

Animals. Adult male ICR mice (10 weeks old) were used. All mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg body weight) before the following experiments.

Probe synthesis. The full-length of TBPIP cDNA was obtained from the pGAD10-TBPIP plasmid, then subcloned into pGEM-T vector (Promega). The sequence of this subcloned cDNA fragment was confirmed prior to Northern blotting analysis and in situ hybridization histochemistry.

Northern blot analysis. Each 20μg of total RNA isolated from mouse testis and spleen was separated on formaldehyde-agarose gels (120 V, for 2 hours) and transferred onto PVDF membrane (Immobilon-N, Millipore). Membranes were hybridized with the ^{32}P-dCTP labelled cDNA fragment of TBPIP.

In situ hybridization histochemistry. The protocol for in situ hybridization histochemistry was based on the published method (22, 23). Adult male ICR mice (10 weeks old) were anesthetized, then perfused transcardially with saline followed by ice-cold 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (PB). After perfusion, the mouse testis was postfixed with the same fixative at 4 °C for 1 day and immersed in 30 % sucrose in 0.1M PB at 4 °C for 2-3 days. They were then frozen with powdered dry-ice and 15 μm thick frozen sections of mouse testis were made on a cryostat. As a pretreatment, tissue sections were fixed with formaldehyde, digested with proteinase K, acetylated with acetic acid anhydride, dehydrated and dried. [α-^{35}S]UTP-labeled single-strand RNA synthesized with the Apa I-digested template plasmid containing the TBPIP fragment (see probe synthesis section) and SP6 RNA polymerase was used as the TBPIP antisense probe. [α-^{35}S]UTP-labeled single-strand RNA synthesized with the Sac I-digested same template plasmid and T7 RNA polymerase was used as the TBPIP sense probe. Hybridization and washing procedures were the same as the published protocols (22). Hybridization signals were visualized by emulsion autoradiography (Irford, UK). On identification of positive hybridization signals, only cells with a grain density at least 3 times higher than the background density were considered to be positive (23).

RT-PCR for mouse lymphocytes. Lymphocytes were prepared from 20 mice spleens by gradient separation using the Ficoll based medium. Enriched CD4+ T cells were recovered by a process of negative selection of the lymphocyte fraction, using an affinity chromatography column for mouse CD4+ T cell (Cedarlane, Canada). Total RNA were isolated from mouse testis and CD4+ T cells using Isogen (Nippongene, Japan). RNA from Kupffer cells was gifted from Dr. H. Tsutsui and it was extracted from the purified Kupffer cells collected by the published method (24). Identification of macrophage origin was confirmed by RT-PCR for IL-12p40. Single strand-cDNA primed with the random primer was synthesized from each 5 μg total RNA using a First-strand cDNA synthesis kit (Pharmacia). The primers used for the RT-PCR analysis were designed as follows: The sense primer for TBPIP was 5′-ATGAGTAAAAGCCGGGCCGA-3′ and the antisense primer was 5′-TCAGGGGTCGGGGAGCAAAA-3′. The sense primer for IL-12p40 was 5′-CGTGCTCATGGCTGGTGG-AAAG-3′ (bp 447-4680) and the antisense primer was 5′-GAACAC-ATGCCCACTTGCTG-3′ (bp 1022-1003). Conditions for PCR were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min.

Antibody production and western blot analysis. Polyclonal antisera were generated against the peptide proteins specific to TBPIP (aa 1-16). Antisera were initially tested for their ability to react with the peptide by dot blotting. Antisera were purified by affinity column of the synthetic peptide conjugated to FMP (2-*f*luolo-1-*m*ethyl*p*yridinium toluene-4-sulfonate) activated Cellulofine (Seikagaku, Japan). Homogenized protein extracts were prepared from the ICR mouse testis tissue with sample buffer (125 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, 2% SDS, 0.1% glycerol). Aliquotes (50 μg) were resolved by electrophoresis on 12 % SDS-polyacrylamide gels by using Mini-PROTEAN II Electrophoresis Cell (200V, 45min) and transferred to nitrocellulose membrane (Trans-Blot Transfer Medium, BioRad). Antigen-bound anti-TBPIP antibody was detected with a goat anti-rabbit IgG-alkaline phosphatase and color development reagent.

Immunohistochemical analysis. The immunohistochemical procedure was carried out with 5 μm tissue section using the avitin-biotin-peroxidase complex method (Vectastain ABC kit). Briefly, the reaction with the anti-TBPIP antibody (1:100 dilution) was done overnight at 4 °C. This was followed by incubation with the biotinylated anti-rabbit IgG (1:200 dilution) for 2 hr at room temperature, then with the avidin-biotin complex (1:1000) and finally with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide to visualize the immunohistochemical reaction.

RESULTS

A Novel Gene Encoding One Such Candidate Protein That Binds to mTBP-1 Was Identified as TBP-1 Interacting Protein (TBPIP)

By the yeast two-hybrid screening, two potential candidate clones of mTBP-1 binding protein were identified. To further characterize these candidate cDNAs, we determined their partial DNA sequences and carried out homology searches using fasta program against DDBJ, GenBank and EMBL data bases. The homology searches indicated that both these clones were found to be novel. One of two candidate genes was studied in this study. We identified this clone as TBP-1 interacting protein (TBPIP), which was composed of 874 bp encoding 217 amino acid residues (651 bp) to be consistent with the size of TBPIP mRNA (approxi-

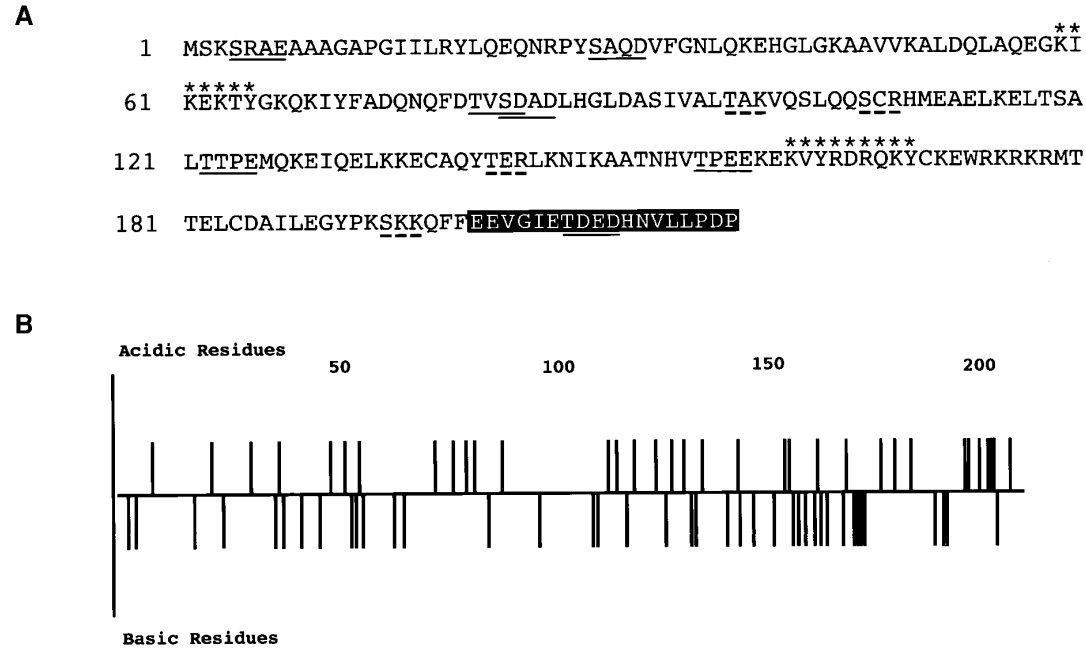

FIG. 1. Deduced amino acid sequence, domain structure and distribution of charged acidic (Glu and Asp) and basic (Lys and Arg) amino acids in TBPIP. (A) The two tyrosine kinase phosphorylation sites (25) are marked with asterisks. The seven casein kinase II phosphorylation sites (26) are underlined and the four protein kinase C phosphorylation sites (27) are underlined with broken lines. The C-terminal acidic region are shown in the shaded area and acidic amino acid residues (Glu and Asp) are rich. (B) TBPIP has a high content (32%) of charged acidic and basic amino acids and shows unusual distribution of these amino acids.

mately 0.9kb) accessed by Northern blot analysis (Fig. 1A, 2A).

The sequence analysis of TBPIP revealed that it contains two tyrosine kinase phosphorylation sites (25) and many serine and threonine residues that are arranged in a short peptide sequence matching consensus sites for phosphorylation by casein kinase II (26) and / or protein kinase C (27) (Fig. 1A). Furthermore, TBPIP has a high content and unusual distribution of charged amino acids (Fig. 1B). TBPIP 1-217 possesses a neutral isoelectric point of 7.8 and contains 35 acidic amino acids (Glu and Asp) and 35 basic amino acids (Arg and Lys); thus, 32% of the total amino acid residues are charged. TBPIP 200-217 is the most acidic region, with an isoelectric point of 3.5 due to the existence of glutamate and aspartate-rich in the sequence of last 18 amino acid residues (Fig. 1A).

Nucleotide sequence of TBPIP shows high similarity (86.2% homology) to that of GT198 sequence, which has been isolated from human breast cancer cell cDNA library (28). However, the length of deduced amino acid sequence of GT198 is 203; 14 amino acid residues shorter than that of full-length TBPIP.

TBPIP mRNA Is Strongly Expressed in the Mouse Testis and Localizes in the Mouse CD4+ Lymphocytes

We investigated whether TBPIP is actually co-localized in the cells with mTBP-1 in the adult mouse testis from which TBPIP cDNA originated. In situ hybridization histochemistry revealed a remarkable expression of TBPIP mRNA in the cells in seminiferous tubules where mTBP-1 is localized (Fig. 2B). Furthermore, we examined whether it was possible that TBPIP coexisted with mTBP-1 in the CD4+ lymphocyte which was a major target of HIV infection. Although Northern blot analysis of TBPIP showed that there was no significant detectable signals in mouse spleen (data not shown) where TBP-1 is expressed, expression of TBPIP mRNA in both CD4+ T cell and macrophage preparations was confirmed by RT-PCR (data not shown); yet its level of expression was not high in either preparation. PCR products were clearly stained by the ethidium bromide after 35 cycles of amplification procedures, while were not distinctly recognized after 25 cycles.

TBPIP Is Highly Expressed in the Nuclei of Germ Cells in Seminiferous Tubules

Polyclonal antibody against TBPIP was characterized by Western blot analysis. A band of approximately 24 KDa in size was visualized in the testis preparation (Fig. 2C). Immunohistochemistry was carried out using an obtained antibody purified with a peptide-conjugated column. In the testis, while Leydig cells are free from positive staining, the nuclei of primary and secondary spermatocytes express TBPIP most abundantly among all germ cells processing the spermatogenesis.(Fig. 2D).

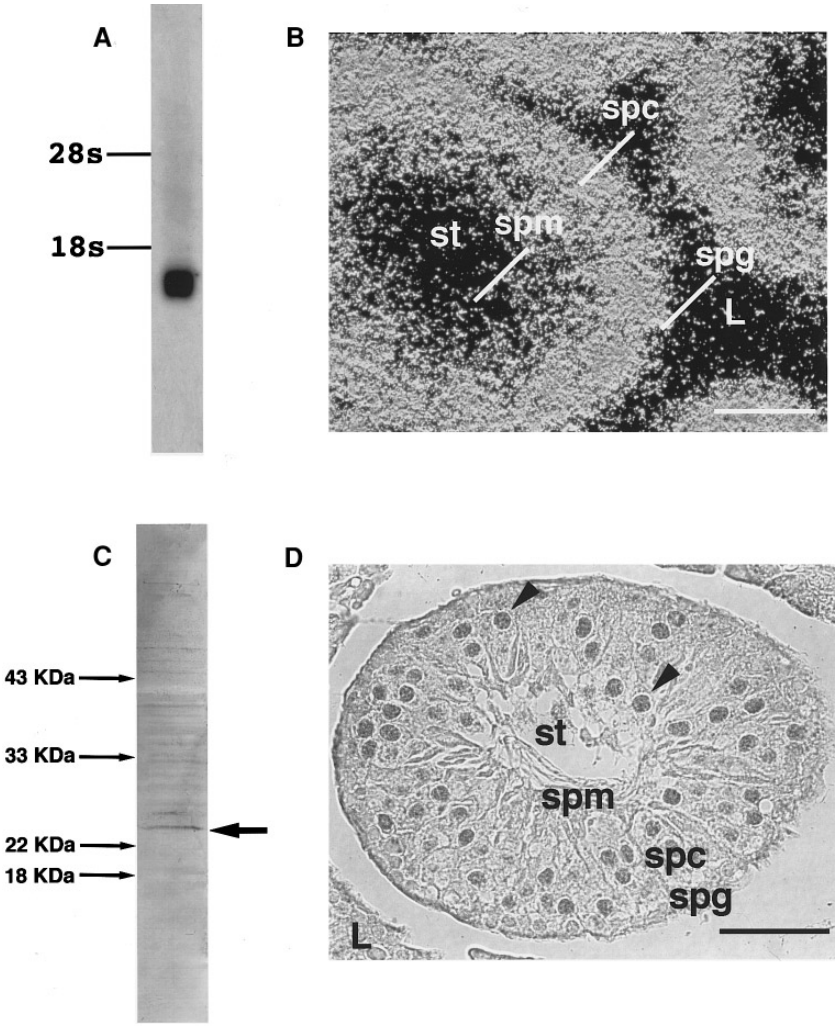

FIG. 2. TBPIP expression in the testis. (A) Northern blot analysis. TBPIP mRNA is approximately 0.9 kb in size. (B) Strong expression of TBPIP mRNA in the seminiferous tubule (st) is observed. spg: spermatogonium, spc: spermatocyte, spm: sperm, L: Leydig cell. Scale bar = 200 μm. (C) Western blot analysis of the raised anti-TBPIP antibody. Immunoblot analysis was performed with purified anti-TBPIP antibody with the testis preparations. The antibody detected a single protein with an apparent molecular mass of approximately 24 KDa, consistent with the size predicted by TBPIP amino acid sequence. (D) Immunohistochemical demonstration of TBPIP in mouse testis. TBPIP is highly expressed in the spermatogenesis-processing cells. The nuclei of primary and secondary spermatocytes (spc) in the seminiferous tubules (st) are immuno-positive for TBPIP. The positive stained examples are shown by arrowheads. spg: spermatogonium, spc: spermatocyte, spm: sperm, L: Leydig cell. Scale bar = 200 μm.

TBPIP Enhances the TBP-1 Inhibitory Action on Tat Activity in Vitro

TBP-1 was reported first as an inhibitory cofactor suppressing Tat-mediated transactivation in vitro (12). We have already examined whether or not the TBP-1 could inhibit Tat activity with a full-length mouse TBP-1 cDNA. Our results reconfirm the previously reported data. Since the histological analysis of TBPIP suggested its close association with TBP-1, CAT assay was carried out to observe whether or not TBPIP could influence the inhibitory action of TBP-1 on Tat activity. When pCI-TBPIP was cotransfected with pCV1-Tat (20), pBennCAT (21) and pCI-mTBP1 into CHO cells, the Tat-mediated expression of the CAT gene was markedly suppressed, as compared to the inhibition of Tat-mediated expression by the pCI-mTBP1 without pCI-TBPIP. On the contrary, Tat-mediated transactivation was not significantly affected in CHO cells cotransfected with pCI-TBPIP, pCV1-Tat and pBennCAT (Fig. 3). These findings suggest that TBPIP can synergistically cooperate with TBP-1 to suppress Tat-mediated transactivation from the HIV LTR promoter.

DISCUSSION

Several reports have raised the controversy as to whether the TBP-1 actually exerts an inhibitory action

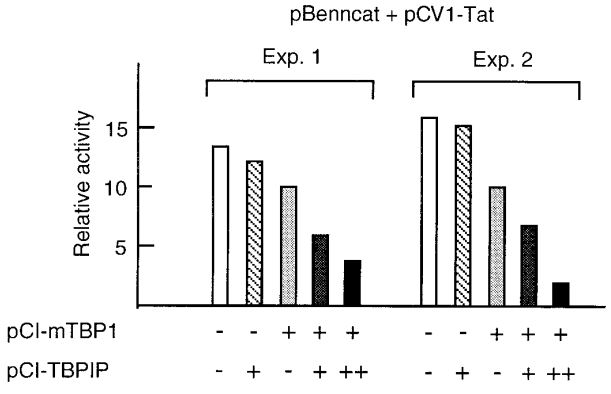

FIG. 3. Enhancement of TBP-1 action by TBPIP accessed by CAT assay. Half μg of pBennCAT, pCV1-Tat and variable amounts of pCI-mTBP1 and pCI-TBPIP plasmids were cotransfected into CHO cells; then chloramphenicol activity was measured in 2 series of experiments. Activities were represented relatively as shown in the graph with the standard of 1 representing the background activity in the CHO cells alone. Tat activity was not substantially inhibited in the presence of TBPIP alone, while that was dramatically inhibited when both the TBP-1 and TBPIP were present. + indicates 0.5 μg, ++ indicates 2.0 μg, while - indicates 0.

on Tat activity or not (16, 17). In this respect, we have already confirmed the suppressive effect of TBP-1 with an apparent full-length mouse TBP-1 cDNA which we have cloned (Nakamura, T. et al., submitted). However, we speculate that the in vitro results obtained by CAT assay may not be biologically relevant to the Tat-mediated transactivation in vivo. We surmise that there is a strong possibility that the Tat-mediated transactivation in vivo is altered by an additional molecule(s) associated with TBP-1. It is noteworthy that TBP-1 is capable of binding to several factors, as TBP-1 could work as a huge protein complex (26S proteasome) (29-31). Therefore we searched for proteins that bind to mTBP-1 using the yeast two-hybrid system in order to find molecules involved in the function of TBP-1 in vivo. As quantities of TBP-1 are expressed in the testis, we employed a mouse testis cDNA library and set mTBP-1 as the bait for this library for the two-hybrid system.

TBPIP contains multiple kinase phosphorylation sites (25-27). Of interest, The C-terminal 200-217 region of TBPIP is rich in acidic amino acids (Glu and Asp) (Fig. 1A), implying that this structural domain is associated with several potent transcriptional activators (32, 33). This region also contains a casein kinase II phosphorylation site. Such phosphorylation could contribute to additional negative charges of this region. Although TBPIP show high sequence similarity to GT198 which is cloned from human breast cancer cDNA (28), it is not apparent whether the GT198 is the human counterpart of TBPIP or not at present.

Several lines of experiments suggest that cofactors exist which mediate interactions between Tat and the cellular transcriptional machinery (9, 34). However, it remains to be determined how these cofactors associated with Tat affect such cellular transcriptional machinery. In addition, it has been shown that (i) one cellular kinase (e.g., TFIIH kinase) affects the Tat action on RNA polymerase II (pol II) processivity, involving the phosphorylation of the carboxyl-terminal domain (CTD) of pol II (35, 36), and (ii) the other cellular kinases which phosphorylate a cofactor (e.g., Tat-SF1) enhance the Tat mediated transactivation (15). Our results show that TBPIP works indirectly and synergistically to suppress Tat activity with TBP-1, although it is still possible that TBPIP acts on other molecules distinct from TBP-1, that are involved in the suppressive events of TBP-1 on Tat activity. More in-depth study is needed regarding how TBPIP contributes to TBP-1 through a protein complex comprising of Tat, basal transcriptional factors and cellular kinases, that is recruited to the transactivation responsive element (TAR).

We have shown that mTBP-1 is strongly expressed in the nuclei of germ cells in seminiferous tubules of mouse testis, where TBPIP is localized. This fact suggests that TBP-1 and TBPIP cooperate for the biological events to occur in the testis (e.g., spermatogenesis). However, the expression of TBPIP is observed in the late maturation stage-specific manner compared to that of mTBP-1; TBPIP is mainly observed in spermatocytes while mTBP-1 is localized in both spermatogonia and spermatocytes. Thus it is possible that TBPIP contributes to the certain stage of spermatogenesis, particularly to the meiotic phase.

In regard to the HIV-1 proliferation in vivo, weak proliferation of HIV-1 in seminiferous tubules is confirmed by in situ PCR (37). It is possible that both TBP-1 and TBPIP coexist with HIV-1 in infected germ cells and function together as a suppressor of virus replication there. In the CD4+ lymphocytes (T cell and macrophage), which are a major target of HIV-1 infection, the level of TBPIP mRNA expression is not high. RT-PCR products of TBPIP were not clearly recognized after 25 cycles of amplification, yet confirmed after 35 cycles of amplification. On the contrary, the RT-PCR products of mTBP-1 were easily identified in both CD4+ T cell and macrophage preparations after 25 cycles of amplification (unpublished observation). These results indicate the coexistence of TBPIP and mTBP-1 in the CD4+ lymphocytes regardless of the difference in amounts of mRNA expression. TBPIP is an encouraging candidate for modulating HIV proliferation in vivo. Hence we speculate that overexpression of TBPIP in CD4+ lymphocytes could enhance the cellular suppressive effect against HIV proliferation.

It has been found that TBP-1 is one such component of 26 S proteasome (29-31), a large multiprotein complex that degrades proteins targeted for degradation by the ubiquitin pathway in an ATP-dependent fashion. It is composed of two functionally independent parts—

20 S proteasome, a core catalytic subunit that can act as a peptidase and a 19 S regulatory complex (so-called PA700) that confers the possibility to bind and process ubiquitinated substrates in an ATP-dependent manner (38). There are ~ 18 different polypeptides in PA700, including some CAD proteins of S4 (39), MSS1 (39), TBP7 (40, 41) and p45 (42, 43). TBP-1 is also identified as a subunit of PA700 (30, 31). Furthermore it has been speculated that TBP-1 is a subunit of both PA700 and the PA700 dependent activator (modulator) (31). It is possible that TBPIP is a component of 26 S proteasome, particularly PA700 and modulator, or TBPIP could operate as a molecule that interferes with 26 S proteasome.

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