Loss of Thioredoxin-Binding Protein-2/Vitamin D3 Up-Regulated Protein 1 in Human T-Cell Leukemia Virus Type I-Dependent T-Cell Transformation: Implications for Adult T-Cell Leukemia Leukemogenesis

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ABSTRACT

Human T-cell leukemia virus type I (HTLV-I) is the causative agent of adult T-cell leukemia (ATL). However, the low incidence of ATL among HTLV-I-infected carriers, together with a long latent period, suggests that multiple host-viral events are involved in the progression of HTLV-I-dependent transformation and subsequent development of ATL. Human thioredoxin (TRX) is a redox active protein highly expressed in HTLV-I-transformed cell lines, whereas the TRX-binding protein-2/vitamin D3 up-regulated protein 1 (TBP-2/VDUP1) was recently identified as a negative regulator of TRX. We report here that expression of TBP-2 is lost in HTLV-I-positive, interleukin-2-independent T-cell lines but maintained in HTLV-I-positive, interleukin-2-dependent T-cell lines, as well as HTLV-I-negative T-cell lines. Ectopic overexpression of TBP-2 in HTLV-I-positive T cells resulted in growth suppression. In the TBP-2-overexpressing cells, a G1 arrest was observed in association with an increase of p16 expression and reduction of retinoblastoma phosphorylation. The results suggest that TBP-2 plays a crucial role in the growth regulation of T cells and that the loss of TBP-2 expression in HTLV-I-infected T cells is one of the key events involved in the multistep progression of ATL leukemogenesis.

INTRODUCTION

Human T-cell leukemia virus type I (HTLV-I) is the causative agent of adult T-cell leukemia (ATL; Refs. 1–6). HTLV-I infection triggers an initial key event in virus-infected cells, followed by as yet undetermined multiple changes, leading to the full development of ATL (7). In an in vitro model, HTLV-I-infected T cells require interleukin-2 (IL-2) to proliferate in the early phase of transformation but subsequently lose cell cycle control in the late phase, as indicated by their continuous proliferative state in the absence of IL-2. The change of cell growth phenotype has been suggested to be one of the oncogenic transformation processes (8, 9). Because the viral oncoprotein Tax, a Mr 40,000 transcriptional activator of HTLV-I, transactivates not only the genes of its own virus but also a set of cellular genes and proteins, including IL-2, the chain of the IL-2 receptor (IL-2R) genes and proteins, such as thioredoxin (TRX; Refs. 10– 13), interleukin-1 (IL-1), granulocyte macrophage colony-stimulating factor, and several immediate early response genes (5, 10, 11), Tax was suggested to play a key role in the events leading to T-cell transformation. However, several lines of evidence have suggested that not just Tax but multiple cellular events are involved in the HTLV-I-mediated cellular transformation (11). Indeed, HTLV-I-infected T cells exhibit augmented production of a variety of cytokines and other inducible proteins, such as thioredoxin (TRX; Refs. 4 and 12). Human TRX was first identified in the HTLV-I-positive T-cell lines as an IL-2rα-inducing factor and designated ATL-derived factor (13, 14). It is a small ubiquitous protein having a redox-active disulfide/dithiold within the conserved active site sequence (15) and is involved in a variety of cellular responses, including cell proliferation, apoptosis, and the activation of transcription factors (16, 17). Recently, we identified TRX-binding protein-2 (TBP-2) as an endogenous molecule interacting with TRX (18). Database analysis revealed that the protein is identical to the vitamin D3 up-regulated protein 1 reported previously (19). The protein stably interacted with TRX in vitro and in vivo and has an inhibitory effect on TRX-dependent reducing activity. Therefore, TBP-2 is considered to be a negative regulator of the biological function of TRX. In this study, we investigated the possible involvement of TBP-2 in HTLV-I-mediated cellular transformation and cell proliferation. The results suggest that loss of TBP-2 expression is one of the key events in the proliferation of HTLV-I-positive cells and that the protein plays a crucial role in the regulation of HTLV-I-dependent transformation.

MATERIALS AND METHODS

Cell Culture and Transfections. Human lymphohematopoietic T-cell lines (Jurkat, Molt4, Hut78, CCRF-HSB2, CCRF-CEM, and HPB-ALL) and HTLV-I-positive T-cell lines (ATL2, MT1, MT2, and Hut102) were cultured in RPMI 1640 containing 10% heat-inactivated FCS and antibiotics (penicillin at 100 units/ml and streptomycin at 100 μg/ml) at 37°C in 5% CO2 in air. HTLV-I-positive T-cell lines ATL-2, ATL-16T, ATL-35T, and ATL-43T, and ED04515 were established by culturing leukemic cells from ATL patients in the presence of IL-2 (7.5 ng/ml). To establish IL-2-independent cell lines, these cell lines were cultured in the absence of IL-2, and the cells that grew were cloned. Each set of IL-2-dependent/independent cell lines was confirmed to be of identical clonal origin, based on T-cell receptor β gene rearrangements and HTLV-I proviral integration sites (20). Stable transfectants were generated by electroporation of 1 × 107 MT2 clone cells, which are IL-2-independent and lack TBP-2, with 10 μg of either pCMV-Tag2 control plasmid or pCMV-Tag2 containing the TBP-2 gene using a Gene Pulser (Bio-Rad, Hercules, CA) at 400 V and 950 μF. After 24 h, cells were plated and selected in medium containing G418 at 2 mg/ml (Nakalai, Kyoto, Japan). The expression of TBP-2 in the transfectants was confirmed by reverse transcription-PCR and Western blotting.

Northern Blot Analysis. Total RNA was extracted using TRIzol (Life Technologies, Inc., Rockville, MD). Twenty micrograms of total RNA per lane were resolved by electrophoresis on a 1% agarose gel containing formaldehyde and then transferred to a nylon membrane by capillary blotting. Hybridization and washing procedures were performed as described previously (18).

Reverse Transcription-PCR. Total RNA from each cell preparation was extracted using TRIZol (Life Technologies, Inc., Rockville, MD). First strand cDNA was prepared using the Superscript preamplification system (Life Technologies) with oligo dT12–18. The primers used for the amplification of the TBP-2 transcript were as follows: TBP-2, 5′-ATGTTGATGGTTCAGAGATCAAG-3′ (forward) and 5′-CTCAGGG-GCATACTAAAGA-3′ (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5′-ATGGGGAAGGTGAGGTGTCGAGTC-3′ (forward), 5′-CCATGC-CAGTGAGCTTCCCGTTC-3′ (reverse). PCR for TBP-2 and glyceraldehyde-3-phosphate dehydrogenase was conducted in a GeneAmp PCR system 9700.
(PE Applied Biosystems) for 30 cycles (denaturing at 94°C for 30 s, annealing at 53°C for 45 s, and extension at 72°C for 1.5 min), respectively. PCR products were then subjected to electrophoresis on a 1% agarose gel containing ethidium bromide.

Immunoprecipitation and Western Blot Analysis. Cell lysates were prepared with lysis buffer [150 mM NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS, 20 mM Tris-HCl (pH 7.5), and protease inhibitors]. An aliquot of the cell lysates was incubated overnight at 4°C with anti-TBP-2-monomonal antibody (Ab; Yat818)-immobilized Sepharose. Immunoprecipitates were resolved on an SDS-10% polyacrylamide gel and then analyzed by Western blotting with anti-TBP-2 (Yat818) or TRX monoclonal antibody (MAb; ADF11).

Preparation of TAT Fusion Proteins. A TAT-mediated protein transduction technique that can allow exogenous recombinant proteins to be transduced into cells was recently introduced by Nagahara et al. (21). TAT-TBP-2 fusion constructs were prepared by PCR cloning and subsequent ligation into the Ncol-EcoRI cloning sites of the vector pTAT-HA vector (a gift from Dr. S. F. Dowdy, Washington University, St. Louis, MO) or the EcoRI-Xhol cloning sites of pGEX-TAT (a gift from Dr. Kizaka-Kondoh, Kyoto University, Kyoto, Japan; Ref. 22). PCR fragments of the full-length TBP-2 were amplified with the primers 5′-CCATGGTGATGTTCAAGAAGATCAAG-3′ and 5′-GAATTCTTCAGTCACATTTGTTG-3′. All constructs were verified by sequencing. pTAT-HA-cDNA plasmids were then introduced into the bacterial strain BL21 (DE3) LysS (Novagen, Madison, WI). The bacterial pellet was dissolved in lysis buffer [20 mM phosphate buffer (pH 7.0), 150 mM NaCl, 10% glycerol, and protease inhibitors], incubated on ice for 30 min, and sonicated. Then, the cell lysate was centrifuged at 13,000 rpm for 30 min. The supernatant was applied to a Ni-NTA column equilibrated with 20 mM imidazol in lysis buffer, and the TAT-fusion protein was eluted with 500 mM imidazol. Endotoxins in the purified samples were removed using Detoxi-gel affinity (Pierce, Rockford, IL). pGEX-TAT and pGEX-TAT-TBP-2 were introduced into BL21Gold(DE3)pLysS, and the fusion proteins were purified using Glutathione Sepharose 4B and PreScission Protease (Amersham Pharmacia Biotech) following the manufacturer’s directions.

Cell Proliferation Assay. Cells (3–10^3 cells/well) were cultured in 96-well, flat-bottomed microtiter plates (Nunc, Inc., Naperville, IL) in triplicates. At different time points of the culture (1–5 days), cell proliferation was assessed based on the formation of formazan using a WST-1 cell proliferation assay kit (TaKaRa, Shiga, Japan). For the experiments with the TAT-mediated protein transduction technique, each TAT-fused protein was added at a specific concentrations at the initiation of culture.

Cell Cycle Analysis. Cells were fixed with 70% ethanol and incubated overnight at 4°C. Fixed cells were subsequently washed, treated with 5 μg/ml RNase A, and stained with 50 μg/ml propidium iodide. The analysis of DNA content was performed in a Beckton Dickinson FACS Calibur with Beckton Dickinson Cell Quest software (Franklin Lakes, NJ).

RESULTS

Loss of TBP-2 Expression in HTLV-I-Positive T-Cell Lines. The expression of TBP-2 mRNA in HTLV-I-positive and -negative T-cell lines was assessed by Northern blot analysis. The mRNA was

Fig. 1. Association of thioredoxin-binding protein-2 (TBP-2) expression in human T-cell leukemia virus type I (HTLV-I)-positive cell lines with interleukin-2 (IL-2) dependence. A, expression of TBP-2 and thioredoxin (TRX) mRNA in HTLV-I-positive (ATL-2, MT1, MT2, and Hut102) and -negative (Jurkat and MOLT4) T-cell lines analyzed by Northern blotting. B, reverse transcription-PCR analysis of TBP-2 mRNA expression in HTLV-I-positive (ATL-2 and MT2) T-cell lines and nonadult T-cell leukemia (ATL) T-cell lines derived from T-cell malignancies (Jurkat, MOLT4, Hut78, CCRF-HSB2, CCRF-CEM, and HPB-ALL). C, protein expression of HTLV-I-negative and -positive T-cell lines detected by immunoprecipitation and Western blotting with anti-TBP-2 (Yat818) or TRX monoclonal antibody (MAb; ADF11).
detected in two HTLV-I-negative cell lines (Jurkat and Molt4) but not in the four HTLV-I-positive T-cell lines (ATL-2, Hut102, MT1, and MT2; Fig. 1A). The loss of TBP-2 expression in the HTLV-I-positive T-cell lines was confirmed by reverse transcription-PCR (Fig. 1B). In addition to Jurkat and Molt4, four other non-ATL malignant T-cell lines derived from T-cell malignancies were found to maintain TBP-2 expression (Fig. 1B). These results indicate a complete loss of TBP-2 expression in the HTLV-I-positive T-cell lines. Protein levels of TBP-2 were analyzed by immunoprecipitation with anti-TBP-2 monoclonal Ab (Yat818) followed by Western blotting with anti-TBP-2 polyclonal Ab. The results shown in Fig. 1C indicated that the expression of TBP-2 paralleled that of TBP-2 mRNA. Consistent with an earlier report (23), the expression of TRX was markedly (ATL-2 and MT2) or moderately (Hut102 and MT1) enhanced in the HTLV-I-positive T-cell lines compared with the HTLV-I-negative T-cell lines. As expected, expression levels of TRX protein, determined by Western blot analysis with anti-TRX monoclonal Ab, paralleled those of TRX mRNA (Fig. 1, A and C).

**Association of IL-2-Independent Phenotype of HTLV-I-Positive Cells with the Loss of TBP-2 Expression.** After infection with HTLV-I, T-cells are easily immortalized in the presence of IL-2, but some of the cells were capable of proliferating in the absence of IL-2. This change of cell growth phenotype is thought to reflect one of the oncogenic transformation processes (8, 9). To investigate the possible association of TBP-2 with HTLV-I-dependent transformation, we assessed the possible correlation between the expression of TBP-2 mRNA in HTLV-I-positive T-cell lines and the IL-2 dependency for their growth using five sets of IL-2-dependent/independent cell lines. In all sets, TBP-2 mRNA was not detectable in the IL-2-independent clones (Fig. 2), whereas all but one (ATL-16T) of the IL-2-dependent cell lines expressed a detectable level of TBP-2 mRNA. These results suggest that the loss of TBP-2 expression is associated with the change of growth phenotype in HTLV-I-positive T cells.

**Augmented Expression of TBP-2 by IL-2 Deprivation on IL-2-Dependent T Cells.** To test the possibility that IL-2 controls TBP-2 expression, we measured TBP-2 mRNA levels in IL-2-dependent ATL-43T in the presence and absence of IL-2. Fig. 3A shows that IL-2 deprivation markedly augmented TBP-2 expression, the level of which was sustained in the absence of IL-2, even after a change of culture medium. However, the TBP-2 level quickly returned to the basal level on supplementation of IL-2. The results indicate that IL-2 suppresses TBP-2 expression and therefore that the basal TBP-2 expression observed in HTLV-I-positive, IL-2-dependent cell lines was not attributed to the presence of IL-2 in the culture medium. TBP-2 expression in IL-2-independent ATL-43T cells, on the other hand, was not induced by IL-2 (Fig. 3C). In addition to the HTLV-I-positive, IL-2-dependent T-cell lines, an augmented expression of TBP-2 caused by IL-2 deprivation was observed in CTLL-2, a murine IL-2-dependent T-cell line (data not shown).
Growth Suppressive Effect and Cell Cycle Arrest Induced by TBP-2. We next determined the effects of TBP-2 on the cell growth of HTLV-I-positive T-cell lines. Stable transfectants were established by introducing pCMV-Tag2-TBP-2 or pCMV-Tag2 control plasmids into MT2 cells, which are IL-2 independent and lack TBP-2. The expression of TBP-2 protein in TBP-2-transfected clones (MT2-T1 and -T2) was confirmed by Western blotting with anti-FLAG antibody. In B, cells (3 × 10^5 cells/well) were cultured in 96-well, flat-bottomed microtiter plates in triplicate. At different time points of culture (1–5 days), cell proliferation was assessed from the formation of formazan using the WST-1 cell proliferation assay kit. Values are means ± SD (n = 3). In C and D, TAT-TBP-2 protein suppressed the cell proliferation of interleukin-2 (IL-2)-independent ATL-43T cells (C), ATL-2, and MT2 (D). Each TAT-fused protein was prepared as described in “Materials and Methods.” Cells (1 × 10^5/ml) were cultured with TAT-fused proteins for the periods indicated. Cell proliferation was determined based on the formation of formazan using the WST-1 cell proliferation assay system. Values are means ± SD (n = 3).

DISCUSSION

The results presented in this study show that the expression of TBP-2 was lost in HTLV-I-positive T-cell lines with the IL-2-independent growth phenotype. The loss of TBP-2 expression in the HTLV-I-positive T cells appears to occur during the transition from the IL-2-dependent growth phenotype to the IL-2-independent phenotype (Fig. 2). Because a change in growth phenotype has been implicated in the proliferation of HTLV-I-positive T cells in an in vitro model of ATL development (4, 9), the loss of TBP-2 expression may be a critical event associated with the leukemogenesis of ATL. Indeed, the present experiments provide evidence that TBP-2 is involved in the regulation of the cell cycle (Fig. 5). It was also found that ectopic overexpression of TBP-2 in IL-2-independent HTLV-I-positive T cells resulted in the suppression of cell growth (Fig. 4). In the TBP-2-transfected clones, we observed a significant accumulation of cells in G1 phase accompanied by an increase in p16 and a reduction in hyperphosphorylated Rb (Fig. 5A). These results are consistent with the finding that inhibition of cyclin-dependent kinases by p16 inhibits phosphorylation of Rb protein, thereby causing G1 arrest (24). Numerous studies have documented deletions and alterations of variable frequency in the p16 gene in different tumors, including ATL (25, 26). Because our data demonstrated that TBP-2 expression results in elevated levels of p16, loss of TBP-2 would provide a growth advantage to the HTLV-I-positive T cells, leading to dysregulation of the cell cycle progression. It was also found in our previous experiments that vitamin D3 promotes the synthesis of
The expression of TBP-2 is down-regulated in various tumors (28, 30), inhibition of cell growth caused by overexpression of TBP-2 was recently reported in the nonlymphocytic tumor cell line HeLa (31). These results collectively suggest that TBP-2 is involved in the growth suppression of various tumor cells, as well as HTLV-I-positive ATL leukemic cells.

An interesting finding obtained in the present study is that the expression of TBP-2 is regulated by IL-2. TBP-2 levels in the cultures of IL-2-dependent T cells increased with depletion of IL-2 and decreased on addition of IL-2. Such results exclude the possibility that the expression of TBP-2 in the IL-2-dependent T cells, but not in the IL-2-independent cell lines, is attributable to the presence of IL-2 in the culture. Because IL-2 is undetectable in the IL-2-independent HTLV-I-positive T-cell lines, an autocrine IL-2 mechanism is not a likely explanation for the loss of TBP-2 in the IL-2-independent cell lines. Nevertheless, suppression of TBP-2 expression by IL-2 might imply a physiological role of TBP-2 for the regulation of IL-2-induced cell cycle progression. If TBP-2 physiologically suppresses T-cell growth, one can hypothesize that IL-2-dependent suppression of TBP-2 is a necessary step for the IL-2-dependent cell growth and that the loss of TBP-2 expression effectively induces the IL-2-independent growth of HTLV-I-positive T cells. In an earlier report, constitutive activation of the Janus-activated kinase-signal transducers and activators of transcription (JAK-STAT) pathway was suggested to be involved in the acquisition of the IL-2-independent growth phenotype of HTLV-I-transformed cells (32). However, no significant difference was observed in the activation of this pathway between the TBP-2-transfected clones (MT2-T1 and -T2) and control clones (MT2-C1 and -C4; data not shown), suggesting that TBP-2 expression is not directly associated with the activation of the JAK-STAT pathway.

The mechanisms for the loss of TBP-2 expression have not been clarified. Because IL-2 did not induce TBP-2 expression in IL-2-independent, HTLV-I-positive T cells (Fig. 3C), IL-2 signaling is not likely involved. To investigate the mechanisms, we determined genomic sequences of the TBP-2 gene, including its promoter regions, in IL-2-independent HTLV-I-positive T-cell lines (data not shown). However, there were no deletions or mutations in the sequence, suggesting that other types of gene silencing mechanisms are involved. Because TRX is highly expressed in HTLV-I-positive T-cell lines, and the reduced form of TRX binds to TBP-2, the possibility that TRX is involved in the loss of TBP-2 in the HTLV-I-positive T-cell lines should be considered. However, TBP-2 expression was maintained in TRX-overexpressing transgenic mice and EBV-transformed B-cell lines, in which TRX expression is markedly augmented (data not shown). The results indicate that loss of TBP-2 expression is not attributable to a high level of expression of TRX. The regulatory mechanism for TBP-2 expression needs further investigation.

We demonstrated previously that TBP-2 is an endogenous negative regulator of TRX, reducing its activity and protein expression (18). Indeed, we observed a decrease in TRX-reducing activity in the TBP-2-transfected clones (data not shown). TRX has been demonstrated to have a variety of biological functions, including the promotion of cell growth (33–35), inhibition of apoptosis (16, 36, 37), and the regulation of transcription factors through its reducing activity (38, 39). A wild-type TRX induced several-fold increase in colony formation in soft agar, whereas a redox-inactive mutant TRX C32S/C35S acted in a dominant negative manner to inhibit cell proliferation (40). These results collectively suggest that the inhibition of TRX reducing activity by TBP-2 is one of the mechanisms involved in the growth inhibitory effect of TBP-2. Moreover, if the loss of TBP-2 in IL-2-independent cells virtually enhances TRX activity, the resultant increase in TRX activity may be related to the altered cell cycle progression in HTLV-I-positive T cells. In our preliminary experiments, TBP-2 was found to localize predominantly in the nucleus, whereas TRX translocated from the cytosol to the nucleus in response to oxidative stimuli, functioning as a transcriptional regulator. The direct association of TRX with Ref-1 in the nucleus has been shown to regulate several transcription factors related to cell growth, such as p53 (39) and AP-1 (38). Thus, the inhibition of TRX-dependent transcriptional activity by TBP-2 may also be involved in the TBP-2-induced growth suppression and cell cycle alteration.

Because lymphocytes from ATL patients show no consistent abnormalities and only 2–5% of infected individuals develop ATL, various cellular changes during the long latent period have been suggested to be involved in the development of ATL, including deletions or mutations of

\[ Y. \text{ Nishinaka, H. Masutani, S. I. Oka, Y. Matsuo, Y. Ishii, and J. Yodoi, unpublished data.} \]
tumor suppressor genes, such as p16 and p53 (4, 7, 11). On the basis of the results of the present study, we postulate that the loss of TBP-2 is a key event in the progression of ATL leukemogenesis. After the initial infection of T cells by HTLV-I, a number of cellular genes and proteins, including IL-2Rα, IL-2, and TRX, are activated, partly through the effect of the viral oncoprotein Tax. The continued activation of the IL-2 signaling pathway in the early phase apparently stimulates the proliferation of HTLV-I-positive T cells. Despite the constitutive expression of IL-2Rα, some of the cells later acquire the IL-2-independent growth phenotype. The acquisition of this phenotype, which is associated with the progression of HTLV-I-dependent transformation, almost coincides with the loss of TBP-2 expression. Because TBP-2 negatively regulates functions of TRX, there is a possibility that the loss of TBP-2 results in the enhancement of cell growth and inhibition of apoptosis through the augmentation of TRX functions in the progression of HTLV-I-dependent leukemogenesis.

Another possibility is that TBP-2 directly interacts with some other molecules, which are related to the cell growth and cell cycle progression. In an attempt to elucidate the functions of TBP-2, we have found several molecules with TBP-2 other than TRX. Accordingly, it is possible that TBP-2 targets other molecules that are involved in the progression of HTLV-I-dependent transformation and the onset of ATL. Further analysis of the functions of TBP-2 may provide insights into the mechanism of development of ATL, because TBP-2 may play a crucial role in the regulation of cell growth and/or death.

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