Thioredoxin-binding protein-2 (TBP-2)/vitamin D₃ up-regulated protein 1 is an endogenous molecule interacting with thioredoxin (TRX), negatively regulating TRX function, and being implicated in the suppression of tumor development and metastasis. We found that TBP-2 ectopically expressed in the breast cancer cell line MCF-7 was localized predominantly in the nucleus exhibiting growth suppressive activity. The nuclear accumulation of endogenous TBP-2 protein was also demonstrated when the cells were treated with an anticancer drug, suberoylanilide hydroxamic acid. To investigate the mechanism underlying the nuclear localization, we performed a yeast two-hybrid screening and identified importin α₁ (Rch1) as a protein interacting with TBP-2. The physical interaction between TBP-2 and Rch1 was confirmed with a glutathione S-transferase pull-down assay. The interaction of TBP-2 was specific to Rch1 among other importin α subfamilies (Qip1 and NPI-1), and amino acids 1–227 of TBP-2 were sufficient for both the interaction with Rch1 and the nuclear localization, although there is no typical nuclear localization signal in this sequence. The expression of short interfering RNA of Rch1 suppressed suberoylanilide hydroxamic acid-induced nuclear accumulation of TBP-2. Collectively, our results strongly suggest that an interaction with importin Rch1 is required for TBP-2 nuclear translocation and growth control tightly associated with TRX-dependent redox regulation of transcription factors.

We recently identified thioredoxin binding protein-2 (TBP-2),¹ identical to vitamin D₃ up-regulated protein 1 (1), as a regulatory molecule interacting with thioredoxin (TRX) (2).

Later, several other reports confirmed the functional association between TBP-2/vitamin D₃ up-regulated protein 1 and TRX (3, 4). TRX, a small ubiquitous protein with conserved redox active sites, plays pivotal roles in regulating cell proliferation, apoptosis, and the activation of transcription factors by maintaining a reducing intracellular microenvironment (5). TBP-2 has been demonstrated to inhibit the reducing activity of TRX in vitro and in vivo (2, 3). Recent reports demonstrated that TBP-2 expression is up-regulated in cells treated with anti-cancer reagents, such as suberoylanilide hydroxamic acid (SAHA) (6), and down-regulated in various tumor cells (6, 7). A regulatory role of TBP-2 in the metastasis associated with CRSP3, a transcriptional co-activator on chromosome 6, was also suggested (8). These findings imply the possible involvement of TBP-2 in the regulation of tumor development, progression, and metastasis. Interestingly, a nonsense mutation of the mouse TBP-2 gene (Hylip1) was identified as a possible cause of hyperlipidemia (9), implicating that TBP-2 is involved in lipid metabolism as well. An immediate up-regulation of TBP-2 mRNA expression by glucose has also been reported (10). Although TBP-2 is involved in a wide variety of biological functions, its physiological functions are not well understood and the underlying mechanisms are unknown.

In this study, we show that TBP-2 predominantly localizes in the nucleus associated with its growth-suppressive activity. In addition, we have identified a member of the importin α family, Rch1, as a protein interacting with TBP-2 based on a yeast two-hybrid screen. The import of many proteins into the nucleus is mediated by the importin system (11). Our results suggest that TBP-2 is translocated into the nucleus by a mechanism involving the importin system to function as a growth suppressor.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—SAHA and doxycycline (Dox) were purchased from ALEXIS Biochemicals (Carlsbad, CA) and Sigma, respectively. The primary antibodies used were as follows: anti-FLAG M2 monoclonal antibody (mAb) from Sigma; anti-GFP mAb from Medical & Biological Laboratories (Nagoya, Japan); and anti-TRX mAb (ADF11) described previously (12).

**DNA Constructs**—The full-length cDNA of human TBP-2 was cloned in-frame into each of the vectors, pGBK7, pEGFP, pTRE2 (Clontech), or pCMV-Tag2 (Stratagene). TBP-2 deletion mutant vectors (pGBK7-TBP-2Δ1–120, pGBK7-TBP-2Δ121–227, and pGBK7-TBP-2Δ121–227) were prepared by PCR cloning and subsequent ligation into TOPO-cloning vector (Invitrogen) followed by ligation into the NdeI-EcoRI cloning sites of pGBK7. The cDNA of Rch1 was cloned in-frame into pCMV-Tag2 to produce pCMV-Rch1. All of the constructs were verified by sequencing. Listed below are primers used for amplification. Each primer contains a new restriction site, NdeI or KpnI (underlined). All of the sequences are in the 5’ to 3’ orientation, and the direction, forward (F) or reverse (R), is indicated as follows: p1F, 5’-CCATATGGTTGTTAAGATC-
AAG-3′; pr391B, 5′-GGGTACCCAGTGGACTGG-3′; pr121F, 5′-TGGATCACTAGTGGTGGAGCATTCAAGAGATGTTCCACCACTGGTATG-3′; and pr227R, 5′-GGGTACCTCGTGGCATTGGAAGTTGA-3′. To construct a series of pEGFP plasmids, the fragments containing deletion mutants, which were prepared using either the appropriate restriction enzyme or PCR with appropriate oligonucleotides, were inserted in-frame into the vector pEGFP. The integrity of all of the constructs was confirmed before use. cDNAs containing importin α1 (Rch1), importin α2 (Qip1), and importin α3 (NPI-1) were kindly provided by Dr. Yoneda (Osaka University) and were cloned in-frame into the vector pACT2. The pSUPER-Rch1 expression vectors were constructed by inserting oligonucleotides into BglII/HindIII site of pSUPER (OligoEngine). Oligonucleotides inserted into pSUPER-Rch1-2 and pSUPER-Rch1-3 were as follows: pSUPER-Rch1-2 (forward) 5′-GGTTACCCGAGTGGTGGAGCATTCAAGAGATGTTCCACCACTGGTATG-3′, and (reverse) 5′-AGCTTTTCCAGAAGGTCGACGTGGTCTGGCCATTGGCAAGGTA-3′; and pSUPER-Rch1-3 (forward) 5′-GATCCCCGAGTGGTGGAGCATTCAAGAGATGTTCCACCACTGGTATG-3′, and (reverse) 5′-AGCTTTTCCAGAAGGTCGACGTGGTCTGGCCATTGGCAAGGTA-3′.  

**Cell Culture and Transfections—** COS-7 and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO₂ vs atmosphere. For transfection, cells were seeded at 70% confluence and transfected with each DNA using FuGENE 6. MCF-7 Tet-On cells (Clontech) were transfected with pTRE2-EGFP-TBP-2 or pTRE2-EGFP. After 24 h, cells were plated and selected in medium containing 2.5 μg/mL puromycin (Sigma). The induction of EGFP-TBP-2 or EGFP expression by Dox in the resistant clones was confirmed by Western blotting with the anti-EGF mAb mA and by fluorescence microscopy.  

**Western Blot Analysis—** Cell lysates were prepared with lysis buffer (150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 20 mM Tris-HCl, pH 7.5, and protease inhibitors). Nuclear extracts were prepared as described previously (15). Cell lysates containing equal amounts of protein were separated by SDS-PAGE, electroblotted to a polyvinylidene difluoride membrane, probed with each antibody, and visualized by chemiluminescent detection (ECL, Amersham Biosciences). Anti-nucleoporin p62 antibody was purchased from Transduction Laboratories.  

**Cell Proliferation Assay—** Cells (10³ cells/well) were cultured in 96-well flat-bottom microtiter plates (Nunc) in triplicate. At different time points of culture (1–3 days), cell proliferation was assessed using a WST-1 cell proliferation assay kit (TaKaRa).  

**Establishment of Anti-TBP-2 mAbs—** Recombinant TBP-2 protein was expressed as described previously (2). Spleen cells from BALB/c mice immunized with recombinant TBP-2 protein were cloned by the limiting dilution procedure twice, and two of them used in this study were designated Yat818 and Yat315. Two selected mAbs, Yat818 and Yat315, specifically detected TBP-2. The decrease in absorbance at 340 nm was recorded and used to calculate the growth of cells with or without 5 μM SAHA for 20 h. Cells were fixed with 4% paraformaldehyde for 30 min. All of the subquent incubations were performed with intermittent irradiation (Microwaveprocessor MI-77, Azumaya, Tokyo, Japan) (14). Nonspecific binding was blocked by incubating the cells in a humidified 5% CO₂ atmosphere for 1 h. Cells were incubated with the reaction mixture without insulin. The calculated values were compared with the standard curve for recombinant TRX to obtain quantitative determinations of the absolute amounts of TRX.  

**Yeast Two-hybrid Assay—** The MATCHMAKER Two-hybrid System 3 from Clontech was used according to the manufacturer’s directions. pGBKTT7-TBP-2 was introduced into yeast strain AH109, and selected transformants were raised and transformed with a human leukemia Jurkat cDNA library (Clontech). Yeast plasmids were prepared from colonies showing growth on a leucine-, adenine-, histidine-, and tryptophan-deficient medium and from those that were positive for β-galactosidase activity. Among the ~1 × 10⁶ colonies of the Jurkat cDNA library, 72 μL DNA-positive clones were selected and introduced into Escherichia coli strain HB101 for sequencing.  

**Glutathione S-transferase (GST) Pull-down Assays—** The expression of full-length Rch1 or TBP-2 as a GST fusion protein from pGEX-4T3 was performed as described previously (2). The 35S-labeled proteins produced using the TnT reticulocyte lysate system (Promega) were incubated with resin containing 4 μg of GST or GST fusion protein. Reactions were carried out in binding buffer (160 mM NaCl, 1% Nonidet P-40, 50 mM Tris-Cl (pH 8.0), and protease inhibitors) at 4°C for 4 h with gentle agitation. TBP-2 or Rch1, which remained attached to the resin-bound GST or GST fusion proteins, was resolved by SDS-PAGE. All of the results were confirmed in at least two independent experiments.  

**RESULTS**  

**Growth-suppressive Activity of TBP-2—** To clarify the functional roles of TBP-2, MCF-7 stable transfectants expressing EGFP-TBP-2 (A1, D1, and D3) were established. The induction of EGFP-TBP-2 expression by Dox was confirmed by Western blot analysis (Fig. 1A). We determined the effects of TBP-2 expression on the cell growth of MCF-7 by measuring succinate-tetrazolium reductase activity. The TBP-2-transfected clones, A1 and D3, showed marked growth inhibition in culture, whereas a control clone, EG1, expressing EGFP alone showed no significant difference in its cell growth rate (Fig. 1B). In the D1 clone, which expressed a lower level of EGFP-TBP-2, cell growth suppression was not seen. These results indicate that overexpression of TBP-2 results in growth suppression.  

**Nuclear Localization of Endogenous TBP-2 Detected with Specific mAbs—** All of the MCF-7 clones expressing EGFP-TBP-2 exhibited a predominant nuclear localization of the protein in a Dox-dependent manner (Fig. 2, A and B). In a minor population, EGFP-TBP-2 showed a speckled nuclear distribution (Fig. 2C). In a minior population, EGFP-TBP-2 showed a speckled nuclear distribution (Fig. 2C). Similar results were observed when COS-7 cells were transiently transfected with pEGFP-TBP-2 (Fig. 2, D and E), whereas EGFP protein was diffusely distributed throughout the cells (Fig. 2F). To determine the subcellular localization of endogenous TBP-2, we established mAbs specific to TBP-2. Two selected mAbs, Yat818 and Yat315, specifically detected TBP-2 protein in HL-60 cells treated with vitamin D₃ and FLAG-TBP-2 ectopically overexpressed in human T-cell lymphophotrophic virus I-positive T cell line MT-2 by Western blotting (Fig. 3A). By immunohistochemical staining with the antibody, endogenous TBP-2 was not detectable in MCF-7 cells (Fig. 3B, left panel). Because SAHA, a histone deacetylase inhibitor, has been demonstrated to induce TBP-2 expression (6), MCF-7 cells treated with 5 μM SAHA were used to detect the expression of TBP-2. The induction of TBP-2 by SAHA was observed in the nuclear compartment but scarcely in the cytoplasm (Fig. 3B, right panel). Consistent with the results, Western blot analysis showed that TBP-2 was detected in the nuclear fraction of SAHA-treated MCF-7 cells (Fig. 3C). The fraction was proven to be nuclear, since the expression of nucleoporin p62 was detected.  

**Association of TBP-2 Expression with TRX—** Because TBP-2 has been demonstrated to interact with TRX, we analyzed the co-localization of TBP-2 and TRX in the TBP-2-transfected MCF-7 clone D3 using confocal microscopy. When D3 cells were cultured in the presence of Dox, TRX was expressed in both the nucleus and cytoplasm, whereas TBP-2 was found predominantly in the nucleus, indicating that TBP-2 and TRX are occasionally co-expressed in the nucleus of some cells (Fig. 4A). To further analyze the functional association with TRX, the expression and reducing activity of TRX was determined in D3 cells.
and/or A1 cells cultured in the presence or absence of Dox. The results show that the reducing activity of TRX was slightly decreased (Fig. 4B), whereas the protein expression levels of TRX were not significantly changed (Fig. 4C).

Identification of Rch1 (Importinα1) as a TBP-2-interacting Protein using a Yeast Two-hybrid Assay—To identify proteins that regulate TBP-2 nuclear expression, we performed a yeast two-hybrid screening of a Jurkat cDNA library. Among 72 positive clones, 18 independent clones encoded a single protein Rch1 (importinα1), which differed only in length. Nine clones (clones 8, 14, 18, 20, 28, 59, 71, 83, and 92) contained an entire open reading frame of Rch1. The other nine clones lacked different sections of the N terminus but were capable of encoding the truncated forms of Rch1 in-frame (Fig. 5). Rch1 protein can be divided into an N-terminal hydrophilic region, the importinα1-binding domain, a hydrophobic central region composed of 8–10 repeats called “armadillo” repeats, and a short hydrophilic C terminus (CAS binding domain). The schematic structures of Rch1 variant clones identified in our screening and the positions at which each isolated clone begins are indicated in Fig. 5. These findings indicated that the armadillo repeats are maintained in all of the positive clones, suggesting

![Image](https://example.com/image.png)

**Fig. 1.** **TBP-2 overexpression exerts growth suppression in breast cancer cell line MCF-7.** A, Western blot analysis of control (EG1) and TBP-2-transfected cells (A1, D3, and D1). MCF-7-Tet-On cells were transfected with either pTRE2-EGFP or pTRE2-EGFP-TBP-2, and the induction of protein expression by Dox (5 μM) was confirmed at 48 h by Western blotting with anti-GFP-mAb. B, cell growth kinetics of MCF-7-transfected cells. Cells (10^2 cells/well) were cultured in 96-well flat-bottom microtiter plates in triplicate. At different time points of culture (1, 2, and 3 days), cell proliferation in the presence and absence of Dox was assessed by WST-1 cell proliferation assay kit. Values are means ± S.D. (n = 3).

**Fig. 2.** **Nuclear localization of EGFP-TBP-2.** MCF-7-transfected D3 cells were cultured in the absence (A) or presence (B and C) of Dox (5 μM) for 3 days. COS-7 cells were seeded at 70% confluency and transfected with either pEGFP-TBP-2 (D and E) or pEGFP (F) using FuGENE 6. The fluorescence of EGFP proteins was analyzed by confocal microscopy.
the importance of the region for the interaction between TBP-2 and Rch1. Other positive clones encoding known proteins in-frame include MAP4K4 (mitogen-activated protein kinase kinase kinase-4) (HGK (hepatocyte progenitor kinase/germinal center kinase-like kinase)) (three independent clones) and HSP40 (DNAJB4) (three independent clones).

**Rch1 Binds to TBP-2 in a GST Pull-down Assay**

To confirm the physical interaction between Rch1 and TBP-2, a GST pull-down assay was carried out. Fig. 6A shows that TBP-2 specifically interacts with GST-Rch1. The specificity of the interaction was confirmed by the observation that TBP-2 did not adhere to GST resin devoid of Rch1. The binding between TBP-2 and GST-TRX was consistent with previous reports (2–4). Similar experiments were performed using [35S]methionine-labeled Rch1 and TBP-2 fused to GST. Rch1 binds to GST-TBP-2 but does not bind to either GST alone or GST-TRX (Fig. 6B).

**Specific Interaction between TBP-2 and Rch1**—In humans, there are at least six importin α family molecules and these can be divided into three subfamilies, Rch1, Qip1, and NPI-1 (an

![Fig. 3. Identification of nuclear localization of endogenous TBP-2 by anti-TBP-2 specific mAbs.](image)

**Fig. 3. Identification of nuclear localization of endogenous TBP-2 by anti-TBP-2 specific mAbs.** A, detection of TBP-2 protein with anti-TBP-2-specific mAbs, Yat818 and Yat315. MT-2 transfectant cell lines were prepared by transfection with pCMV (lane 1) or pCMV-TBP-2 (lane 2). TBP-2 protein expression in HL-60 cells was induced by treatment with vitamin D₃ (100 nM, 3 days) (2). Lane 3, untreated; lane 4, vitamin D₃-treated. B, immunohistochemical detection of endogenous TBP-2 in MCF-7 cells treated with SAHA. MCF-7 cells were cultured in the presence or absence of 5 μM SAHA for 20 h, and endogenous TBP-2 was detected by immunohistochemical staining with Yat315 mAb. C, Western blot (WB) analysis of endogenous TBP-2 protein (upper panel) and nucleoporin p62 (lower panel) in the cytoplasm and nucleus of MCF-7 cells. MCF-7 cells were cultured in the presence or absence of 5 μM SAHA for 20 h, and cytoplasmic and nuclear fractions of MCF-7 cells were prepared. DMSO, Me₂SO.

**Importin α-mediated Nuclear Translocation of TBP-2**

![Fig. 4. Association of TBP-2 with TRX.](image)

**Fig. 4. Association of TBP-2 with TRX.** A, MCF-7-transfected cells were cultured for 3 days in the presence of Dox (5 μM), and the fluorescence of EGFP-TBP-2 (a, green) was analyzed by confocal microscopy. TRX was stained with anti-TRX mAb (ADF11) and secondary Alexa 568-labeled anti-mouse antibodies (b, red). Co-localization is seen in yellow (c). B, TRX-reducing activity in MCF-7-D3 cells determined by insulin-reducing assay. Cells were cultured for 3 days either in the presence or absence of Dox (5 μM). C, protein expression of TRX in MCF-7-transfected cells. Cells were cultured for 3 days either in the presence or absence of Dox (5 μM). TBP-2 and TRX expression in each cell lysate was determined by Western blotting with Yat315 and anti-TRX (ADF11) mAb, respectively.

![Fig. 5. Domain structures of the positive clones encoding Rch1 (importin α) isolated in the yeast two-hybrid assay.](image)

**Fig. 5. Domain structures of the positive clones encoding Rch1 (importin α) isolated in the yeast two-hybrid assay.** All of the clones encoded full-length or N-terminal-truncated Rch1 protein in-frame. IBB domain, importin β binding domain.

~50% amino acid sequence identity) (11). Thus, we examined the binding specificity between TBP-2 and the importin α subfamilies using a yeast two-hybrid assay. As shown in Fig. 7A, TBP-2 interacted with Rch1 but not with Qip1 or NPI-1. We further examined whether Rch1 mediates the nuclear import of TBP-2 using different constructs of Rch1 RNAi expression vec-
Discussion

Here, we demonstrated that when TBP-2 was overexpressed, cell growth was markedly suppressed in a breast cancer cell line, MCF-7 (Fig. 1B). The growth-suppressive activity of TBP-2 in 293 cells is associated with cell cycle arrest at the G0/G1 phase (15). Our recent observation also revealed that the overexpression of TBP-2 in human T-cell lymphotrophic virus I-transformed cell lines induced G1 arrest in association with an accumulation of the p16 protein (16). However, the p16 protein has been reported not to be functional in MCF-7 cells. The mechanism involved in the tumor-suppressive activity may vary depending on cell type.

TBP-2 is a negative regulator of TRX (2), and recent reports have unequivocally suggested that the effects of TBP-2 expression are closely associated with TRX activity (6, 9, 17, 18). Thus, we analyzed the effect of TBP-2 overexpression on the protein expression and the reducing activity of TRX. Although the protein expression of TRX was not significantly changed, the reducing activity was slightly decreased in MCF-7 D3 treated with Dox. Because TRX has a variety of biological functions including cell growth-promoting activity, the inhibition of TRX functions by TBP-2 may at least partly explain the growth-suppressive effect of TBP-2.

An interesting finding of the present study is that TBP-2 and TRX are not always co-localized (Fig. 4A). TRX has been shown to be translocated into the nucleus in response to various oxidative stimuli (19). These findings suggest that the formation of complexes between TBP-2 and TRX in the nucleus is a transient phenomenon, probably depending on the redox status of cells. Furthermore, it is possible that the induction of the nuclear accumulation of TRX, whereby the interaction of TBP-2 and TRX is enhanced, augments the growth-suppressive effect of TBP-2.

We observed nuclear localization of TBP-2 in MCF-7 and COS-7 cells transfected with pEGFP-TBP-2 (Fig. 2). EGFP protein, fused to TBP-2, might alter the localization of TBP-2. Therefore, we established mAbs specific to TBP-2 (Yat315 and Yat818) to identify the localization of the endogenous TBP-2 protein. The antibodies recognize the C-terminal region of TBP-2 (data not shown) and were shown to specifically detect TBP-2 induced by vitamin D3 in HL-60 cells or ectopically expressed FLAG-TBP-2 in MT2 cells (Fig. 3A). We failed to determine the localization of endogenous TBP-2 in MCF-7 cells by immunohistochemical staining. SAHA is a potent inhibitor of histone deacetylase and is known to induce growth arrest tors (pSUPER-Rch1). Reduced Rch1 expression was shown using pSUPER-Rch1–2 and pSUPER-Rch1–3 (Fig. 7B). The suppression of Rch1 expression by overexpression of these vectors suppressed SAHA-induced nuclear expression of TBP-2 (Fig. 7C, upper panel), whereas it augmented the cytoplasmic expression of TBP-2 (Fig. 7C, lower panel).

Domains of TBP-2 Interacting with Rch1—There seems to be no classical nuclear localization signal (NLS) in TBP-2. We used a series of deletion mutants of TBP-2 to assess interacting domains. First, we determined the possible domains of TBP-2 interacting with Rch1 with a yeast two-hybrid assay. The results revealed that TBP-2(1–227) was capable of interacting with Rch1. However, TBP-2(Δ1–120), TBP-2(Δ121–227), and TBP-2(121–227) showed no interaction with Rch1 (Fig. 8A). We next constructed a series of pEGFP-TBP-2 deletion mutants and analyzed the localization of their proteins using immunofluorescence microscopy. Consistent with the above results on Rch1 interaction, EGFP-TBP-2(1–227) maintained predominantly a nuclear localization but EGFP-TBP-2(Δ1–120) showed a diffused localization throughout the cytoplasm and nucleus (Fig. 8B). The results are summarized in Fig. 8C.
and apoptosis in many tumor types, and it has been reported to enhance TBP-2 expression in several tumor cell lines (6). Therefore, MCF-7 cells were treated with SAHA and subjected to an analysis of TBP-2 expression by immunohistochemical staining and Western blotting. Using anti-TBP-2 mAb, endogenous TBP-2 expression was found to be up-regulated predominantly in the nucleus of SAHA-treated MCF-7 cells (Fig. 3, B and C). These results suggest that enhanced TBP-2 expression in the nucleus is associated with growth suppression in response to SAHA.

To further investigate the mechanism of TBP-2 nuclear expression, we performed a yeast two-hybrid screening of a Jurkat cDNA library and identified Rch1 (importin β1) as a molecule interacting with TBP-2. In a different yeast two-hybrid screening using a K562 cDNA library, several clones encoding Rch1 were also selected positively (data not shown). These results suggest that TBP-2 interacts with Rch1 in various types of cells. Importin α/β heterodimers are known as major nuclear transport systems. Importin α proteins first trap proteins bearing the classical NLS through the armadillo repeats and bind to importin β, thus mediating translocation through the nuclear pore complexes (11, 20). Our results demonstrated that amino acids 1–227 of TBP-2 were sufficient for the interaction with Rch1, although there seems to be no classical NLS in the sequence of TBP-2. Because TBP-2-Δ(1–120) and TBP-2-Δ(121–227) did not interact with Rch1, the interaction between

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**Fig. 8. Determination of the TBP-2 regions required for its nuclear localization and interaction with Rch1.** A, deletion mutants of TBP-2 fused to the GAL4 DNA binding domain were cloned in pGBK7 and used to locate the domain of TBP-2 interacting with Rch1 in a two-hybrid assay. Yeasts were transformed with the indicated constructs of TBP-2 in pGBK7 and full-length Rch1 in pACT2. The growth of yeast transformants on a selective synthetic medium (SD) with histidine and adenine (left panel) or without histidine and adenine (right panel) is shown. B, COS-7 cells were transiently transfected with pEGFP, pEGFP-TBP-2, or pEGFP-TBP-2-(1–227) or pEGFP-TBP-2-Δ(1–120), and the localization of each product was analyzed by confocal microscopy. C, summary of the results with schematic structures of the deletion mutants. +, positive interaction (growth without histidine and adenine); −, negative (no growth without histidine and adenine); N.D., not determined.
Importin α-mediated Nuclear Translocation of TBP-2

TBP-2 and Rch1 may require a conformational structure or multiple NLS-like sequences in amino acids 1–227. The analysis of EGFP-fused TBP-2 deletion mutants also revealed that amino acids 1–227 of TBP-2 were sufficient for the nuclear localization, consistent with the results for the Rch1-interacting domain.

Although only one gene for importin β is found, several importin α genes exist and are classified into subfamilies, such as importin α1 (Rch1), importin α3 (Qip1), and importin α5 (NPI-1). These subfamilies show considerable similarity in sequences and often import the same protein. According to previous reports on importin α proteins (21, 22), it is probable that other importin α cDNAs are present in the Jurkat human T cell line. It has also been suggested that different importin α proteins have both different and overlapping specificities for the various NLSs. hnRNP K and pCAF are imported by most of the importin α proteins including Rch1, Qip1, and NPI-1 (23), whereas STAT1 (24) and RCC1 (23) are specifically imported by NPI-1 and Qip1, respectively. However, little information is available regarding the range of sequences that can be utilized by each importin α protein. Thus, we determined the specificity for TBP-2 of the major three importin α subfamilies (Rch1, Qip1, and NPI-1). Our results clearly showed that TBP-2 interacted with Rch1 but not with Qip1 or NPI-1. The overexpression of Rch1 RNAi expression vectors suppressed SAHA-treated with Rch1 but not with Qip1 or NPI-1. The overexpression of Rch1 RNAi expression vectors suppressed SAHA-induced nuclear expression of TBP-2 and augmented the cytoplasmic expression of TBP-2 (Fig. 7C). These results suggest that the nuclear transport of TBP-2 is mediated by Rch1. The structural basis of the interaction between TBP-2 and Rch1 needs further investigation.

We demonstrated here that TBP-2 protein induced by treatment with SAHA or by ectopic transfection of the TBP-2 gene is predominantly localized to the nucleus. These observations suggest that TBP-2 is enhanced and imported into the nucleus to exert its physiological functions including growth-suppressive activity. Of note, we have occasionally observed a unique speckled distribution of TBP-2 in the nucleus (Fig. 2, C and E). The speckled distribution increases as the induction of TBP-2 expression becomes stronger (data not shown). A recent report by Han et al. (15) shows the interaction and co-localization of TBP-2 with transcriptional repressors, such as Pafanci anemia zinc finger, promyelocytic leukemia zinc finger, and histone deacetylase 1, and that the co-localization exhibited a speckled pattern in the nucleus. We also have isolated several nuclear proteins that are important for the regulation of transcriptional events as partners of TBP-2 (data not shown). These observations collectively suggest that the interaction of those nuclear proteins following increased nuclear expression of TBP-2 is associated with the growth-suppressive activity of TBP-2. In our preliminary studies, Rch1 inhibited the interaction between TBP-2 and TRX. Because the functions of several TRX binding partners are regulated by a redox-dependent interaction with TRX, TRX may play a role in regulating the nuclear translocation of TBP-2 mediated by Rch1 and growth suppression in a redox-dependent fashion as well.

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REFERENCES

1. Chen, K. S., and DeLuca, H. F. (1994) Biochem. Biophys. Acta 1219, 26–32
2. Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, T., Sono, H., Gen, Y., and Yodoi, J. (1999) J. Biol. Chem. 274, 21577–21580
3. Junn, E., Han, S. H., Im, J. Y., Yang, Y., Cho, E. W., Um, H. D., Kim, D. K., Lee, K. W., Han, P. L., Rhee, S. G., and Choi, I. (2000) J. Immunol. 164, 6287–6295
4. Yamanaka, H., Maelihi, F., Oshiro, M., Asato, T., Yanagawa, Y., Takei, H., and Nakashima, Y. (2000) Biochem. Biophys. Res. Commun. 271, 796–800
5. Nishinaka, Y., Masutani, H., Nakamura, H., and Yodoi, J. (2001) Redox. Rep. 6, 289–295
6. Butler, L. M., Zhou, X., Xu, W. S., Scher, H. I., Riffkind, R. A., Marks, P. A., and Richon, V. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11700–11705
7. Ikazaki, M., Takahashi, Y., Ishii, Y., Nagata, T., Asai, S., and Ishikawa, K. (2002) Anticancer Res. 22, 4035–4048
8. Goldberg, S. F., Miele, M. E., Hatta, N., Takata, M., Paquette-Straub, C., Freedman, L. P., and Welch, D. R. (2003) Cancer Res. 63, 432–440
9. Böndar, J. S., Chatterjee, A., Castellani, L. W., Ross, D. A., Ohmen, J., Cavalcoli, J., Wu, C., Daines, K. M., Catanese, J., Chu, M., Sheth, S. S., Charugundla, K., Demant, P., West, D. B., de Jong, P., and Laszlo, A. J. (2002) Nat. Genet. 30, 110–116
10. Hirota, T., Okano, T., Kukame, K., Shiratori-Ikejima, H., Miyata, T., and Fukada, Y. (2002) J. Biol. Chem. 277, 44244–44251
11. Yoneda, Y. (2000) Genes Cells 5, 777–785
12. Kogaki, H., Fujiwara, Y., Yoshiki, A., Kitajima, S., Tanimoto, M., Tsvi, A., Shimamura, T., Hamuro, J., and Ashihara, Y. (1996) J. Clin. Lab. Anal. 10, 257–261
13. Andrews, N. C., and Faller, D. V. (1991) Nucleic Acids Res. 19, 2499
14. Toyota, K., Kawaguchi, W., Akatsu, S., Hiyosu, Y., and Hiai, H. (2003) Pathol. Int. 53, 259–261
15. Han, S. H., Jeon, J. H., Ju, H. R., Jung, U., Kim, K. Y., Yoo, H. S., Lee, Y. H., Song, K. S., Hwang, H. M., Na, Y. S., Yang, Y., Lee, K. N., and Choi, I. (2003) Oncogene 22, 4035–4046
16. Nishinaka, Y., Nishiyama, A., Masutani, H., Oka, S., Ahsan, K. M., Nakayama, Y., Ishii, Y., Nakamura, H., Maeda, M., and Yodoi, J. (2004) Cancer Res. 64, 1287–1292
17. Schulze, P. C., De Kleinenauer, G. W., Yoshioka, J., Kassik, K. A., and Lee, R. T. (2004) Circ. Res. 94, 659–665
18. Wang, Y., De Kleinenauer, G., and Lee, R. (2002) J. Biol. Chem. 277, 26496–26500
19. Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K., and Yodoi, J. (1999) J. Biol. Chem. 274, 27891–27897
20. Marelli, M., Dilworth, D. J., Wozniak, R. W., and Aitchison, J. D. (2001) Biochem. Cell Biol. 79, 603–612
21. Nadler, S. G., Trischler, D., Haffar, O. K., Blake, J., Bruce, A. G., and Cleaveland, J. S. (1997) J. Biol. Chem. 272, 4310–4315
22. Priee, M. G., Guttridge, K. L., Munguia, J. E., and Waterman, M. L. (1996) J. Biol. Chem. 271, 7654–7658
23. Kohler, M., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., Haller, H., Gorlich, D., and Hartmann, E. (1999) Mol. Cell. Biol. 19, 7762–7771
24. Sekimoto, T., Imamoto, N., Nakajima, K., Hirano, T., and Yoneda, Y. (1997) EMBO J. 16, 7067–7077