Identification of Thioredoxin-binding Protein-2/Vitamin D₃ Up-regulated Protein 1 as a Negative Regulator of Thioredoxin Function and Expression*

(Received for publication, March 19, 1999, and in revised form, May 3, 1999)

Akira Nishiyama, Minoru Matsui‡, Satoshi Iwata, Kiichi Hirota§, Hiroshi Masutani, Hajime Nakamura, Yasushi Takagi, Hiroshi Sono, Yasuhiro Gon, and Junji Yodoi†

From the Department of Biological Responses, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

Thioredoxin (TRX) is a ubiquitous 12-kDa protein. It has two redox-active cysteine residues in its consensus sequence (Trp-Cys-Gly-Pro-Cys) and serves as a general disulfide oxidoreductase. The two cysteine residues can be reversibly oxidized to form a disulfide bond and reduced by the action of TRX reductase and NADPH (3). TRX catalyzes the reduction of disulfide bonds in multiple substrate proteins.

Recent works have shown the importance of reduction/oxidation (redox) regulation in various biological phenomena. Thioredoxin (TRX) is one of the major components of the thiol reducing system and plays multiple roles in cellular processes such as proliferation, apoptosis, and gene expression. To investigate the molecular mechanism of TRX action, we used a yeast two-hybrid system to identify TRX-binding proteins. One of the candidates, designated as thioredoxin-binding protein-2 (TBP-2), was identical to vitamin D₃ up-regulated protein 1 (VDUP1). The association of TRX with TBP-2/VDUP1 was observed in vitro and in vivo. TBP-2/VDUP1 bound to reduced TRX but not to oxidized TRX nor to mutant TRX, in which two redox active cysteine residues are substituted by serine. Thus, the catalytic center of TRX seems to be important for the interaction. Insulin reducing activity of TRX was inhibited by the addition of recombinant TBP-2/VDUP1 protein in vitro. In COS-7 and HEK293 cells transiently transfected with TBP-2/VDUP1 expression vector, decrease of TRX-reducing activity of TRX and diminishment of TRX expression was observed. These results suggested that TBP-2/VDUP1 serves as a negative regulator of the biological function and expression of TRX. Treatment of HL-60 cells with 1α,25-dihydroxyvitamin D₃ caused an increase of TBP-2/VDUP1 expression and down-regulation of the expression and the reducing activity of TRX. Therefore, the TRX-TBP-2/VDUP1 interaction may be an important redox regulatory mechanism in cellular processes, including differentiation of myeloid and macrophage lineages.

Thioredoxin (TRX) is a 12-kDa ubiquitous protein that has
1α,25-dihydroxyvitamin D$_3$ (28). Although several homologous sequences of VDU1 from mammalian species have been reported, the function of VDU1 remains unclear. In this paper, we report how TBP-2/VDU1 interacts with TRX and modulates the function and the expression of TRX in vitro and in vivo.

MATERIALS AND METHODS

**Plasmids**—Standard methods were used for DNA and RNA manipulations (29). TRX mutant C32S/C35S, which lacks reducing activity, was made by substituting two reducing cysteine residues for serine residues (30, 31). A cDNA of TRX or TRX C32S/C35S was fused in-frame to pGBT9 (CLONTECH) or pGEX4T-2 (Amersham Pharmacia Biotech). TRX cDNA fragment was excised by EcoRI from agt10-TRX vector and ligated into pcDNA3 (Invitrogen) (9). pACT-cl.13 and pACT-cl.29 were isolated from positive colonies of yeast two-hybrid screening and contained partial coding sequences of TBP-2. The open reading frame of the TBP-2 cDNA was amplified by polymerase chain reaction using the following oligonucleotide primers: 5′-GGAATTCCGATGGTTGA-3′ and 5′-CCGCTGAGTCACTGACATTGTGTTGA-3′. To prepare protein expression vectors, the TBP-2/VDU1 open reading frame was ligated in-frame to pGADGH (CLONTECH), pcDNA3.1/His B (Invitrogen), pGEX4T-2, pRSET B (Invitrogen), or pEGFCT-1 (CLONTECH), pcDNA3.1/His B-IacN was purchased from Clontech. A 2.9-kilobase pair cDNA in pBlueScript was sequenced on both strands.

**Screening of TBP-2 cDNA**—The ZAP II human placenta cDNA library (Stratagene) was screened with [α-³²P]dCTP-labeled DNA probes derived from the XhoI fragment of pACT-cl.29. Positive plaques were phagemid-rescued by VCSM13 helper phage (Stratagene) according to the manufacturer’s instruction. A 2.9-kilobase pair cDNA in pBluescript was sequenced on both strands.

**Preparation of Recombinant Proteins**—In vitro translated proteins were prepared using a TNT-coupled rabbit reticulocyte translation system (Promega) and 55S Semithinhead (Amersham Pharmacia Biotech). Bacterially expressed His$_6$-tagged recombinant protein was prepared under denaturing conditions according to the instructions provided in the QIAexpressionist booklet (Qiagen). E. coli strain XL1 Blue MR6 transformed with pGEX expression vector was treated with phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 5 μg/ml aprotinin). After 2 h, samples were centrifuged and washed five times with degassed Nonidet P-40 buffer by batch method. Then in vitro translated TRX protein was incubated for 2 h in the presence of Nonidet P-40. The precipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (36).

**Total RNA from cultured cells** was extracted using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s instruction. Electrophoresis and Northern blotting were performed as described previously (37).

**Insulin Reducing Assay**—To estimate the reducing activity of TRX, insulin reducing assay was performed according to a previous report (36) with slight modifications. In our assay, yeast TRX reductase was used and was able to reduce recombinant human TRX. Yeast TRX reductase was provided by Oriental Yeast Co. Ltd. (Tokyo, Japan). The decrease in absorbance at 340 nm was recorded by use of a THERMOMAX micro plate reader (Molecular Devices) to detect maximal NADPH consumption rate. Each value was calculated according to a method previously reported (36).

**RESULTS**

**Screening of Genes Encoding TRX-binding Protein by Yeast Two-hybrid System**—We used the yeast two-hybrid system to clone genes encoding TRX-binding protein using a cDNA library of B cell population of Epstein-Barr virus-transformed human lymphoblastoid cell lines. Among approximately 1.8 × 10$^8$ yeast transformants screened, nine colonies showed histidine prototrophy and β-galactosidase activity. Isolated plasmids were classified by restriction enzyme excision or DNA sequencing into three groups that were designated as thioridoxin-binding proteins. Because double transformants with pGBT-TRX and each plasmid belonging to one group, TBP-2, showed strongly positive phenotypes of histidine prototrophy and β-galactosidase activity, we chose TBP-2 for further study (Fig. 1).
has homology with TRT407–2 and vitamin D₃ up-regulated protein 1 (VDUP1). TRT407–2 was reported as a gene screened by an RNA fingerprinting method in mink Mv1Lu cells (38), and VDUP1 was reported as an up-regulated gene in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃ (28). Another candidate (TBP-1) was identical to human p40phox candidate (TBP-1) was identical to human p40phox candidate (TBP-1) was identical to human p40phox.* 

**Interaction between TRX Protein and TBP-2/VDUP1 Protein**—To examine the interaction between TRX protein and TBP-2/VDUP1 protein, an *in vitro* binding assay was performed. †<sup>35</sup>S-Labeled proteins were prepared by *in vitro* translation. ‡<sup>35</sup>S-Labeled His₆-TBP-2/VDUP1 protein was co-immunoprecipitated with TRX by anti-TRX monoclonal antibodies (Fig. 2A, lane 1) but not by control mouse IgG1 (Fig. 2A, lane 2). ‡<sup>35</sup>S-Labeled His₆-β-galactosidase protein was not co-immunoprecipitated with TRX (Fig. 2A, lane 3). In addition, ‡<sup>35</sup>S-labeled TRX was co-immunoprecipitated with *in vitro* translated His₆-TBP-2/VDUP1 protein by anti-Xpress antibody but not by control mouse IgG1 (Fig. 2B).

We then analyzed whether TRX interacts with TBP-2/VDUP1 *in vivo*. Cell lysates from human Jurkat cells were applied to an affinity column of either anti-TRX monoclonal antibody or control IgG1. Eluted proteins were subjected to Western blotting analysis with affinity purified anti-TBP-2/VDUP1 antibody. TBP-2/VDUP1 was detected in eluates from the anti-TRX antibody column (Fig. 2C, lane 1) but not from the control IgG1 column (lane 2). Therefore, these results demonstrated the interaction of TRX with TBP-2/VDUP1 in *vitro* and *in vivo*.

**Effect of Redox Status of TRX on Interaction between TRX and TBP-2/VDUP1**—Because TRX is a redox active protein, we next tested whether TRX-TBP-2/VDUP1 interaction is influenced by the redox status of TRX. GST-fused TRX was pretreated with reducing/oxidizing reagents, diithiothreitol, hydroxyl peroxide, or diamide (a sulfhydryl-specific oxidant) and subjected to *in vitro* binding assay. Whereas the TRX-TBP-2/VDUP1 interaction was unaffected by treatment with diithiothreitol, the interaction was markedly inhibited by treatment with hydrogen peroxide or diamide, suggesting that the reduced form of TRX is critically important for the interaction (Fig. 3).
Yeast strain HF7c was used in this experiment. Using His6-TBP-2/VDUP1 expression vector (data not shown). Similar results were obtained in experiments transiently transfected with GFP-TBP-2/VDUP1 expression vector (Fig. 6). Similar results were obtained in experiments using His6-TBP-2/VDUP1 expression vector (Fig. 6). Western blotting analysis demonstrated that expression of TRX protein was significantly down-regulated in COS-7 cells transiently transfected with GFP-TBP-2/VDUP1 expression vector (Fig. 5A), in comparison with those of cells transfected with a GFP expression vector (Fig. 5A, lane 1). Similar results were obtained in experiments using Hist6-TBP-2/VDUP1 expression vector (data not shown).

To confirm the inhibitory effect of TBP-2/VDUP1 on the reducing activity of TRX, we tested recombinant GST-TBP-2/VDUP1 protein in the insulin reducing assay. The reducing activity of TRX was repressed to less than 50% by the addition of 1 μM GST-TBP-2/VDUP1 protein, indicating that TBP-2/VDUP1 protein inhibits the disulfide reducing activity of TRX in vitro (Fig. 5B).

Effects of TBP-2/VDUP1 on TRX Expression—We examined the effect of TBP-2/VDUP1 on TRX activity by the insulin reducing assay (36, 40). In our experiments, yeast TRX reductase was used and was able to reduce recombinant human TRX (data not shown). As shown in Fig. 5, a significant decrease of the reducing activity of TRX was observed in cellular extracts of COS-7 or HEK293 cells transiently transfected with a green fluorescent protein (GFP)-fused TBP-2/VDUP1 protein expression vector (Fig. 5A), in comparison with those of cells transfected with a GFP expression vector (Fig. 5A, lane 1). Similar results were obtained in experiments using Hist6-TBP-2/VDUP1 expression vector (data not shown).

FIG. 4. Interaction of TBP-2/VDUP1 with mutant TRX. A, GST fusion proteins immobilized on the beads were used for an in vitro binding assay with 35S-labeled His6-TBP-2/VDUP1 protein. The assay was performed as described under "Materials and Methods." Lane 1, GST; lane 2, GST-TRX; lane 3, GST-TRX C32S/C35S. B, yeast two-hybrid analysis using TRX mutant. pGBT9-TRX or pGBT-TRX C32S/C35S was co-transformed with pGADGH or pGADGH-TBP-2/VDUP1. The growth of yeast transformants on selective synthetic medium without histidine (upper panel) or with histidine (lower panel) is shown. Yeast strain HF7c was used in this experiment.

FIG. 5. Effect of TBP-2/VDUP1 for the reducing activity of TRX. A, TRX activity in cells transiently transfected with TBP-2/VDUP1 expression vector. TRX activity of the cell extract (10 μg of COS-7 (left panel) or HEK293 cells (right panel) transiently transfected with the indicated amount of GFP-TBP-2/VDUP1 expression vector was determined by use of an insulin reducing assay. Plasmids (total 10 μg/plate) were introduced to cells cultured in 10-cm dishes. Total amount of plasmid was adjusted to 10 μg with pEGFP-C1. Activities for samples are shown relative to Vmax of control (pEGFP-TBP-2/VDUP1 0 μg, lane 1), which is assigned as 100%. Data shown are representative of two independent experiments. The results are the means ± S.D. of three samples. B, the effect of recombinant TBP-2/VDUP1 protein on TRX reducing activity. TRX activities of each sample were determined by use of an insulin reducing assay. Reduced TRX (0.5 μM) was incubated with the indicated concentration of GST (open bar) or GST-TBP-2/VDUP1 protein (closed bar) for 15 min at 25 °C. The reducing activity of TRX was measured in 0.1 M Tris-HCl (pH 8.0), 2 mM EDTA, 0.2 mM NADPH, 9.9 units/ml yeast TRX reductase, 140 μM insulin, 1 mM glutathione at 25 °C. Activities for samples are shown relative to Vmax of control (lane 1), which is assigned as 100%. Data shown are representative of two independent experiments. The results are the means ± S.D. of three samples.

FIG. 6. Expression of TRX protein in TBP-2/VDUP1 overexpressed cells. Each lane contains 10 μg of cell lysate that was isolated from cells transfected with the indicated amount of GFP-TBP-2/VDUP1 expression vector. Plasmids (total 2 μg/well) were introduced to cells cultured in a 6-well plate. The total amount of plasmid was adjusted to 2 μg with pEGFP-C1. Western blotting was performed using a monoclonal anti-TRX antibody (TRX-11 mAb).

Effects of TBP-2/VDUP1 on TRX Expression—We examined the effect of TBP-2/VDUP1 on TRX expression. As shown in Fig. 6, Western blotting analysis demonstrated that expression of TRX protein was significantly down-regulated in COS-7 cells transiently transfected with GFP-TBP-2/VDUP1 expression vector (Fig. 6). Similar results were obtained in experiments using Hist6-TBP-2/VDUP1 expression vector (data not shown). Thus, TBP-2/VDUP1 protein down-regulated TRX protein expression as well.

TRX and TBP-2/VDUP1 expression in HL-60 cells differentiated with 1α,25-dihydroxyvitamin D3—Because TBP-2/VDUP1 was originally reported as an up-regulated gene in 1α,25-dihydroxyvitamin D3 treatment in HL-60 cells (28), we analyzed TBP-2/VDUP1 and TRX expression in the differentiation of 1α,25-dihydroxyvitamin D3-induced HL-60 cells. There was a gradual increase of TBP-2/VDUP1 mRNA after treatment with 1α,25-dihydroxyvitamin D3 (Fig. 7A). After 72 h, the TBP-2/VDUP1 mRNA was enhanced 9-fold over that before the treatment. In contrast, 72 h after the treatment, the TRX mRNA level markedly declined to less than 20% compared with that before the treatment. This inverted expression pattern was also observed in protein expression. The expression of TBP-2/VDUP1 protein was enhanced after treatment of 1α,25-dihydroxyvitamin D3 (Fig. 7B). In contrast, 48 h after the treat-
regulated in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃ (28). Thus, TRX expression and its reducing activity were downregulated to 70% of the control value within 24 h and to 60% by 72 h (Fig. 7A). Each lane contains 20 μg of total RNA that was isolated from cells treated with 1α,25-dihydroxyvitamin D₃. The blot was hybridized with ³²P-labeled probes for TBP-2/VDUP1, TRX or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, Western blotting analysis of TBP-2/VDUP1 and TRX expression. Each lane contains 20 μg of cell lysate from cells treated with 1α,25-dihydroxyvitamin D₃. Western blotting was performed using an anti-TBP-2/VDUP1 antibody or a monoclonal anti-TRX antibody (TRX-11 mAb).

ment, the amount of TRX protein was reduced to half of that before treatment (Fig. 7B). We then analyzed the reducing activity of TRX in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃. Insulin reducing activity of the cell lysates decreased to 70% of the control value within 24 h and to 60% by 72 h (Fig. 8). Thus, TRX expression and its reducing activity were downregulated in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃, whereas TBP-2/VDUP1 expression was up-regulated.

DISCUSSION

In our search for interacting molecules with TRX, we isolated VDUP1 as a TBP-2 using a yeast two-hybrid system. We characterized TBP-2/VDUP1 as a TRX-binding protein. The interaction was dependent on the redox status of TRX. Moreover, the inhibitory effect of TBP-2/VDUP1 on the reducing activity of TRX and the expression was observed in TBP-2/VDUP1-overexpressed cells as well as HL-60 cells treated with 1α,25-dihydroxyvitamin D₃.

VDUP1 was originally reported as an up-regulated gene in HL-60 cells stimulated by 1α,25-dihydroxyvitamin D₃ (28). The function of VDUP1 is still unclear, although several homologous sequences from mammalian species have been reported. The rat VDUP1 homologue was isolated as a down-regulated gene by N-methyl-N-nitrosourea in rat mammary tumor (41). There are two transcripts homologous to VDUP1, TRT407–2 and TRT407–9, whose expressions were induced by cycloheximide and repressed by transforming growth factor-β in mink Mv1Lu cells (38).

The interaction of TRX with TBP-2/VDUP1 was demonstrated both in vitro and in vivo. TRX treated with oxidizing reagents was incapable of binding with TBP-2/VDUP1. It should be noted that the effect of oxidizing reagents was detectable at a low concentration (10 μM), in which aggregation of TRX was avoided (42). Thus, only the reduced form of TRX appears to interact with TBP-2/VDUP1. There is a possibility that the presence of unreacted reagents has effects on the interaction between TRX and TBP-2/VDUP1. However, it seems unlikely because the GST-TRX beads used in the experiments were intensively washed after treatment with reducing/oxidizing reagents. In addition, the interaction was hardly inhibited by direct addition of the low concentration (10 μM) of reducing/oxidizing reagents to the TRX and TBP-2/VDUP1 re-

A Vitamin D₃-inducible Negative Regulator of TRX

In the TBP-2/VDUP1-overexpressed cells, decrease of the reducing activity of TRX was observed. This result raised the possibility that TBP-2/VDUP1 affects enzymatic action of TRX or TRX expression. Therefore, we examined the effect of TBP-2/VDUP1 on insulin reducing activity of TRX and TRX protein expression. The insulin reducing assay using recombinant GST-TBP-2/VDUP1 protein clearly demonstrated the inhibitory effect of TBP-2/VDUP1 on the reducing activity of TRX. Structural interference caused by GST seems unlikely, because experiments using cell lysates transfected with His₉-TBP-2/VDUP1 expression vector also showed the decreased TRX activity. Although recombinant TBP-2/VDUP1 protein demonstrated an inhibitory effect, the effect was not complete, probably because the TBP-2/VDUP1 protein concentration was insufficient. Additionally, suppression of TRX protein expression was observed in cells transiently transfected with TBP-2/VDUP1 expression vector. In addition to its inhibitory effect,

![Fig. 7. Expression of TBP-2/VDUP1 and TRX in 1α,25-dihydroxyvitamin D₃-stimulated HL-60 cells. A. Northern blotting analysis of TBP-2/VDUP1 and TRX expression. Each lane contains 20 μg of total RNA that was isolated from cells treated with 1α,25-dihydroxyvitamin D₃. The blot was hybridized with ³²P-labeled probes for TBP-2/VDUP1, TRX or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, Western blotting analysis of TBP-2/VDUP1 and TRX expression. Each lane contains 20 μg of cell lysate from cells treated with 1α,25-dihydroxyvitamin D₃. Western blotting was performed using an anti-TBP-2/VDUP1 antibody or a monoclonal anti-TRX antibody (TRX-11 mAb).](image)

![Fig. 8. The reducing activity of TRX in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃. Cell extracts (10 μg) of HL-60 cells treated with 1α,25-dihydroxyvitamin D₃ were collected at indicated hours. The TRX activities were determined by use of the insulin reducing assay. Activities for samples are shown relative to Vₚₘₜ of control (0 h), which is assigned as 100%. Data shown are representative of two-independent experiments. The results are the means ± S.D. of three samples.](image)

[^2]: A. Nishiyama and J. Yodoi, unpublished data.
A Vitamin D₃-inducible Negative Regulator of TRX

TBP-2/VUDP1 may be involved in TRX protein expression, and the interaction of TBP-2/VUDP1 with TRX might be required for the suppression mechanism of TRX expression. Based on these findings, we hypothesize that TBP-2/VUDP1 inhibits the redox-regulatory action of TRX.

We observed the decrease of TRX expression and the reducing activity in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃ whose TBP-2/VUDP1 expression was enhanced as described in a previous report (28). An intriguing possibility is that up-regulation of TBP-2/VUDP1 expression is involved in the decrease of TRX expression and reducing activity. The decrease of TRX mRNA also was observed in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃. The decrease of TRX mRNA also was observed in HL-60 cells treated with 1α,25-dihydroxyvitamin D₃ and the involvement of TBP-2/VUDP1 should be further analyzed.

1α,25-Dihydroxyvitamin D₃ is an essential biologically active molecule and is important for regulation of calcium homeostasis and secretion of hormone (44, 45). 1α,25-Dihydroxyvitamin D₃ also is a potent inducer of myeloid leukemic cell differentiation (46, 47) and can inhibit the growth of cancer cells from several different tissues (48, 49). TRX has been reported to inhibit the growth of cancer cells from several different tissues (48, 49). TRX has been reported to play a role in several different tissues (48, 49). TRX has been reported to inhibit the growth of cancer cells from several different tissues (48, 49). TRX has been reported to inhibit the growth of cancer cells from several different tissues (48, 49). TRX has been reported to inhibit the growth of cancer cells from several different tissues (48, 49).

In conclusion, we identified TBP-2/VDUP1 as a new TRX-regulatory factor from human T-cell lymphotropic virus-I transformed cells. TBP-2/VDUP1 interaction may be one of the mechanisms through which 1α,25-dihydroxyvitamin D₃ exerts its growth inhibitory effect (54).

Accordingly, the decrease of TRX function due to the TRX-D3 interaction may be one of the mechanisms through which 1α,25-dihydroxyvitamin D₃ exerts its growth inhibitory effect (54).