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Thioredoxin-Binding Protein-2-Like Inducible Membrane Protein Is a Novel Vitamin D3 and Peroxisome Proliferator-Activated Receptor (PPAR)γ Ligand Target Protein that Regulates PPARγ Signaling

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Thioredoxin binding protein-2 (TBP-2), which is identical with vitamin D3 (VD3) up-regulated protein 1 (VDUP1), plays a crucial role in the integration of glucose and lipid metabolism. There are three highly homologous genes of TBP-2/vitamin D3 up-regulated protein 1 in humans, but their functions remain unclear. Here we characterized a TBP-2 homolog, TBP-2-like inducible membrane protein (TLIMP). In contrast to TBP-2, TLIMP displayed no significant binding affinity for thioredoxin. TLIMP exhibited an inner membrane-associated pattern of distribution and also colocalized with transferrin and low-density lipoprotein, indicating endosome- and lysosome-associated functions. VD3 and ligands of peroxisome proliferator-activated receptor (PPAR)-γ, an important regulator of energy metabolism and cell growth inhibition, induced the expression of TLIMP as well as TBP-2. Overexpression of TLIMP suppressed both anchorage-dependent and -independent cell growth and PPARγ ligand-inducible gene activation. These results suggest that TLIMP, a novel VD3- or PPARγ ligand-inducible membrane-associated protein, plays a regulatory role in cell proliferation and PPARγ activation. (*Endocrinology* 147: 733–743, 2006)

IT IS WELL established that vitamin D3 (VD3) acts as a modulator of cell growth, differentiation, maintenance of extracellular calcium levels, bone mineralization, and lipid metabolism. VD3 exerts its actions through nuclear VD3 receptor (VDR)-mediated signal transduction and gene transcription (1). Another well-established nuclear receptor subfamily, peroxisome proliferator-activated receptors (PPARs), comprising PPARα, -δ (β), and -γ, binds fatty acids and play important roles in energy homeostasis. Thiazolidine derivatives, such as troglitazone and pioglitazone, which are selective PPARγ agonists, reduce hyperlipidemia in obese and diabetic animals (2–5). VDR and/or PPARs regulate gene transcription to modulate uptake of calcium, phosphate, lipids, and glucose from plasma (6, 7). In addition, several reports have suggested that VDR and PPARs have a regulatory role in the uptake of plasma proteins (8–11). However, the regulatory role and mechanisms of the membrane-associated function of VDR and PPARs largely remains to be clarified.

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Abbreviations: DTT, Dithiothreitol; EF, elongation factor; EGFP, enhanced green fluorescent protein; FABP4, fatty acid-binding protein 4; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; LDL, low-density lipoprotein; LPL, lipoprotein lipase; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; TBP-2, thioredoxin binding protein-2; TLIMP, TBP-2-like inducible membrane protein; TRX, thioredoxin; VD3, vitamin D3; VDUP1, vitamin D3 up-regulated protein 1.

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In the course of our study of thioredoxin (TRX), an important redox regulator (12, 13), we identified TRX-binding protein (TBP)-2 (14), which is identical with VD3 up-regulated protein 1 (VDUP1), a VD3-inducible gene in HL-60 cells (15). Several reports show that loss of TBP-2 expression is associated with cell growth or transformation. TBP-2 is down-regulated in human T cell leukemia virus I-transformed cells and human cancer tissues (16–19), whereas overexpression of TBP-2 induces cell growth suppression (18–20). Interestingly, HcB-19 mice, which have a nonsense mutation in the TBP-2 gene, exhibit hyperlipidemia characterized by elevated plasma triglyceride and/or cholesterol levels (21). The analyses of the HcB-19 mice (22) or TBP-2 knockout mice (23) revealed that TBP-2 plays a critical role in the integrated regulation of glucose and lipid metabolism in fasting. The molecular mechanisms underlying transformation and hyperlipidemia caused by loss of TBP-2 function remain to be elucidated, as does the mechanism behind the physiological function of TBP-2.

In this paper, we report that there are three homologous TBP-2/VDUP1 genes in humans. The TBP-2 homologs constitute a family and are preserved in *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* but not *Escherichia coli*, indicating that the genes have evolutionarily conserved roles in the eukaryotic system. However, the functions of these family members have not been clarified. Characterization of members of the human TBP-2 family will provide new insights into the biological roles of these genes. We have cloned a human TBP-2 homolog, TBP-2-like inducible membrane protein (TLIMP),

and show that TLIMP and TBP-2 are VD3/PPARγ ligand-inducible genes. To investigate the biological role of the TBP-2 gene family and understand the molecular mechanism of the VD3/PPAR-mediated cellular function, we characterized TLIMP and investigated the role of TLIMP in cellular growth regulation and PPARγ ligand-induced gene activation.

Materials and Methods

Reagents and materials

Phorbol 12-myristate 13-acetate (PMA), clofibrate, and prostaglandin J2 were purchased from Sigma (St. Louis, MO). Blasticidin was purchased from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Complete protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). Total RNAs from human cultured preadipocytes and adipocytes were purchased from Zen-Bio, Inc. (Research Triangle Park, NC). The human adipocytes were differentiated from preadipocytes by culturing with insulin and dexamethasone for 14 d. Troglitazone and pioglitazone were kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan) and Takeda Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. L165,041 was purchased from Calbiochem (La Jolla, CA). Alexa-633-labeled transferrin, BODIPY FL-labeled low-density lipoprotein, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, Alexa 568-conjugated antimouse IgG, and LysoTracker Blue were purchased from Molecular Probes (Eugene, OR). Human 12-lane multiple tissue and cancer cell line Northern blots were obtained from CLONTECH (Mountain View, CA).

Amino acid sequence alignment

Amino acid sequences of TBP-2 family proteins were aligned using the ClustalW program (EMBL-European Bioinformatics Institute) and the BOXSHADE program (Swiss Institute of Bioinformatics) was used for formatting the results.

Plasmids

The open reading frame of TLIMP was amplified by PCR using the oligonucleotide primers 5′-AATGGATCCATGGTGCTGGGAAAGGTGAA-3′ and 5′-CGAATTCAACGAGAGGGGCAGGAT-3′. The cDNAs were subcloned into pCMV-Tag2B (CLONTECH) and pGEX6P1 (Amersham Biosciences, Piscataway, NJ) to generate pCMV-Flag-TLIMP and pGST-TLIMP, respectively. pEF-BSR, a blasticidin-resistant mammalian expression vector driven by the elongation factor (EF) 1 promoter, was kindly provided by Dr. Ishii (Riken Research Center for Allergy and Immunology, Yokohama, Japan). The cDNA containing a Kozak sequence in the 5′ side of the TLIMP gene lacking a stop codon was obtained by PCR using oligonucleotide primers 5′-GGAATTCGCCACCATGGTGCTGGGAAAGGT-3′ and 5′-CGGGATCCACGAGAGGGGCAGGATGGTCTA-3′. The TLIMP cDNA was ligated into pEF-BSR downstream of the EF promoter, and then a DNA fragment encoding the Flag epitope or enhanced green fluorescent protein (EGFP; pEGFP-N3; CLONTECH) was inserted into the 3′ end of TLIMP to generate pEF-BSR-TLIMP-Flag or pEF-BSR-TLIMP-EGFP, respectively. The N-terminal Flag epitope-tagged TBP-2 cDNA was ligated into pEF-BSR to generate pEF-BSR-Flag-TBP-2. Yeast expression vectors, pGBKT7-TLIMP and pGBKT7-TBP-2, for the expression of TLIMP or TBP-2 fused with the GAL4 DNA-binding domain were constructed by insertion of the open reading frames of TLIMP and TBP-2 into pGBKT7 (CLONTECH). The cDNA of TRX was ligated into pACT2 (CLONTECH) to generate pACT2-TRX, an expression vector for the GAL4 activation domain fused with TRX. Luciferase reporter plasmids containing five GAL4 binding sites in the promoter region (pGAL4-luc), and plasmid expressing PPARγ2 fused to the GAL4 DNA binding domain (pM-PPARγ2) and HA-tag (pcDNA3-HA-PPARγ2) were kindly provided by Dr. Ohshima (24).

Cell culture and transfection

HL-60 cells were cultured in RPMI 1640 medium. HeLa S3, COS7, and 293 cells were cultured in DMEM. CHO cells were cultured in F-12 medium. Heat-inactivated fetal calf serum (FCS) and antibiotics were added to the media. Cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Stable transfectants were generated by transfection of HeLa S3 cells with either pEF-BSR-TLIMP-Flag or pEF-BSR control plasmid. After 24 h, cells were plated and selected in medium containing 4 μg/ml blasticidin. The expression of TLIMP in the transfectants was confirmed by Western blotting analyses. Reporter assay were performed as described previously (25). NIH3T3 cells were transfected with the TLIMP and GAL4-PPARγ2 expression vectors and luciferase reporter plasmids containing five GAL4 binding sites in the promoter region. Cells were then treated with troglitazone and luciferase reporter assay were performed (24).

Northern blotting analysis

Total RNA from cells was extracted using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Total RNA (10–20 μg/lane) was fractionated by denaturing agarose gel electrophoresis and transferred to a nylon membrane (Hybond N^+; Amersham Biosciences). The blots were hybridized with a [^{32}P]-labeled probe prepared using the BcaBest labeling kit (Takara, Shiga, Japan) overnight at 68 C.

Semiquantitative RT-PCR

Total RNAs from cells and tissues were extracted using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was performed with a SuperScript III first-strand synthesis system kit (Invitrogen). PCRs were carried out using the following oligonucleotide primers: human TLIMP forward primer, 5′-ATGGTGCTGGGAAAGGTGAA-3′, and reverse primer, 5′-TCAACGAGAGGGGCAGGAT-3′; human TBP-2 forward primer, 5′-AAGGTGCTGACTCAGAAG-3′, and reverse primer, 5′-CTCACTGCACATTGTTGTTG-3′; human glyceraldehyde-3-phosphate dehydrogenase forward primer, 5′-ATGGGGAAGGTGAAGGTCGGAGTC-3′, and reverse primer, 5′-CCATGCCAGTGAGCTTCCCGTTC-3′; mouse TLIMP forward primer, 5′-ACAGTTACAGTGCCTGAGAAGACTCGG-3′, and reverse primer, 5′-GTGCCCTCAGGTGTTACGTCAAG-3′; mouse TBP-2 forward primer, 5′-GTGATGGATCTAGTGGATGTC-3′, and reverse primer, 5′-TCACTGCACGTTGTTGTTG-3′; mouse fatty acid-binding protein 4 (FABP4) forward primer, 5′-ACAAAATGTGTGATGCCTTTGTGGGAAC-3′, and reverse primer, 5′-TCCGACTGACTATTGTAGTGTTTGATGCAA-3′; mouse lipoprotein lipase (LPL) forward primer, 5′-GGGGTACCTGCCCACCACTTGTCCCCTGGAG-3′, and reverse primer, 5′-CGGGATCCCGGTGCACCCTTCTGCTTTGCTGC-3′; and mouse β-actin forward primer, 5′-ATGGATGACGATATCGCTGCGCT-3′, and reverse primer, 5′-TAGAAGCACTTGCGGTGCACGAT-3′. Amplification of the products is not saturated with the number of cycles performed.

Western blotting analysis

Cell lysates or immunoprecipitates were fractionated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). Western blot analysis was performed using an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences), according to the manufacturer's instructions.

Subcellular fractionation

Cells (at confluence in a 3.5 cm dish) were lysed with lysis buffer [25 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol (DTT), 10% glycerol, 1% Trition X-100, and 1× complete protease inhibitor cocktail] and the cell lysate was centrifuged at 100,000 × *g* for 10 min. The supernatant, the Triton X-100 soluble fraction (100 μl) was transferred to a new tube, and 25 μl of 5× sample buffer [500 mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, 25% glycerol, 12.5% 2-mercaptoethanol, and 0.25% bromophenol blue] was added. The pellet, the Triton X-100 insoluble fraction, was resuspended with 125 μl of 1× sample buffer (5-fold dilution of 5× sample buffer with lysis buffer) before sonication. The fractionated samples were subjected to Western blotting analyses.

Immunofluorescent staining

Cells were cultured on glass-bottom dishes, fixed with 4% paraformaldehyde in PBS for 5 min at room temperature, and permeabilized for

5 min using 1% Nonidet P-40 in PBS. The permeabilized cells were incubated in PBS containing 1.5% FCS and then with anti-Flag antibody (Sigma) or anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by FITC- or Alexa 568-conjugated antimouse IgG. The immunostained cells were examined with a confocal microscope (Leica Microsystems, Mannheim, Germany).

Preparation of recombinant proteins and in vitro binding assay

In vitro-translated proteins were prepared using a TNT-coupled rabbit reticulocyte translation system (Promega, Madison, WI). The [^{35}S]methionine-labeled translated products were analyzed by SDS-PAGE after performance of a glutathione-S-transferase (GST) pull-down assay. GST-TRX was prepared as described previously (14). The precipitates were detected by autoradiography using a Bio-image analyzer BAS2000 (Fuji Film Co. Ltd., Tokyo, Japan) or Coomassie Brilliant Blue staining.

Insulin-reducing assay

COS7 cells were transiently transfected with the GFP-TBP-2 or TLIMP expression vector. After 3 d, the cells (at confluence in a 10 cm dish) were collected and lysed by the freeze and thaw method. The cell lysate (10 mg/ml, 10 μl) was preincubated with 2.5 μl DTT activation buffer [0.1 M Tris-HCl (pH 7.5), 2 mM EDTA, 1 mg/ml BSA, and 2 μM DTT] for 15 min at 37 C. The preincubated samples were mixed with 110 μl reaction buffer [0.1 M Tris-HCl (pH 7.5), 2 mM EDTA, 0.2 mM nicotinamide adenine dinucleotide phosphate reduced, and 0.4 U/ml yeast TRX reductase], and then 10 μl insulin solution [50 mM Tris-HCl (pH 7.5), 10 mg/ml insulin] were added to the mixture. The decrease in nicotinamide adenine dinucleotide phosphate reduced absorbance at 340 nm was recorded (maximal velocity, millioptical density at 340 nm/min) at room temperature. The calculated values were compared with the standard curve for recombinant TRX to obtain a quantitative determination of the absolute amounts of TRX.

Yeast two-hybrid analysis

The yeast two-hybrid analysis was performed using the yeast MATCHMAKER two-hybrid system (CLONTECH), according to the manufacturer's directions. *S. cerevisiae* strain AH109 transformed with pGBKT7-TLIMP or pGBKT7-TBP-2, together with either pACT2 or pACT2-TRX, respectively. Transformed colonies were cultured in synthetic medium with or without histidine.

Cell proliferation assay

Blasticidin-resistant mammalian expression vectors were introduced into CHO cells, and the cells were cultured with 8 μg/ml of blasticidin-containing medium for 2 or 3 d to eliminate the nonresistant cells. The blasticidin-resistant cells (3–5 × 10^3 cells in 100 μl of culture medium containing 8 μg/ml of blasticidin) were cultured in 96-well flat-bottom microtiter plates. Cell proliferation was measured as the formation of formazan using SF cell-counting reagents (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions.

Colony formation assay

Blasticidin-resistant mammalian expression vectors were introduced into HeLa S3 cells, and the cells were cultured with 4 μg/ml of blasticidin-containing medium for 3 d. The cells (1 × 10^6 cells) were plated in 0.35% agar containing DMEM, 10% FCS, and 4 μg/ml of blasticidin on a 0.5% agar base layer containing DMEM. The total number of foci was scored after 10 d.

Statistical methods

Results are expressed as means ± SD. Statistical comparisons were made using Student's *t* test or ANOVA coupled with a Fisher's test. A statistically significant difference was defined as $P < 0.05$, which is represented by an *asterisk* in the data presentation.

Results

TBP-2 protein family

As shown in Fig. 1A, BLAST searches were used to identify human genes homologous to TBP-2. DRH1 and two other genes (GenBank accession no. AF193051 and BC015928) were identified. DRH1 has been reported as a gene that is down-regulated in advanced human hepatocellular carcinoma (26). Here we refer to BC015928 as TLIMP, which was originally identified as KIAA1376 when it was found in the full-length human cDNA sequencing project (27). The function of AF193051 has not been reported. TLIMP has 40% identity and over 80% similarity with TBP-2 at the amino acid level, and the other genes also exhibit high levels of identity and similarity. All the genes included eight exons, each of which encodes a corresponding region of the protein (Fig. 1B and supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Therefore, it is likely that this mammalian gene family was generated by gene duplications. The members of the TBP-2 gene family also have some homology to arrestin proteins. For example, TLIMP has 25% similarity with β-arrestin-1 at the amino acid level. TBP-2 and arrestins have similar numbers of amino acids (~400) and show overall similarity, rather than just a highly conserved region.

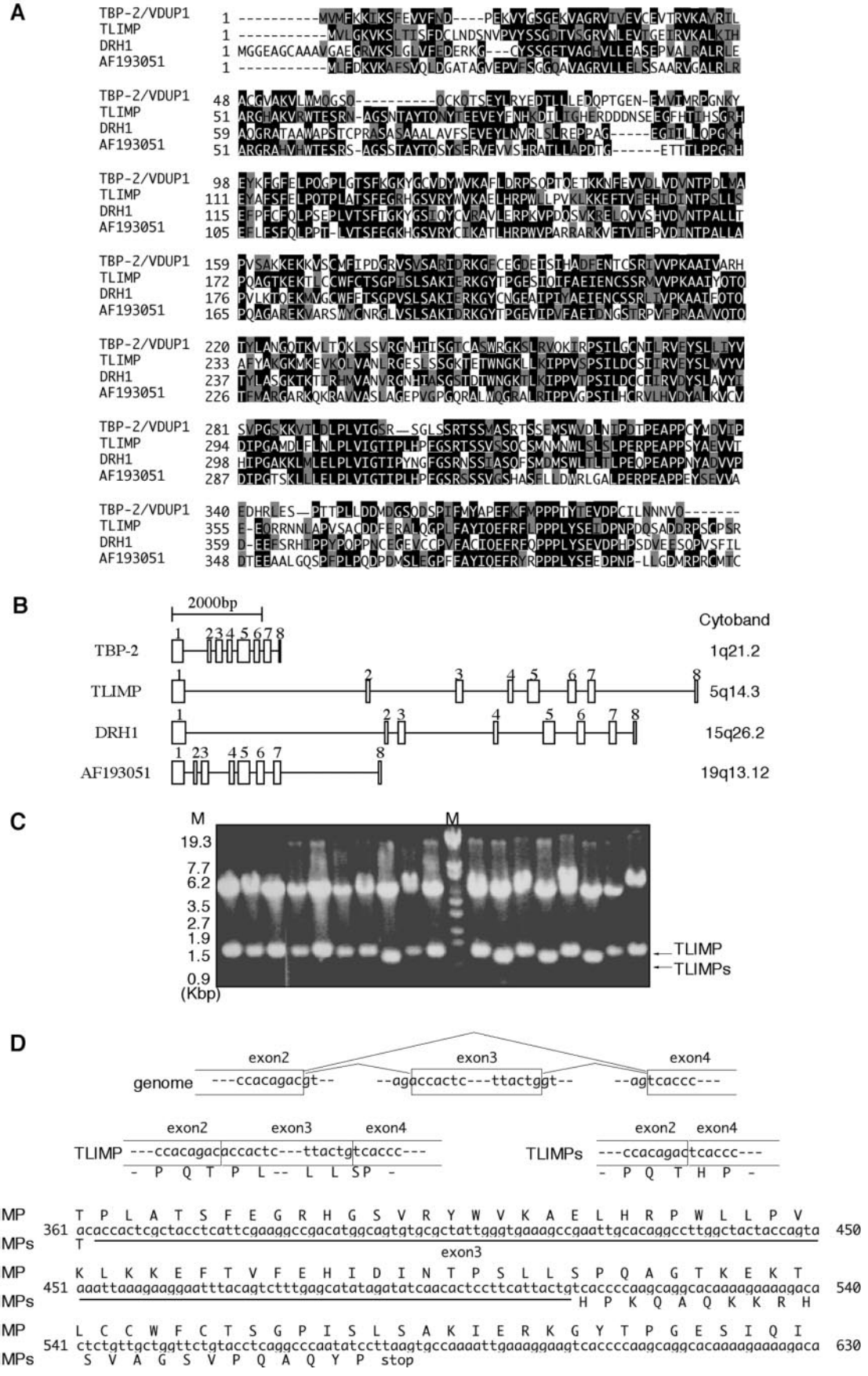
TLIMP cDNA was cloned from a 293-cell cDNA library by PCR and then sequenced. Two isoforms of TLIMP cDNA with different lengths were found (Fig. 1C). The longer cDNA encoded full-length TLIMP, producing a protein of 414 amino acids. The shorter TLIMP splice variant, referred to as TLIMPs, lacks 148 bp corresponding to exon 3, generating a 24-amino-acid C-terminal tail and a translational stop codon and producing a shorter protein of 144 amino acids (Fig. 1D).

Tissue distribution of TLIMP expression

The expression of TLIMP was analyzed in normal human tissues and human cancer cell lines, and as shown in Fig. 2A, the size of TLIMP mRNA was about 4.5 kb in both normal and cancer cells. Strong expression of TLIMP was detected in normal skeletal muscle, placenta, kidney, adrenal gland, lymph node, mammary gland, thyroid, and trachea, but only very weak expression was detected in normal colon, thymus, spleen, small intestine, bladder, and bone marrow. In human cancer cell lines, TLIMP was strongly expressed in the lung adenocarcinoma cell line A549, whereas low expression levels were observed in other cancer cell lines. The expression of TBP-2 was strong in normal heart, skeletal muscle, spleen, and peripheral blood lymphocytes, and weak in normal brain and liver (Fig. 2B). The distribution of TLIMP was clearly different from that of TBP-2 in some tissues.

TLIMP does not interact with TRX

TBP-2 binds to TRX *in vivo* and *in vitro* (14), but no interaction of TLIMP with TRX was detected in a yeast two-hybrid analysis, as shown in Fig. 3A. The same analysis showed binding of TRX with TBP-2. Similar results were obtained in *in vitro* binding assays, in which TBP-2 coprecipitated with GST-TRX, but TLIMP failed to do so (Fig. 3B). Overexpres-


A
TBP-2/VDUP1
TLIMP
DRH1
AF193051
B
2000bp
Cytoband
TBP-2
1q21.2
TLIMP
5q14.3
DRH1
15q26.2
AF193051
19q13.12
C
M
19.3
7.7
6.2
3.5
2.7
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TLIMP
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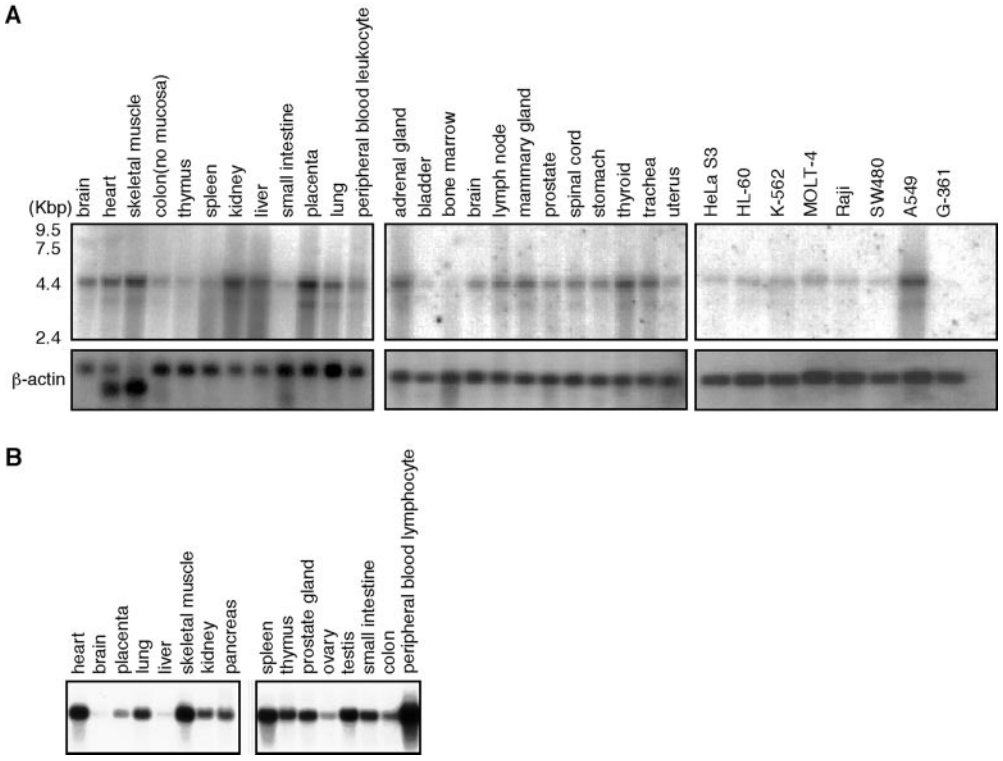

FIG. 2. Analysis of TLIMP expression: tissue distribution of TLIMP (A) and TBP-2 (B). RNA blots containing poly (A)+ RNA from multiple human tissues or cancer cell lines were hybridized with full-length TLIMP or TBP-2 cDNA as a probe. Human β-actin was used as a control to determine the relative amount of RNA from each of the tissues or cells.

sion of TBP-2 has been shown to suppress the reducing activity of TRX (14), and the effect of TLIMP on this activity was examined in an insulin-reducing assay. TRX-reducing activity was not significantly changed in cellular extracts transiently expressing TLIMP, whereas it was significantly suppressed in cellular extracts transfected with TBP-2 (Fig. 3C). These results indicate that TRX interacts with TBP-2 but not with TLIMP.

Intracellular localization of TLIMP

To determine the intracellular distribution of TLIMP, Flag-tagged TLIMP (TLIMP-Flag) was expressed in HeLa S3, 293, COS7, and CHO cells. The cellular distribution of TLIMP-Flag is shown in Fig. 4A. Immunofluorescent analysis using anti-Flag antibody showed that TLIMP is present in the plasma membrane and small vesicular structures scattered throughout the cytoplasm. As reported previously, TBP-2 was detected mainly in the nucleus and partly in the cytoplasm in COS7 cells (19). To determine whether TLIMP is expressed on the inner or outer side of the plasma membrane, TLIMP-Flag was expressed in HeLa S3 cells and stained by Nonidet P-40 without permeabilization. TLIMP-Flag was not stained in the nonpermeabilized cells but significantly stained in the permeabilized cells, indicating that TLIMP is expressed on the inner side of the plasma membrane (Fig. 4B). The vesicular pattern of TLIMP protein expression in cytoplasm is similar to that of endosomes or lysosomes. To analyze whether TLIMP is present in the cellular compartments, we examined the distribution of EGFP-tagged TLIMP with Alexa633-conjugated transferrin and LysoTracker Blue, which are markers for endosomes and lysosomes, respectively. As shown in Fig. 4C, TLIMP-EGFP showed a similar distribution to TLIMP-Flag, and the fluorescence merged with that of transferrin or LysoTracker in intracellular vesicular structures. Merging of all three fluorescent signals was observed in several areas. Whereas internalized transferrin is predominantly recycled to the plasma membrane through recycling of endosomes, low-density lipoprotein (LDL) is internalized and transported into lysosomes. To validate whether TLIMP is present in the lysosomal pathway, the cellular localizations of TLIMP and LDL were examined. As shown in Fig. 4D, the fluorescence of internalized LDL partly merged with TLIMP. These results suggest that TLIMP expression is associated with the plasma membrane, endosomes, and lysosomes during endocytosis.

FIG. 1. The TBP-2 protein family. A, ClustalW alignment of the amino acid sequences of the human TBP-2 family. The numbers on the *left* refer to the amino acids of each protein. B, Intron-exon structures of the TBP-2 gene family. The coding exons of the proteins are depicted as *boxes*. *Upper numbers* indicate the order of each exon. The *signatures on the right* indicate the cytoband of each protein. The *scale below* defines the proportional length of the genomic sequences. C, Alternatively spliced form of TLIMP. The amplified cDNA of TLIMP was subcloned into pCMV-Tag2 (Stratagene, La Jolla, CA) and the length of the insert was checked by restriction enzyme (*Hin*dIII) digestion. A DNA marker is shown in lane M. D, Summary of the two spliced isoforms. The shorter isoform, TLIMPs, lacks 148 bp corresponding to exon 3 (*underlined*), with a translational stop codon after a short 24-amino-acid C-terminal tail.

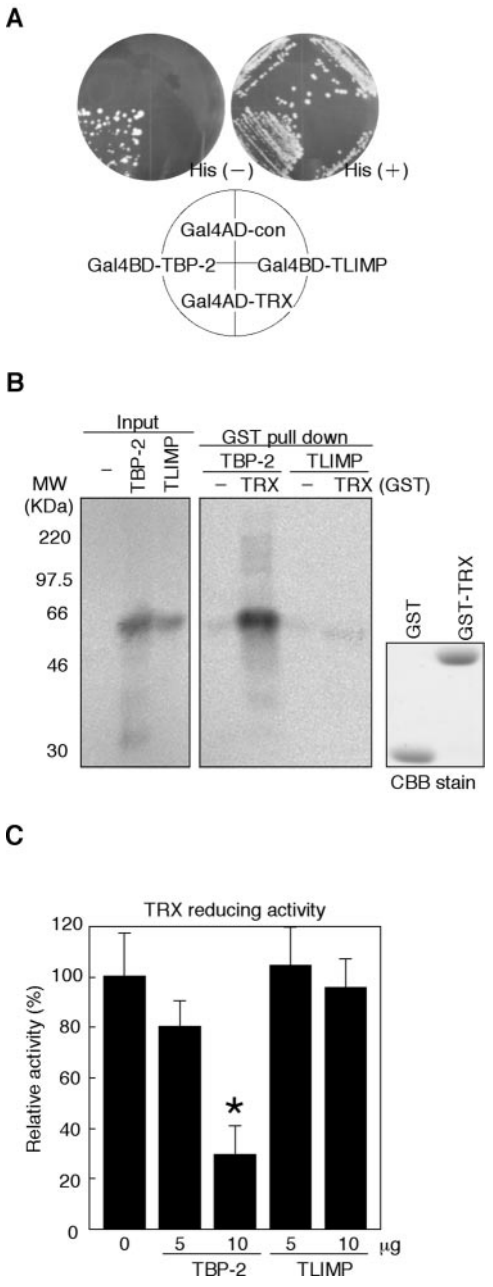

FIG. 3. TLIMP did not associate with TRX. A, Yeast two-hybrid analysis of the interaction of TLIMP or TBP-2 with TRX. pGBKT7-TLIMP or pGBKT7-TBP-2 was introduced into the yeast strain AH109 with either pACT2 or pACT2-TRX. The growth of the yeast transformants on a selective synthetic medium with or without histidine is shown. B, GST pull-down analysis of the interaction of TLIMP or TBP-2 with TRX. ^{35}S-labeled TLIMP or TBP-2 precipitated with GST-TRX or GST immobilized on GSH beads. The *right panel* shows that equal amounts of GST-fusion proteins were used in the pull-down assay, as determined by Coomassie Brilliant Blue staining. C, The effect of TRX activity on TLIMP expression. The TRX activity of the extract of COS7 cells transiently transfected with the indicated amount of expression vector for GFP-TBP-2 or GFP-TLIMP fusion proteins was determined in an insulin reducing assay. Expression vectors (10 μg/plate) were introduced into cells cultured in 10-cm dishes. The total DNA concentration was kept constant using pEGFP-C1. Activities of samples are shown relative to the control value (lane 1), which is taken as 100%. The results are shown as the mean ± SD of three samples.

Regulation of TLIMP expression

Because TBP-2 was first identified as a gene, the expression of which was induced by VD3 in HL-60 cells, TLIMP expression in VD3-treated HL-60 cells was examined. Expression of TLIMP was also induced when HL-60 cells were cultured in the presence of VD3, in a time- and dose-dependent manner (Fig. 5A). The effects of retinoic acid (RA) and PMA on the expression of TBP-2 and TLIMP were then tested. RA and PMA are known to induce differentiation of HL-60 cells into granulocytes and macrophage-like cells, respectively. As shown in Fig. 5B, specific morphological changes in HL-60 cells were observed on treatment with RA, VD3, and PMA. RA did not induce TBP-2 or TLIMP expression, whereas PMA significantly induced the expression of TLIMP but not TBP-2. The extent of induction of TLIMP expression by PMA was less than that by VD3. TRX expression decreased in cells treated with VD3, RA, and PMA.

PPARγ has been implicated in the regulation of cell growth and differentiation of cells or tissues, such as adipose tissues, breast, and colon (28). Similarly to VD3, PPAR ligands induce monocytic differentiation and concomitantly inhibit cell proliferation in HL-60 cells (29, 30). These findings led us to hypothesize that members of the TBP-2 family have a role in the biological responses elicited by not only VD3 but also PPAR ligands. Therefore, the effects of PPAR ligands on the expression of TBP-2 and TLIMP were examined in HL-60 cells. Troglitazone, a selective PPARγ agonist, significantly induced both TBP-2 and TLIMP expression in a time- and dose-dependent manner (Fig. 5C). These genes were also up-regulated by pioglitazone, another PPARγ agonist. Clofibrate, a selective PPARα agonist, markedly induced TBP-2 expression but had no effect on TLIMP expression. These results suggest that TBP-2 and TLIMP are both up-regulated by PPARγ ligands but differentially regulated by PPARα ligands. TRX expression was decreased with all the PPAR agonists tested, showing a reciprocal pattern to TBP-2 expression (Fig. 5D). To further study the effect of PPAR ligands on the expression of these genes, we examined the effects of L165,041, a PPARβ/δ ligand, and prostaglandin J2, an endogenous ligand of PPARγ, on the levels of TLIMP and TBP-2 expression (31). As shown in Fig. 5E, both TLIMP and TBP-2 are up-regulated by prostaglandin J2 but not L165,041. Collectively, the above results indicate that both TLIMP and TBP-2 are downstream regulatory proteins of VD3 and PPARγ.

Because PPARγ plays an important role in differentiation in adipocytes as well as monocytes, the expression levels of TLIMP and TBP-2 were examined during differentiation of adipocytes *in vitro*. As shown in Fig. 6, both TLIMP and TBP-2 are strongly expressed in adipocytes, compared with preadipocytes, indicating that these genes are up-regulated in association with differentiation of adipocytes. Expression of TLIMP and TBP-2 was also detected in brown and white adipose tissues *in vivo* (Fig. 6B), and collectively these results suggest that both TLIMP and TBP-2 play a role in the differentiation and function of adipocytes.

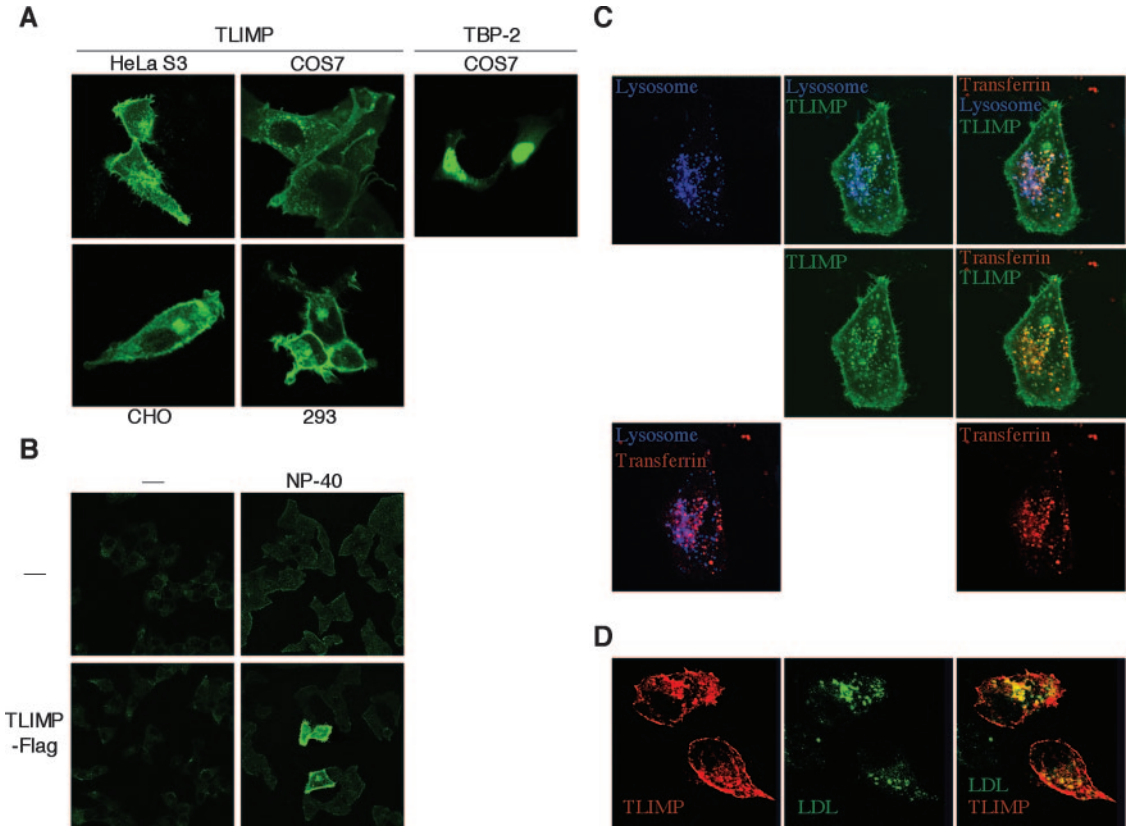

FIG. 4. Cellular localization of TLIMP. A, Expression of TLIMP. Flag epitope-tagged TLIMP was transiently expressed in HeLa S3, 293, COS7, and CHO cells. C-terminal Myc epitope-tagged TBP-2 was transiently expressed in COS7 cells. The cells were permeabilized by 1% Nonidet P-40 and then immunostained using anti-Flag or anti-Myc antibody, and FITC-conjugated antimouse IgG, as described in *Materials and Methods*. B, Detection of TLIMP protein in permeabilized cells. HeLa S3 cells transfected with TLIMP-Flag were immunostained after permeabilization with 1% Nonidet P-40 or after no permeabilization. A and B, The fixed and immunostained cells were analyzed using confocal microscopy. C, Colocalization of TLIMP, transferrin, and LysoTracker. HeLa S3 cells expressing C-terminal EGFP-tagged TLIMP were incubated with 50 nM LysoTracker Blue for 1 h and then 20 μg/ml Alexa633-labeled transferrin for 15 min. All combinations of merged colors are indicated. D, Colocalization of TLIMP and LDL. HeLa S3 cells expressing C-terminal Flag-tagged TLIMP were incubated with 50 μg/ml BODIPY FL-labeled LDL for 1 h and then immunostained using anti-Flag antibody and Alexa 568-conjugated antimouse IgG, as described in *Materials and Methods*. Merged colors are indicated.

Suppression of cell growth by TLIMP

VD3 and PPAR ligands are known to have strong cell growth-inhibitory activity in HL-60 cells, and previous reports have shown that TBP-2 also inhibits cell proliferation (18, 19). To investigate whether TLIMP also has this inhibitory activity, the effect of overexpression of TLIMP on cell proliferation was examined. In TLIMP transfectants, TLIMP was mainly detected in the Triton X-100-insoluble fraction (Fig. 7A, *upper panel*), indicating that the majority of TLIMP was localized in the detergent-insoluble membrane fraction. CHO cells transiently overexpressing TLIMP showed retardation of cell growth (Fig. 7A). It has been reported that TBP-2 suppresses anchorage-dependent and anchorage-independent cell growth (32), and to test the role of TLIMP in anchorage-independent cell growth, the growth of TLIMP-overproducing HeLa S3 cells was examined in culture in a semisolid medium. The number and size of foci was reduced in TLIMP- and TBP-2-transfected cells (Fig. 7B). These results demonstrate that both TLIMP and TBP-2 suppress anchorage-dependent and -independent cell growth.

TLIMP suppresses ligand-induced PPARγ activation

We next hypothesized that up-regulation of TLIMP by PPARγ ligands plays a role in PPARγ signaling through feedback regulation. To test this hypothesis, we analyzed the effect of overexpression of TLIMP on PPARγ transactivation in NIH3T3 cells, with a reporter assay using a GAL4-fused PPARγ2 expression vector and a luciferase reporter plasmid containing GAL4 binding sites in the promoter region. As shown in Fig. 8, troglitazone-induced reporter activation was suppressed by overexpression of TLIMP, suggesting that TLIMP negatively regulates ligand-induced PPARγ activation.

PPARs induce the expression of responsive target genes such as FABP4 and LPL through direct binding to the peroxisome proliferator-responsive element in their promoter regions (33, 34). To investigate the effect of TLIMP on PPARγ-induced endogenous gene expression, the levels of FABP4, LPL, TLIMP, and TBP-2 were examined in cells overexpressing PPARγ and TLIMP. As shown in Fig. 8B, expression of the target genes in cells expressing PPARγ increased with treatment with troglitazone. The induction of expression was

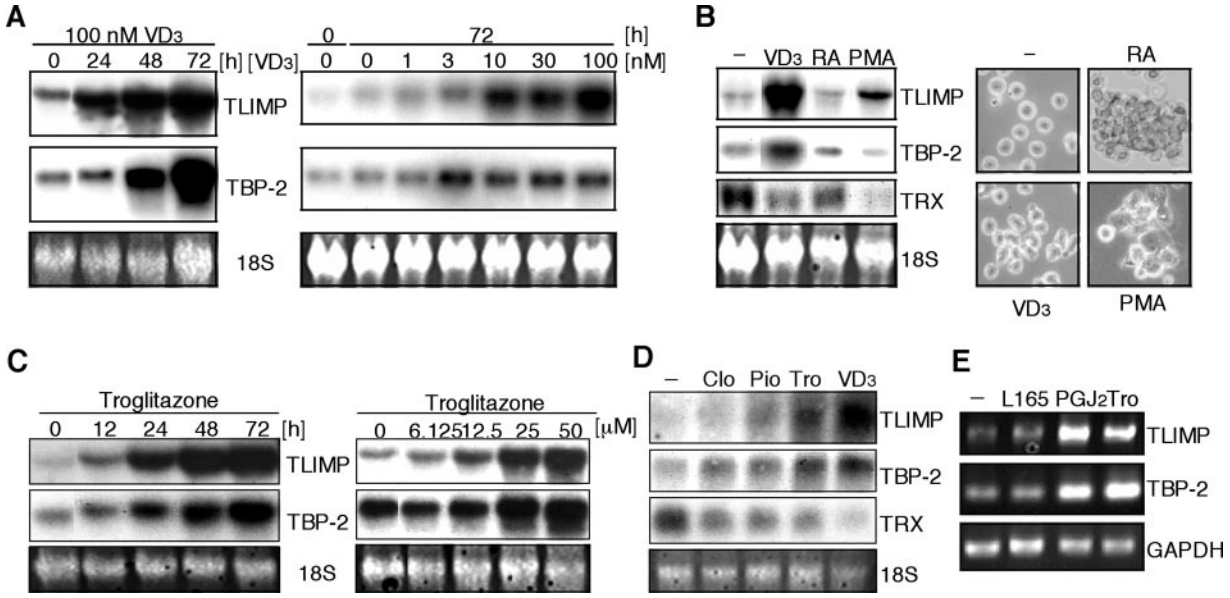

FIG. 5. The effect of inducers of differentiation on TLIMP expression. A, Expression of TLIMP and TBP-2 in VD3-stimulated HL-60 cells. At the indicated time points, total RNA was isolated from cells treated with 100 nM (*left*) or various concentrations (*right*) of VD3. B, Expression of TLIMP, TBP-2, and TRX in HL-60 cells treated with VD3, RA, PMA. Cells were cultured with 100 nM VD3, 10 μM RA, and 100 ng/ml of PMA for 76 h. Morphological changes of the HL-60 cells are shown in the photographs on the *right*. C, Expression of TLIMP and TBP-2 in troglitazone-stimulated HL-60 cells. At the indicated time points, total RNA was isolated from cells treated with 50 μM (*upper*) or various concentrations (*lower*) of troglitazone for 72 h. D, Expression of TLIMP, TBP-2, and TRX in HL-60 cells treated with troglitazone, pioglitazone, and clofibrate. Cells were cultured with 100 μM clofibrate (Clo), 50 μM pioglitazone (Pio), 50 μM troglitazone (Tro), and 100 nM VD3 for 72 h. The isolated total RNA (20 μg/lane) was fractionated and transferred to a nylon membrane, as described in *Materials and Methods*. The same membrane was hybridized with a [^{32}P]-labeled probe using cDNA encoding TLIMP, TBP-2, or TRX. E, Expression of TLIMP and TBP-2 in HL-60 cells treated with L165,041 and prostaglandin J2. Cells were cultured with 10 μM L165,041 (L165), 10 μM prostaglandin J2 (PGJ2), or 50 μM troglitazone (Tro) for 72 h. The expression levels of TLIMP and TBP-2 were determined by RT-PCR using cDNA prepared using RNA from the cells.

suppressed by overexpression of TLIMP, suggesting that TLIMP negatively regulates PPARγ-dependent gene activation.

Discussion

In the present study, we found that there are four familial TBP-2 genes, TBP-2/VDUP1, TLIMP, AF193051, and DRH1 in humans. Members of the human TBP-2 protein family share some common features but also have different features. TBP-2 and AF193051 were predominantly concentrated in the nucleus (20) (Tan, A., S. Oka, M. Mochizuki, H. Masutani, and J. Yodoi, unpublished observation), whereas TLIMP was found in the plasma membrane, lysosomes, and endosomes. The cellular distribution of DRH1 is similar to that of TLIMP; EGFP-tagged DRH1 has been found to be predominantly distributed in small vesicular structures at the periphery of the nucleus in COS7 cells (26), suggesting the localization in endosomes and/or lysosomes. TBP-2 familial proteins may coordinately regulate common cellular functions in different subcellular compartments. TBP-2 proteins have remote homology to the arrestin family of proteins, which play an important role in the internalization of G protein-coupled receptors and transforming growth factor-β, thereby quenching their signal transduction (35, 36). Based on the structural homology, TLIMP may have a similar biological role such as protein trafficking during endocytosis as well as scaffold or adaptor function similar to arrestin (37). Indeed, overexpression of TLIMP suppressed the uptake of transferrin (data not shown). It could be also postulated that TLIMP itself is associated with endocytosis of VD3 or endogenous ligands of PPARγ.

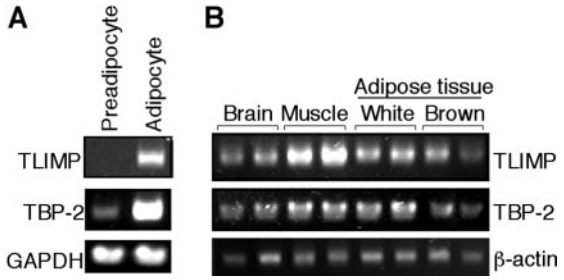

FIG. 6. Expression of TLIMP and TBP-2 in adipose cells and tissues. A, Expression of TLIMP and TBP-2 in association with differentiation of adipocytes. The expression levels of TLIMP and TBP-2 were determined by RT-PCR using cDNA prepared using RNA from human adipocytes and preadipocytes. B, Expression of TLIMP and TBP-2 in brown and white adipose tissues *in vivo*. RT-PCR was performed using cDNA prepared using RNA from the indicated tissues in C57BL/6 mice.

Expression levels of TLIMP and TBP-2 were significantly up-regulated by VD3 and PPARγ agonists, rather than by RA and PMA, in HL-60 cells (Fig. 5). These results indicate that the induction of TLIMP and TBP-2 expression in HL-60 cells is specifically associated with differentiation of monocytes and not with granulocytes. In addition, TLIMP and TBP-2 were up-regulated in association with differentiation of adipocytes (Fig. 6), indicating that these genes play an important role in PPARγ-mediated adipogenesis or lipid metabolism.

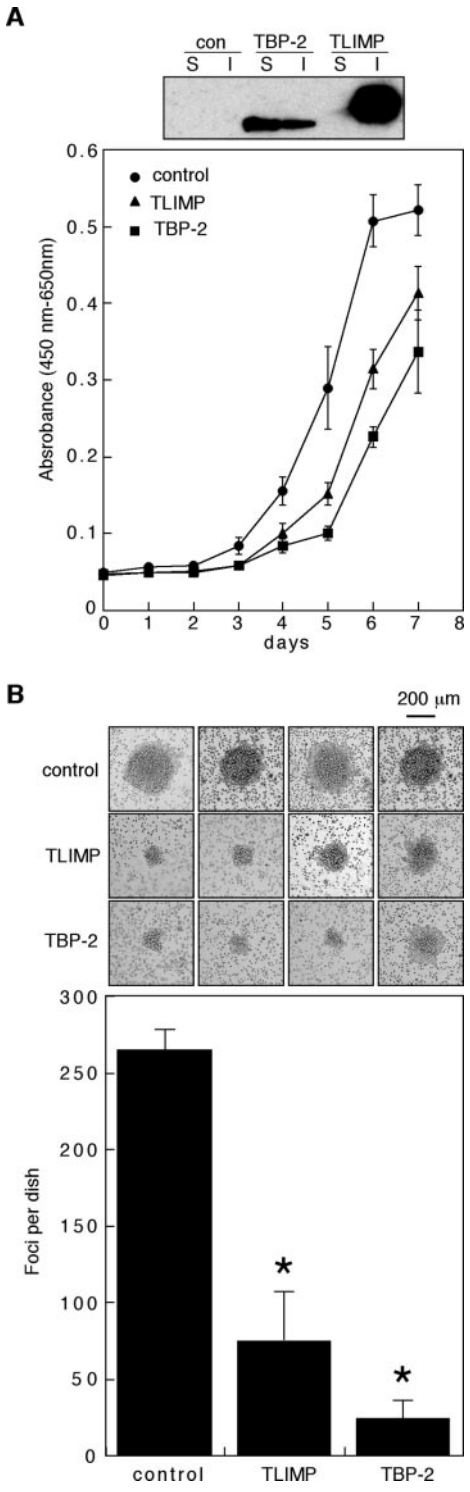

FIG. 7. Cell growth suppressive effect of TLIMP. A, Suppression of cell proliferation by TBP-2 and TLIMP. The cell proliferation rate was analyzed in CHO cells transiently transfected with pEF-BSR, pEF-BSR-TBP-2-Flag, or pEF-BSR-TLIMP-Flag vectors. B, Suppression of anchorage-independent cell proliferation by TBP-2 and TLIMP. Blasticidin-resistant expression vectors, pEF-BSR, pEF-BSR-TBP-2-Flag, and pEF-BSR-TLIMP-Flag, were used to transiently transfect HeLa S3 cells. A colony formation assay was performed as described in *Materials and Methods*. The results are shown as the mean ± SD of triplicate cultures. Foci are shown in the *upper photographs*.

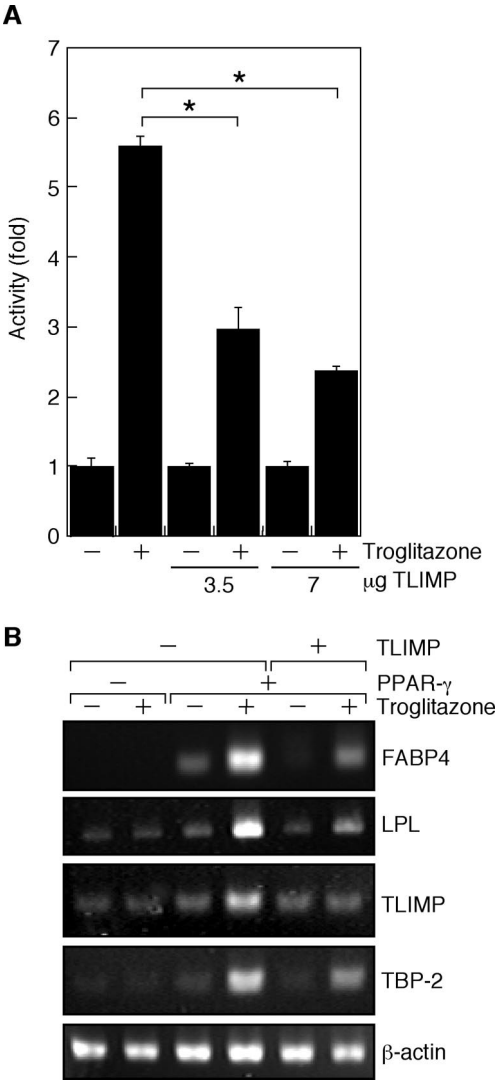

FIG. 8. TLIMP suppresses troglitazone-induced PPARγ activation. A, PPARγ-dependent transactivation is suppressed by overexpression of TLIMP. NIH3T3 cells were cotransfected with 1 μg pGAL4-luciferase reporter plasmid (pGAL4-luc) and 2 μg of plasmid expressing PPARγ2 fused to the GAL4 DNA binding domain (pM-PPARγ2) with or without the indicated amount of Flag-tagged TLIMP expression plasmid (pEF-BSR-TLIMP-Flag). Twenty-four hours after transfection, cells were treated with 10 μM troglitazone and cultured for 18 h. Luciferase activities were then measured. B, TLIMP inhibits PPARγ-dependent gene expression. NIH3T3 cells were transfected with 2 μg HA-tagged expression plasmid (pcDNA3-HA-PPARγ2) with or without 2 μg of Flag-tagged TLIMP expression plasmid (pEF-BSR-TLIMP-Flag). Twenty-four hours after transfection, the cells were treated with 10 μM troglitazone and cultured for 18 h. The expression levels of the indicated genes were determined by RT-PCR.

The promoter regions of the TLIMP and TBP-2 genes do not have a classical VDR or PPAR response element. The mechanism of the regulations should be further investigated.

We have several pieces of evidence that the expressions of TBP-2 familial genes are differentially regulated. VD3 and PPARγ agonists failed to induce the expression of both AF193051 and DRH1 in HL-60 cells (data not shown). In addition, suberoylanilide hydroxamic acid, a histone deacetylase

inhibitor, induced expression of TBP-2 expression but not TLIMP in HeLa S3 cells (supplemental Fig. 2, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org), although a nuclear factor-Y-binding site is conserved in the TLIMP and TBP-2 promoter regions. It is reported that suberoylanilide hydroxamic acid induces the expression of TBP-2 through activation of nuclear factor-Y (17).

TLIMP and TBP-2 suppressed both anchorage-dependent and -independent cell proliferation (Fig. 7). These activities may contribute to the growth-suppressive effects of VD3 and PPAR agonists, which also inhibit anchorage-dependent and -independent cell growth (38, 39).

The mechanism of TLIMP-induced growth inhibition is currently unclear. Inhibition of the TRX-reducing activity or an effect on the nuclear compartment has been suggested as a mechanism of TBP-2-induced cell growth inhibition (20, 40). However, it is unlikely that TLIMP suppresses cell growth through inhibition of TRX-reducing activity because it failed to bind to TRX or inhibit its reducing activity (Fig. 3). There are many important cell growth-regulating molecules including receptor tyrosine kinases, nonreceptor tyrosine kinases, and (small) G-proteins in the plasma membrane, whose endocytosis leads to a quenching of the signal transduction elicited by the molecules (41). VD3 modulates signal transduction proximal to the plasma membrane for phospholipase C, protein kinase C, and calcium channels (1). In addition, it is reported that 15-deoxy-Δ-12, 14-prostaglandin J2, a natural ligand for PPARγ, prevents cell proliferation through inhibition of ErbBs, a receptor tyrosine kinase family (42). It is possible that TLIMP suppresses cell growth through the modulation of signal transduction in the plasma membrane and/or endosomes. On the other hand, TLIMP suppresses ligand-induced PPARγ activation (Fig. 8), suggesting the negative feedback loop between TLIMP and PPARγ. In addition to ligand binding, PPARγ activity is modulated by MAPK through direct phosphorylation (43). TLIMP may modulate the activity of PPARγ through the regulation of up-stream signaling of these kinases in proximal plasma membrane. The mechanism of the effect of TLIMP in cell proliferation and PPARγ activation requires further study.

In conclusion, we have shown that TLIMP, a VD3 or PPARγ target protein, regulates cell proliferation and PPARγ activation. The cellular localization associated with endocytotic membrane trafficking suggests that TLIMP is a molecule connecting the PPARγ signal and endosomal functions, providing a novel mechanism of regulation by VD3 or PPARγ signals .

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