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Vitamin D_3 Up-Regulated Protein 1 Mediates Oxidative Stress Via Suppressing the Thioredoxin Function[1]

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As a result of identifying the regulatory proteins of thioredoxin (TRX), a murine homologue for human vitamin D_3 up-regulated protein 1 (VDUP1) was identified from a yeast two-hybrid screen. Cotransfection into 293 cells and precipitation assays confirmed that mouse VDUP1 (mVDUP1) bound to TRX, but it failed to bind to a Cys^{32} and Cys^{35} mutant TRX, suggesting the redox-active site is critical for binding. mVDUP1 was ubiquitously expressed in various tissues and located in the cytoplasm. Biochemical analysis showed that mVDUP1 inhibited the insulin-reducing activity of TRX. When cells were treated with various stress stimuli such as H_2O_2 and heat shock, mVDUP1 was significantly induced. TRX is known to interact with other proteins such as proliferation-associated gene and apoptosis signal-regulating kinase 1. Coexpression of mVDUP1 interfered with the interaction between TRX and proliferation-associated gene or TRX and ASK-1, suggesting its roles in cell proliferation and oxidative stress. To investigate the roles of mVDUP1 in oxidative stress, mVDUP1 was overexpressed in NIH 3T3 cells. When cells were exposed to stress, cell proliferation was declined with elevated apoptotic cell death compared with control cells. In addition, c-Jun N-terminal kinase activation and IL-6 expression were elevated. Taken together, these results demonstrate that mVDUP1 functions as an oxidative stress mediator by inhibiting TRX activity. *The Journal of Immunology,* **2000, 164: 6287–6295.**

Thioredoxin (TRX)[3] is a 12-kDa thiol reductase with many cellular functions (1, 2). It is found in many species, such as bacteria, plants, and mammals. The redox-active disulfide/dithiol active site of TRX is completely conserved across all the species, constituting the sequence -Trp-Cys-Gly-Pro-Cys-. The two cysteine residues provide the sulfhydryl groups involved in TRX-dependent reducing activity. The oxidized form is reduced to a dithiol by NADPH and the flavoprotein TRX reductase. Thus, the TRX system is composed of TRX, TRX reductase, and NADPH (3, 4).

Mammalian TRX has many biological functions. A classic function is to act as a hydrogen donor for ribonucleotide reductase, which is essential for DNA synthesis (5). Human TRX is identical to adult T cell leukemia-derived factor, which has been characterized as a growth factor secreted by human T lymphotropic virus I-transformed leukemic cell lines (6). TRX is secreted from cells using a leaderless pathway (7–9) and stimulates the proliferation of lymphoid cells, fibroblasts, and a variety of human solid tumor cell lines (10–14). Furthermore, the stable transfection of the human TRX gene increases cell proliferation (15). In addition, TRX is an essential component of the early pregnancy factor (16) and inhibits HIV expression in macrophages (17). TRX has been found to modulate the DNA-binding activity of transcription factors, including TFIIIC, BZLF1, NF-κB, and glucocorticoid receptor, and to indirectly modulate AP-1 activity through Ref-1 (18–23). In addition, TRX is also known to act as a powerful antioxidant by reducing reactive oxygen species (ROS), and protects against H_2O_2-, TNF-α-induced cytotoxicity, in which generation of ROS is thought to participate (24, 25). Recently, TRX was found to be a physiological inhibitor for apoptosis signal-regulating kinase 1 (ASK-1), which is a pivotal component in cytokine- and stress-induced apoptosis (26). While the present study was in progress, Nishiyama et al. (27) reported that human TRX-binding protein-2/vitamin D_3 up-regulated protein 1 (VDUP1), originally reported as an up-regulated gene in HL-60 cells treated with 1α,25-dihydroxyvitamin D_3 (28), is a negative regulator of TRX function. They also demonstrated that TRX-binding protein-2/VDUP1 inhibits the reducing activity of TRX via interacting catalytic active center of TRX.

In the present study, we report the isolation and characterization of the mouse homologue for human VDUP1 (hVDUP1) (28). To date, little has been known about the cellular function of VDUP1. Mouse VDUP1 (mVDUP1) interacted with TRX to modulate its biological functions. The possible roles of mVDUP1 in oxidative stress were investigated.

Materials and Methods

Yeast two-hybrid screen

A yeast two-hybrid screen was performed according to the methods of Gyuris et al. (29). To construct the bait plasmid, a DNA fragment coding human TRX was amplified by RT-PCR from human keratinocyte cDNA library (30) and subcloned between the *Eco*RI and *Bam*HI sites of pEG202 in the correct frame. After confirming that the bait plasmid itself could not activate transcription from the GAL1-GAL10 promoter in the reporter

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[3] Abbreviations used in this paper: TRX, thioredoxin; ASK-1, apoptosis signal-regulating kinase 1; VDUP1, vitamin D_3 up-regulated protein 1; hVDUP1, human VDUP1; JNK, c-Jun N-terminal kinase; mVDUP1, mouse VDUP1; PAG, proliferation-associated gene; PI, propidium iodide; ROS, reactive oxygen species.

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plasmid pSH18–34, we screened a mouse A20 B cell cDNA library constructed in pYESTrp (Invitrogen, Groningen, The Netherlands). Approximately three million colonies were screened. The isolation of positive clones and subsequent analyses were conducted, as described elsewhere (29).

cDNA cloning

The retrieved sequence for partial mVDUP1 in the yeast two-hybrid screen was subcloned into mammalian expression vector pFLAG-CMV, and named Flag-mVDUP1$_{(134–395)}$. For the construction of full-length mVDUP1 cDNA, the 5′ missing part of the sequence was deduced from EST database search, and amplified by RT-PCR from A20 B cell cDNA library using the following oligonucleotide primers: 5′-GCCAAGCTTAT GGTGATGTTCAAGAAGAT-3′ and 5′-TTCACTAGATCTCACTTCG-3′. The amplified product digested with *Hin*dIII and NspV was inserted into Flag-mVDUP1$_{(134–395)}$ in the correct orientation and frame, which was confirmed by DNA sequencing, and named Flag-mVDUP1. Also, the full-length mVDUP1 was subcloned into pLXSN (Clontech, Palo Alto, CA). To obtain cDNA for human TRX, its open reading frame was amplified by RT-PCR from human keratinocyte cDNA library using the following primers: 5′-GCCGAATTCAAGATGGTGAAGCAGATC-3′ and 5′-GGCG GATCCTTAGACTAATTCATTAATGGTG-3′. The amplified product digested with *Eco*RI and *Bam*HI was subcloned into pBluescript KS (+). Again, the cDNA for TRX was subcloned into pEBG vector (31), which codes for GST-fused TRX or pFLAG-CMV (Eastman Kodak, Rochester, NY), respectively, thus named GST-TRX and Flag-TRX. TRX mutant lacking reducing activity was made by substituting two redox-active cysteine for serine (23) using the following primers: 5′-CCACGTGGTCT GGGCCTTCCAAAATGAT-3′ and 5′-ATCATTTTGGAAGGCCCAGAC CACGTGG-3′, and named GST-TRX (CS). The mutagenesis was done using an ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol.

Cell culture and transfection

NIH 3T3 and mouse lung fibroblast cells were maintained in DMEM supplemented with 10% FBS, L-glutamine, penicillin, and streptomycin. Then the cells were placed into the quiescent state by reducing the serum concentration to 0.5% for 1 day before stimulation with TGF-β1 or H_2O_2. The T cell hybridoma cells, KMls-8 (32), and 293 cells were maintained in RPMI supplemented with 10% FBS. For transient transfection assays, cells grown in 60-mm dishes were transfected with appropriate expression plasmids using the calcium phosphate method. For stable transfection (NIH 3T3), cells grown in 35-mm dishes were transfected with pLXSN-mVDUP1 as well as pLXSN using lipofectamine (Life Technologies, Grand Island, NY). Forty-eight hours after transfection, we began to select stable transfectants (0.5 mg/ml of G418).

Northern blot and RT-PCR analysis

KMls-8 cells were exposed to various treatments, as follows: PMA (50 nM), ionomycin (1 μg/ml), H_2O_2 (200 μM), γ-ray (20 Gy), and UV (100 J/m^2) for the indicated times. NIH 3T3 cells were exposed to heat shock at 42°C for the indicated times. Mouse lung fibroblast cells were incubated with 1 ng/ml TGF-β1 for the indicated times. To prepare the tissues blot, male BALB/c mice were sacrificed. Total RNAs were isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Thirty micrograms/lane of total RNA was fractionated by denaturing agarose gel electrophoresis and transferred to nylon membranes (GeneScreen^{Plus}; NEN Life Science Products, Boston, MA). The blots were hybridized overnight at 65°C in the ExpressHyb solution (Clontech), and properly washed. For RT-PCR, aliquots (3 μg) of total RNA were transcribed into cDNA at 37°C for 1 h in a total volume of 25 μl with 2.5 U of Moloney murine leukemia virus reverse transcriptase. PCRs were then performed with 1/20 vol of the reverse-transcription reaction for amplification. Amplifications were performed in a total volume of 30 μl containing 0.5 U of *Taq* DNA polymerase and 10 pmol of primers specific for mVDUP1 (5′-GCCGAATTCAAGATGG TGAAGCAGATC-3′ and 5′-GGCGGATCCTTAGACTAATTCATTAAT GGTG-3′), TRX (5′-GTGGTGGACTTCTCTGCTAC-3′ and 5′-GCTGG TAGCTGGTTACACTT-3′), IL-6 (5′- CTCAGCCCTGAGAAAGGAGA-3′ and 5′-AAAGCTGCGCAGAATGAGAT-3′), TGF-β1 (5′-CGGGAGGCC AGCCGCGGGAC-3′ and 5′-GGGTTGTGTTGGTTGTAGAGGGC-3′), ASK-1 (5′-CGTGGACTTCTGGATGGATT-3′ and 5′-GACCTGGTTGCT CAGGTCTC-3′), or β-actin (5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′). Amplifications were performed with 25 cycles for β-actin and 30 cycles for the others. The amplification profile included denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. After PCR, reaction mixtures were analyzed by agarose gel electrophoresis.

Immunocytochemical analysis

Forty-eight hours after transfection, HeLa cells were detached from dishes, and transferred onto sterile glass slides. Cells grown on glass slides were washed with PBS, dried, and fixed for immunostaining using a freshly prepared solution of cold methanol:acetone (1:1) for 20 min. After fixation, cells were dried and incubated in blocking buffer (1% BSA in PBS) for 20 min. Cells were incubated with either anti-Flag M2 mAb (Eastman Kodak) or normal mouse IgG at 1/100 dilution in blocking buffer for 1 h, and washed five times with PBS. Cells were incubated with goat FITC-conjugated anti-mouse IgG (Becton Dickinson, San Jose, CA) at 1/200 dilution in blocking buffer for 30 min, and were extensively washed again with PBS. Cells were mounted with glycerol for examination on a confocal laser-scanning microscope. The confocal microscope system was composed of a Leica TCS 4D connected to a Leica DAS upright microscope (Leica Lasertech, Heidelberg, Germany).

Precipitation of GST-fusion proteins and Western blot analysis

The 293 cells were transfected with various combinations of expression vectors, as indicated in the text. Twenty-four hours after transfection, cells were harvested in PBS, pelleted, and lysed in lysis buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40, plus protease inhibitors) for 1 h. After lysis, aliquots of cell lysates were incubated with glutathione-Sepharose (Pharmacia, Piscataway, NJ) for 2 h at 4°C. These beads were then washed five times with lysis buffer. The proteins were recovered by boiling in SDS-PAGE sample buffer. The eluted proteins were separated on SDS-PAGE, and either Coomassie stained or transferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The blot was subjected to Western analysis with anti-Flag Ab M2, or anti-hemagglutinin Ab 12CA5 (Boehringer Mannheim, Mannheim, Germany) using an enhanced chemoluminoscence (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ).

In vitro kinase activity assay

Aliquots (500 μg) of total cell lysates were immunoprecipitated with Ab to JNK or ASK (PharMingen, San Diego, CA), and incubated with GST-c-Jun (1–79) or myelin basic protein as a direct substrate in the presence of [γ-^{32}P]ATP. After the reaction, the reaction mixtures were separated in a 12% polyacrylamide gel electrophoresis and autoradiographed.

TRX-reducing assay

The insulin disulfide reduction assay was essentially performed as described elsewhere (27), with a slight modification. Transiently transfected cells were lysed in lysis buffer (20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40, plus protease inhibitors). Cell extracts (20 μg) were preincubated at 37°C for 20 min with 2 μl of DTT activation buffer composed of 50 mM HEPES (pH 7.6), 1 mM EDTA, 1 mg/ml BSA, and 2 mM DTT in a total volume of 70 μl to reduce TRX. Then 40 μl of reaction mixture containing 200 μl of 1 M HEPES (pH 7.6), 40 μl of 0.2 M EDTA, 40 μl of NADPH (40 mg/ml), and 500 μl of insulin (10 mg/ml) was added. The reaction began with the addition of 10 μl of rat TRX reductase (100 A412 U/ml), and incubation continued for 20 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 6 M guanidine-HCl and 1 mM DTNB (3-carboxy-4-nitrophenyl disulfide), and the absorbance at 412 nm was measured.

Flow-cytometric analysis of apoptosis

NIH 3T3 cells stably transfected either with pLXSN-mVDUP1 or pLXSN were plated in 24-well plates (2×10^5/well), allowed to adhere overnight, and then given the indicated concentrations of H_2O_2. At the end of indicated times of incubation, attached cells were harvested by trypsinization. The cells then received propidium iodide (PI, 5 μg/ml), followed by flow cytometry analysis to simultaneously monitor PI uptake (FL-2 channel) and cell size (forward light scatter). The cells that displayed both a reduction in cell size and a high permeability to PI were understood to be dead, as defined previously (33).

DNA fragmentation assay

After treatment with 5 μg/ml of anisomycin for the indicated time, KMls-8 cells (5×10^6 cells) were lysed in 0.5 ml of 10 mM Tris-Cl (pH 8), 0.1 M EDTA, 20 μg/ml pancreatic RNase, and 0.5% SDS for 30 min at 37°C. Proteinase K (final concentration is 100 μg/ml) was then added to the cell lysate, and the lysate was further incubated at 50°C for 3 h. After cooling the cleared lysate to room temperature, the lysate was extracted once with an equal volume of phenol/chloroform (1:1), and the aqueous phase was precipitated with an equal volume of isopropanol in the presence of 0.5 M

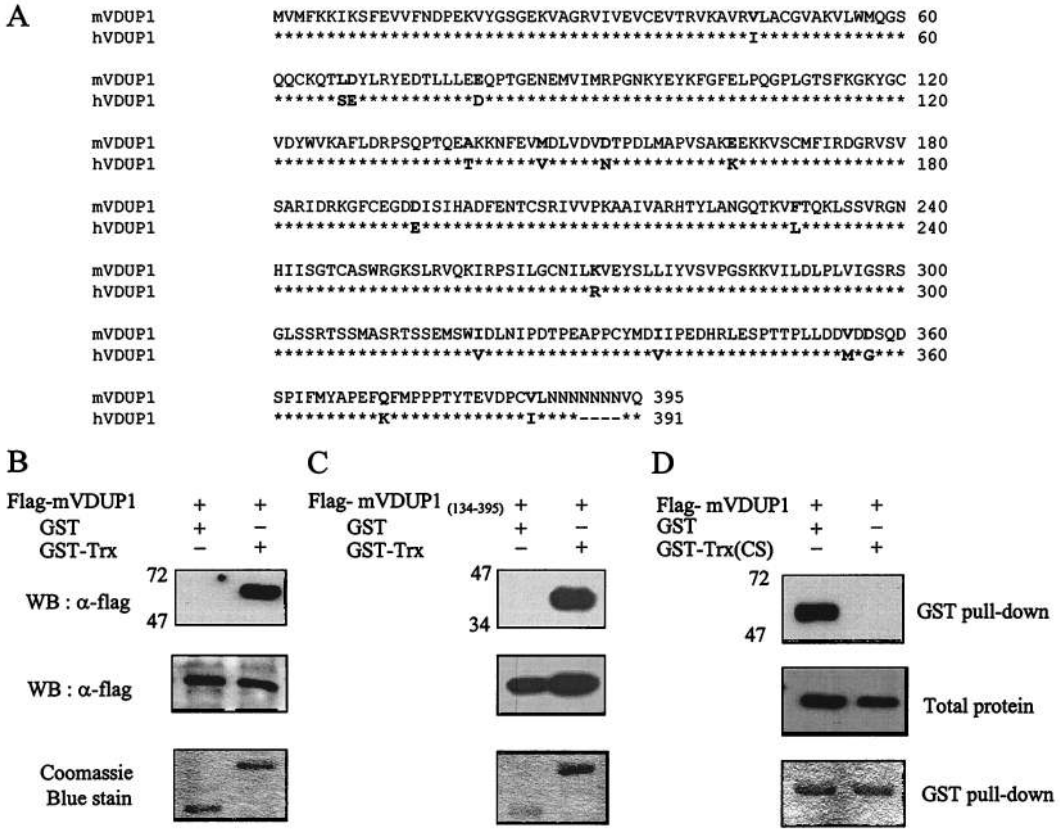

FIGURE 1. Identification of mVDUP1, which interacts with TRX. *A*, Predicted amino acid sequence of mVDUP1. The full-length amino acid sequence of mVDUP1 is compared with hVDUP1 (28). Conserved amino acids are marked with an ∗, and different amino acids are in bold. Dashed bars (−) in hVDUP1 indicate that there are no corresponding amino acids. *B*, An expression vector encoding the Flag-tagged full-length form of mVDUP1 was cotransfected into 293 cells with an expression vector encoding either GST or GST-TRX fusion protein. After 24 h, cell lysates were prepared and subjected to isolation with glutathione-Sepharose beads. Proteins coprecipitated with GST or GST fusion proteins as well as total protein were analyzed by Western blot analysis with anti-Flag Ab. Also, coprecipitated complexes were subjected to Coomassie staining to check the proper expression of GST or GST fusion protein. *C*, A partial form of Flag-mVDUP1$_{(134-395)}$ was used instead of the full-length form of mVDUP1. *D*, An expression vector encoding Flag-tagged mVDUP1 was cotransfected with vector encoding GST-TRX or GST-TRX (CS) mutant.

NaCl. The precipitate was spun down by centrifugation and the pellet was washed with 70% ethanol, dried, and solubilized in 100 μl of 10 mM Tris (pH 7.5), 1 mM EDTA, and 50 μg/ml RNase A. The genomic DNA solution was incubated at 37°C for 1 h, and 5-μl aliquot was separated on a 1% agarose gel, stained with ethidium bromide, and photographed.

Results

Identification of mVDUP1 as a TRX binding and inhibiting factor

Previous studies have suggested that TRX may function as a modulator of kinase activity such as ASK-1 in the redox-signaling process (26). To get an insight into the roles of TRX in signal transduction, we searched for additional TRX-interacting proteins using a yeast two-hybrid screen. By screening cDNA libraries derived from mouse A20 B cells, multiple cDNA clones were isolated (data not shown). Although they were classified into two groups on the basis of *Alu*I and *Hae*III digestion patterns, all of the cloned genes were identified to be a single gene only differing in size, as demonstrated by the DNA sequence analysis.

Comparative analysis of the DNA sequence of the cloned gene based on the GenBank query revealed that it had the partial form of the mouse homologue for a previously known gene as VDUP1 (28), whose function is not known. To construct the complete mVDUP1 cDNA, the 5′ missing sequence was deduced from EST database, amplified by PCR, and then connected to partial cDNA of mVDUP1. mVDUP1 mRNA is predicted to encode a protein of 395 aa that is overall 94% identical to its human counterpart (Fig. 1*A*, GenBank accession number AF173681). Database searches indicated that mVDUP1 does not contain any significant functional motifs and patterns.

Because mVDUP1 interacted strongly with TRX in the two-hybrid system, we tested whether this protein interacted with TRX in mammalian cells. Expression vectors encoding the Flag-tagged full length of mVDUP1 were cotransfected into 293 cells in the presence of an expression vector encoding either GST alone or GST-TRX fusion protein. GST-TRX in total cell lysates was precipitated with glutathione-Sepharose beads, and analyzed by Western blot analysis using anti-Flag Ab. Consistent with the data in the yeast two-hybrid assay, GST-TRX was coprecipitated with mVDUP1 (Fig. 1*B*). To determine whether the partial form of mVDUP1$_{(134-395)}$ lacking the N-terminal portion interacts with TRX in mammalian cells, GST precipitation assay was performed. GST-TRX successfully coprecipitated the partial form of mVDUP1, demonstrating that the portion of mVDUP1 covering aa 134–395 involves in its binding to TRX (Fig. 1*C*). We also examined the interaction between mVDUP1 and GST-TRX (CS), a Cys^{32} and Cys^{35} mutant that has lost reducing activity due to the mutation of cysteine to serine in the redox-active

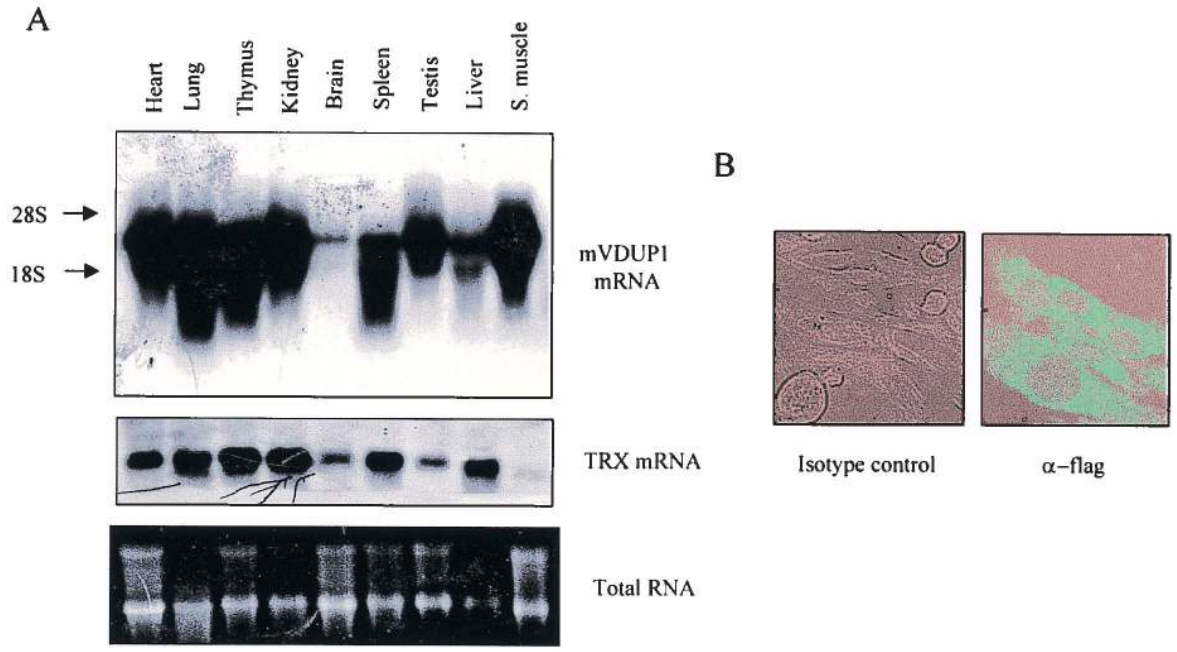

FIGURE 2. Tissue distribution and subcellular localization of mVDUP1. *A*, Total RNAs isolated from various tissues were hybridized with a mVDUP1-specific or TRX-specific probe. Positions of 18S and 28S rRNA are indicated. Total RNA loaded in each lane was stained with EtBr. S. muscle, skeletal muscle. *B*, HeLa cells were transfected with an expression vector encoding Flag-mVDUP1, and then indirect immunofluorescence staining using either normal mouse IgG (Isotype control) or anti-Flag mAb was conducted, as described in *Materials and Methods*.

center. As shown in Fig. 1*D*, mVDUP1 did not interact with GST-TRX (CS) mutant, suggesting that their interaction is made via the active site of TRX.

A recent report by Nishiyama et al. (27) demonstrated that overexpression of hVDUP1 by transfecting cells with VDUP1 cDNA or by treating cells with 1α,25-dihydroxyvitamin D_3 inhibits the reducing activity of TRX. Furthermore, they showed that recombinant hVDUP1 directly inhibits the reducing activity of TRX. Consistent with these observations, transfection of mVDUP1 cDNA into 293 cells reduced the endogenous reducing activity of TRX or the activity of cotransfected TRX cDNA (data not shown). In either case, the activity of TRX was decreased to nearly 50% when mVDUP1 was overexpressed, and its activity was more decreased when TRX reductase was omitted from the assay system. These data all indicate that mVDUP1 acts as an inhibitor of TRX-reducing activity via directly interacting catalytic active center of TRX.

Tissue distribution and subcellular localization of mVDUP1

To determine the tissue distribution of mVDUP1, we performed a Northern blot analysis using male BALB/c mouse tissues.

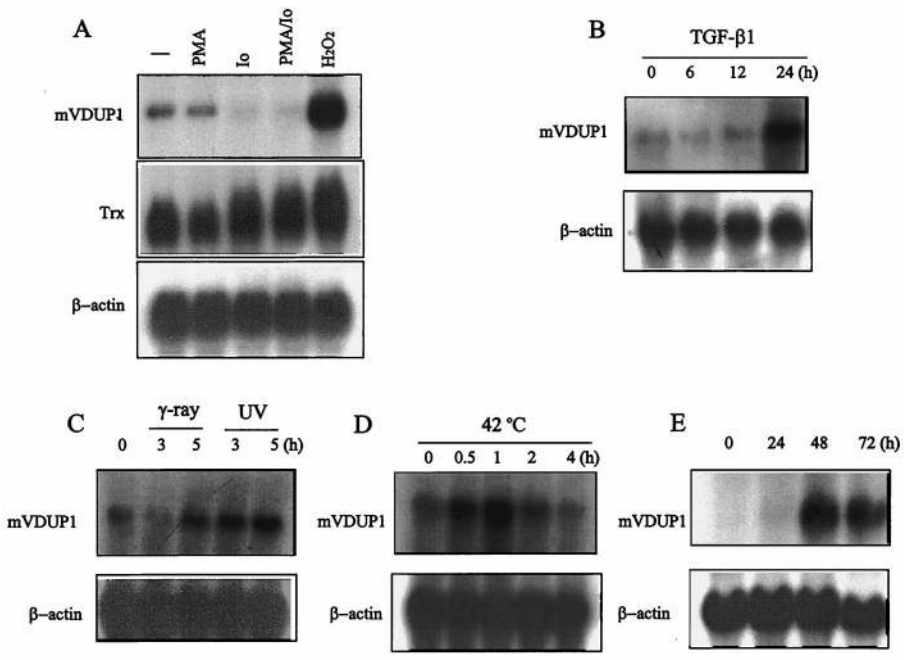

FIGURE 3. Expression regulation of mVDUP1 gene. *A*, The murine T cell hybridomas KMls-8 were treated with indicated stimuli for 12 h. Northern blot analysis was done as described in *Materials and Methods*. Io: ionomycin. *B*, The primary mouse lung fibroblast cells were incubated with TGF-β1 (1 ng/ml) for indicated times (60–70% confluent at 24 h). *C*, The murine T cell hybridomas KMls-8 were exposed to γ-ray (20 Gy) and UV (100 J/m^2) and after the indicated times, cells were harvested. *D*, NIH 3T3 cells were exposed to heat shock at 42°C for indicated times. *E*, NIH 3T3 cells (<30% confluent at 0 h) were spontaneously incubated in DMEM plus 10% serum for indicated times (>90% confluent at 48 h).

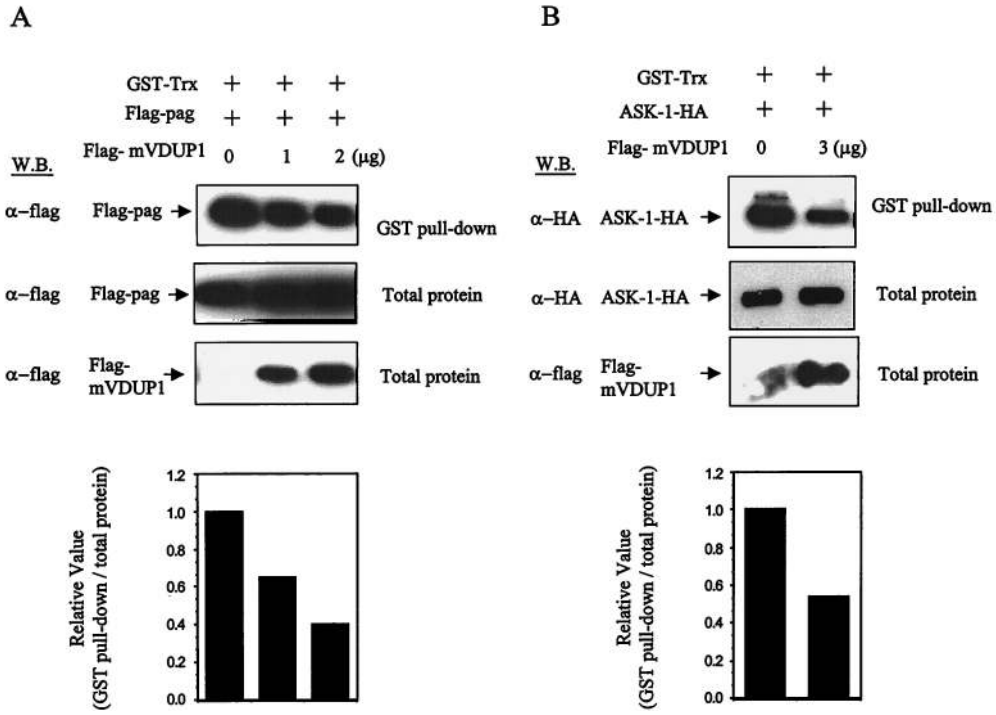

FIGURE 4. mVDUP1 competes with PAG or ASK-1 for binding to TRX. *A*, mVDUP1 overexpression interferes with TRX-PAG interaction. The 293 cells were cotransfected with indicated vectors. The amounts of vectors used were as follows: GST-TRX (1 μg), Flag-PAG (1 μg), and Flag-mVDUP1 (indicated amounts). In each experiment, total DNA amounts were set to equal with pcDNA3.1. After 24 h, cell lysates were prepared and subjected to isolation with glutathione-Sepharose beads. Proteins coprecipitated with GST-TRX as well as total protein were analyzed by Western blot analysis with anti-Flag Ab (1:1000). *B*, mVDUP1 overexpression interferes with TRX-ASK-1 interaction. The 293 cells were cotransfected with GST-TRX (1 μg), ASK-1 (1 μg), and Flag-mVDUP1 (indicated amounts). Cell lysates were precipitated with glutathione-Sepharose beads, and analyzed by Western analysis using anti-Flag Ab or anti-hemagglutinin Ab (1:1000). Relative interaction was quantitated by densitometry (*lower panels*).

mVDUP1 transcript was detected in various tissues, including heart, lung, thymus, spleen, kidney, testis, and skeletal muscle (Fig. 2*A*). mVDUP1 was expressed in relatively low amounts in the brain and liver, while TRX was significantly expressed in the liver. mVDUP1 is predicted to localize in the cytoplasm by computational analysis (PSORT II, National Institute of Basic Biology, Genomenet). To confirm the localization, we transiently transfected the vector, pFlag-mVDUP1 into the HeLa cell and probed it with Flag Ab. mVDUP1 is mainly found in the cytoplasm (Fig. 2*B*, *right panel*), which is consistent with the location of TRX (21).

Expression regulation of mVDUP1 gene

Because the TRX is one of the key components in redox regulation and its gene expression is regulated by a variety of stress stimuli, we tested the effects of oxidative stress, including H_2O_2 on mVDUP1 gene expression. The mVDUP1 mRNA expression was

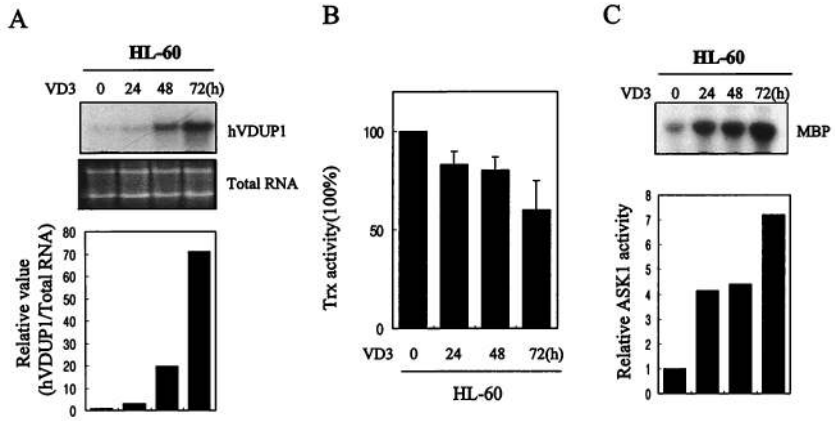

FIGURE 5. Effects of 1α,25-dihydroxyvitamin D_3 on the activities of TRX and ASK-1 in HL-60 cells. HL-60 cells were treated with 100 nM 1α,25-dihydroxyvitamin D_3 (VD3) for the indicated time. *A*, Northern blot analysis of hVDUP1. Relative value (1 = hVDUP1/total RNA at 0 h) was analyzed by densitometry (*lower panel*). *B*, TRX-reducing activity. The insulin disulfide reduction assay was essentially performed as described in *Materials and Methods*. Activities are shown relative to the control (0 h), which is assigned as 100%. The results are the means + SD of four samples. *C*, ASK-1 activity. After immunoprecipitation with anti-ASK-1 Ab, the immunocomplexes were assayed for ASK-1 activity using myelin basic protein as a substrate, as described in *Materials and Methods*.

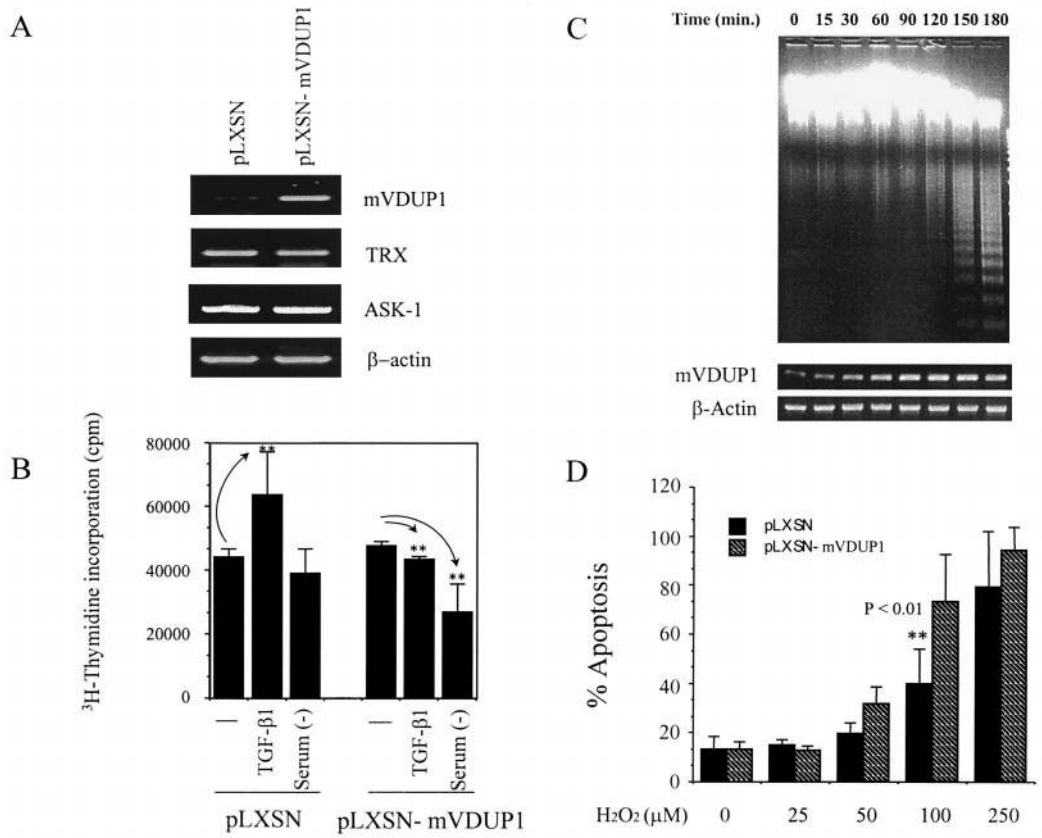

FIGURE 6. Effects of mVDUP1 on cell proliferation and apoptosis. *A*, RT-PCR analysis showing mVDUP1 overexpression. The vector control (pLXSN) and mVDUP1 transfectant (pLXSN-mVDUP1) were subjected to RT-PCR analysis to check the expression level of mVDUP1, TRX, ASK-1, and β-actin. *B*, Effects of mVDUP1 on cell proliferation. The thymidine incorporation assays were conducted as described in *Materials and Methods*. The cells were incubated with TGF-β1 (1 ng/ml) and deprived of serum (serum (−)). Data represent ±SD of three separate experiments (**, $p < 0.01$). *C*, Correlation of mVDUP1 expression and DNA fragmentation induced by anisomysin treatment. KMls-8 cells (5×10^6 cells) treated with 5 μg/ml of anisomycin for the indicated time. The genomic DNA was prepared as described in *Materials and Methods*, and separated on a 1% agarose gel, stained with ethidium bromide, and photographed. mVDUP1 and β-actin expression were monitored by RT-PCR. *D*, Effects of mVDUP1 on apoptosis. The NIH 3T3 transfectants were treated with indicated concentration of H_2O_2 for 4 h, and apoptosis was determined by PI uptake. Values are the means of six separate experiments with an error bar representing SD (**, $p < 0.01$).

strongly induced by 200 μM H_2O_2 treatment in murine T cell hybridoma, KMls-8, at 12 h, but calcium ionophore down-regulated the basal expression of mVDUP1, while TRX gene expression was up-regulated by both treatments (Fig. 3*A*). There was a marked increase of mVDUP1 mRNA at 24 h after TGF-β1 treatment (Fig. 3*B*), and exposing cells to γ-rays or UV increased mVDUP1 gene expression moderately until 5 h after exposure (Fig. 3*C*). Meanwhile, heat shock elevated mVDUP1 gene expression significantly at 1 h (Fig. 3*D*). Interestingly, when NIH 3T3 cells were overgrown (>90% confluent), mVDUP1 expression was rapidly increased (Fig. 3*E*). Based on these data of gene express regulation, mVDUP1 gene expression seems to be readily regulated by a variety of stress, suggesting that mVDUP1 may be actively involved in the stress responses.

mVDUP1 overexpression inhibits the interaction between TRX and PAG or TRX and ASK-1

Binding mVDUP1 with TRX requires a TRX active site, indicating that mVDUP1 might compete with other TRX-binding proteins for TRX binding. The PAG has been initially identified by differential cloning between untransformed and *ras*-transformed cells (34). Its expression is higher in cells having a higher level of proliferation. PAG is also known to be a thiol-specific antioxidant that reduces hydrogen peroxide in the presence of TRX as an immediate electron donor (35), thus renamed as peroxiredoxin I. As shown in Fig. 4*A*, the interaction between TRX and PAG was reduced up to 40% when mVDUP1 was overexpressed. Another well-known TRX-interacting protein is ASK-1, which has been identified as a mitogen-activated protein kinase kinase kinase and is required for oxidative stress-induced apoptosis (36). TRX is a physiological inhibitor of ASK-1, thus seeming to be essential for survival (26). The binding between TRX and ASK-1 was significantly reduced when mVDUP1 was overexpressed, suggesting that mVDUP1 can compete with ASK-1 for binding to TRX (Fig. 4*B*). To confirm the effects of endogenous VDUP on the binding of TRX and ASK-1, HL-60 cells were treated with 1α,25-dihydroxyvitamin D_3 to induce the VDUP1 gene expression. As reported previously (27, 28), 1α,25-dihydroxyvitamin D_3 treatment increased the hVDUP1 expression (Fig. 5*A*) and decreased the reducing activity of TRX (Fig. 5*B*). In this situation, ASK-1 activity was increased (Fig. 5*C*), further indicating that excess binding of VDUP1 to TRX induces the release of ASK-1 from its inhibitor, TRX, to become activated.

Effects of mVDUP1 on the H_2O_2-mediated stress responses

The physiological function of PAG was suggested to protect against apoptosis induced by serum deprivation, ceramide, and etoposide (37). This antiapoptotic function might be due to the peroxidase activity of PAG with the use of electrons from TRX.

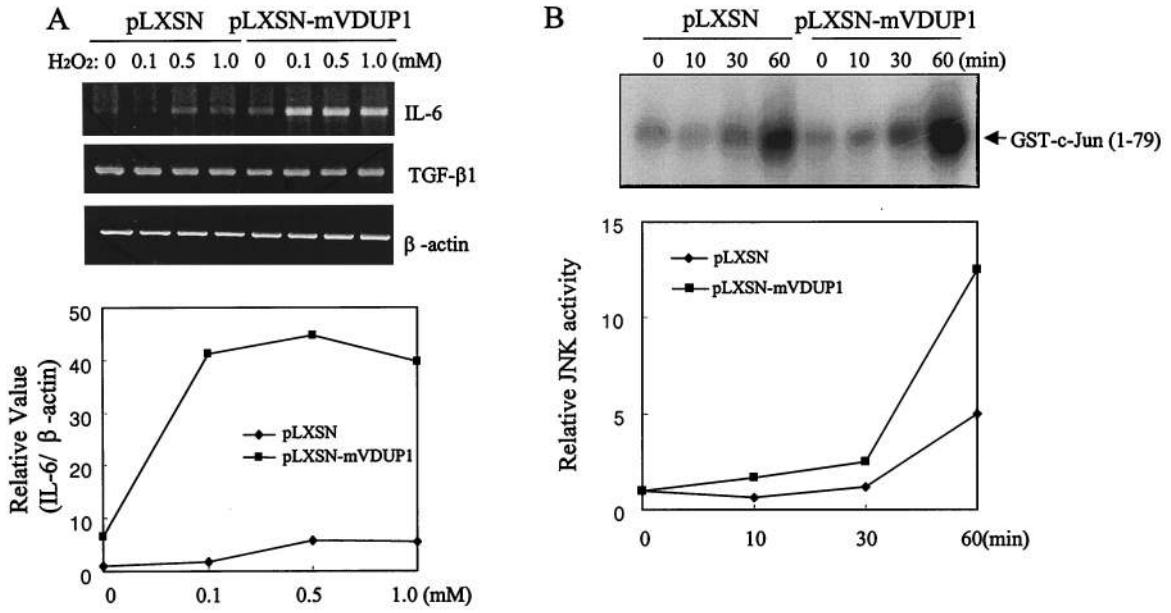

FIGURE 7. Effects of mVDUP1 on IL-6 expression and JNK activation. *A*, Effects of mVDUP1 on IL-6 expression. The transfectants were incubated with indicated concentration of H_2O_2 for 4 h. Total RNAs were isolated and subjected to RT-PCR analysis, as described in *Materials and Methods*. Relative value (1 = IL-6/β-actin at 0 mM) was analyzed by densitometry (*lower panel*). *B*, Effects of mVDUP1 on JNK activation. Serum-deprived transfectants were incubated with H_2O_2 (1 mM) for indicated periods. Aliquots (500 μg) of total lysates were immunoprecipitated with anti-JNK Ab, and kinase assays with GST-c-Jun (1–79) were performed (*upper panel*). The intensities of bands were calculated by phosphor imager (*lower panels*).

Thus, we speculate that cells would be more vulnerable to oxidative stress if mVDUP1 prevents the interaction between TRX and PAG. In addition, mVDUP1 also interferes with the interaction between TRX and ASK-1, probably rendering cells more sensitive to oxidative stress. To investigate this functional property of mVDUP1 in oxidative stress, we established a stable mVDUP1-transfected NIH 3T3 cell line. The overexpression of mVDUP1 was confirmed by RT-PCR analysis (Fig. 6*A*). There was no difference in cell morphology or spontaneous cell growth between the vector-transfected cell line and mVDUP1-transfected cell line in the presence of 10% serum. However, mVDUP1 transfectants grew more slowly than vector controls in the presence of TGF-β1 or in the absence of serum (Fig. 6*B*), implying that overexpression of mVDUP1 makes cells more susceptible to growth inhibition in response to stress. When murine KMls-8 T cells were treated with anisomycin, which is known to induce JNK activation and apoptosis in T cells (38), the correlation between DNA fragmentation and mVDUP1 expression was observed (Fig. 6*C*). In line with these observations, more apoptotic cell death was observed in mVDUP1 transfectants than in vector controls when cells were treated with H_2O_2 (Fig. 6*D*: at 50 μM, control 19.6 $\pm$ 4.6%, mVDUP1 31.9 $\pm$ 6.5%; at 100 μM, control 39.8 $\pm$ 14.4%, mVDUP1 73.3 $\pm$ 19.4%, respectively).

Several studies reported that ROS is involved in the expression of proinflammatory cytokines such as IL-6 (39). When cells were treated with different concentrations of H_2O_2, the induction of IL-6 expression was increased in mVDUP1 transfectants (Fig. 7*A*). It has been reported that JNK is activated by ASK-1 (36), and its activity is sensitive to H_2O_2 treatment (40). In this regard, JNK activity was assayed after H_2O_2 treatment. As shown in Fig. 7*B*, mVDUP1 transfectants showed elevated JNK activity compared with vector controls. Taken together, these results indicate that VDUP1 expression rendered cells more vulnerable to oxidative stress.

Discussion

There is growing evidence that thiol-disulfide redox regulation is one of the key events in biological responses, including signal transduction, apoptosis, and gene expression. Disulfide bond formation is important to regulate protein activity, gain of function, or loss of function, in response to environmental changes such as stress responses. The two most important systems of maintaining the reducing thiol-disulfide status of cytosol are the TRX-thioreductase system and glutathione-glutaredoxin system. TRX exerts its action by a disulfide exchange reaction utilizing an active site. Multisite location of TRX implies its multifunctional roles as a biological regulator. In cytosol, TRX has cytoprotective effects against oxidative stress (24, 25) and regulates signal transduction (2, 36). In the nucleus, it regulates the binding of transcription factors such as NF-κB and AP-1 (20, 23). Secreted TRX has cytokine-like functions and functions as a potent costimulatory molecule outside cells (11, 40, 41).

In an effort to find the regulatory factor of TRX, mVDUP1 was found to interact with TRX through its active site of TRX. This interacting with active site of TRX implies the biological significance of mVDUP1 in regulating TRX function. This study has demonstrated two functional aspects of mVDUP1 in this regard. First, mVDUP1 inhibits TRX functions by blocking the active site, as shown in the case of hVDUP1 (27). Insulin-reducing assay demonstrated that mVDUP1 inhibits TRX-reducing activity (data not shown), which interacts with the active site of TRX (Fig. 1). Second, mVDUP1 renders cells more sensitive to oxidative stress probably by competing with cellular factors, such as ASK-1 and PAG for binding to TRX. Saitoh et al. (26) reported that TRX is known as an inhibitor of ASK-1, a mammalian mitogen-activated protein kinase kinase kinase that delivers the apoptotic signal by activating JNK and p38 pathways. TRX (CS) mutant or oxidized TRX failed to bind to ASK-1. Furthermore, they demonstrated that

H_2O_2 treatment (0.5–5 mM) decreases the TRX-ASK-1 interaction, but increases ASK-1 activity and apoptosis, which is inhibited by *N*-acetyl-L-cysteine treatment (26). PAG, originally described as a proliferating associated gene, is reported as an antioxidant and antiapoptotic gene that requires TRX to carry out its peroxidase function. mVDUP1 also competes with PAG for the binding site of TRX, which can inhibit the antiapoptotic function of PAG. mVDUP1, as an inhibitor of TRX-ASK-1 or TRX-PAG interaction, inhibits cell growth due to inducing apoptosis in the presence of oxidative stress. However, mVDUP1 has no peroxidase activity, and structural and functional similarity with PAG or ASK-1 (data not shown). Biochemical analysis is required for the regulation of ASK-1 and PAG enzymatic activity by mVDUP1-TRX interaction. Moreover, overexpression of mVDUP1 made cells more sensitive to IL-6 expression and JNK activation in response to oxidative stress (Fig. 6), further confirming the prooxidant functions of mVDUP1.

mVDUP1 is a homologue for the hVDUP1 cDNA, which was originally reported as an up-regulated gene by 1α,25-dihydroxyvitamin D_3 (27). 1α,25-dihydroxyvitamin D_3 induces myeloid cell differentiation (42), and inhibits fibroblast proliferation (43). In addition, mVDUP1 expression was decreased in rat mammary tumors, and up-regulation of mVDUP1 by 1α,25-dihydroxyvitamin D_3 treatment inhibited tumor cell growth, having suggested the critical roles of mVDUP1 in mediating the inhibitory effects of 1α,25-dihydroxyvitamin D_3 on tumor cell growth (44). In addition, a dominant-negative mutant TRX reverses the transformed phenotype of human breast cancer cells (15). Taken together, mVDUP1, as an inhibitor of TRX, may have an antitumor effect in certain types of tumors.

Another important aspect of mVDUP1 in response to stress is that mVDUP1 mRNA is readily inducible by various stress stimuli, including H_2O_2, TGF-β1, and heat shock. Comparing the regulation of TRX gene expression, mVDUP1 is more dramatically induced by 200 μM H_2O_2 treatment (Fig. 3), which induced cell death at this concentration (Fig. 5). Based on our and Saitoh's (26) observations, it seems that H_2O_2 induces cell death or stress response at least by inducing the dissociation of ASK-1 from TRX and by increasing the gene expression of mVDUP1 more than that of TRX.

Collectively, our observations and previous reports suggest that mVDUP1 induced by stress mediates stress responses by suppressing TRX functions. It has been known that TGF-β1 can stimulate as well as inhibit cell proliferation, depending on cell types and culture condition (45). The effects of TGF-β1 on the proliferation of murine lung fibroblast cells were also dependent on serum concentration. At serum-reduced condition (<2% serum), TGF-β1 increased cell proliferation. However, at higher serum concentration (>5% serum), it did not increase it (data not shown). The effects of TGF-β1 on mVDUP1 expression were dependent on cell types. TGF-β1 up-regulated VDUP-1 expression in murine lung fibroblasts (Fig. 3*B*) and in TGF-β1-induced differentiated erythroid cells, but had no effects in KMls-8 cells (data not shown). In addition, up-regulation of mVDUP1 gene expression occurred at 24 h after TGF-β1 treatment in murine lung fibroblasts, while other stimuli such as H_2O_2, UV, and heat shock induced mVDUP1 gene expression within 12 h after treatment, implying that TGF-β1-induced mVDUP1 gene expression may display the indirect regulatory responses, i.e., feedback regulation, of TGF-β1 on cell function. Recently, it was reported that TGF-β1-induced SnoN oncoprotein participates in negative feedback regulation of TGF-β1 signaling (46). In addition, 1α,25-dihydroxyvitamin D_3, an inducer of VDUP1, can modulate TGF-β1 signaling both positively and negatively, depending on molecular interplay of smad proteins (47). It has been reported that vitamin D_3 modulates intracellular calcium flux and protein kinase C activity (48, 49). In thyroid cells, vitamin D_3 decreases calcium uptake (50), but it increases intracellular calcium level in promyelocytic HL-60 cells to differentiate into monocytic cells (48). Calcium ionophore suppressed the gene expression of mVDUP1 in KMls-8 cells, indicating intracellular calcium level is critical factor for mVDUP1 expression, as shown in calcium regulation by vitamin D_3. More detailed studies on the roles of mVDUP-1 in TGF-β1 and vitamin D_3 signaling will be required.

In summary, we identified mVDUP1 as an interacting factor with TRX. It locates in the cytosol and is strongly induced by stress responses. mVDUP1 inhibits TRX functions, including insulin-reducing activity and TRX interaction with other factors such as PAG and ASK-1. These characters of mVDUP1 render cells more vulnerable to oxidative stress. Its anti-TRX function and expression pattern imply that mVDUP1 is a key modulator for stress responses to modify the redox status in the cells.

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