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Involvements of mitochondrial thioredoxin reductase (TrxR2) in cell proliferation

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Abstract

Mammalian cells contain two forms of thioredoxin reductase (TrxR), cytosolic TrxR1 and mitochondrial TrxR2. To investigate the biological roles of TrxR2, we generated stable HeLa cell lines expressing a dominant negative form of TrxR2 (TrxR2DN) under the control of the tetracycline-off system. We observed that TrxR2DN-induced cells, following stimulation with EGF, produced more hydrogen peroxide than uninduced cells. The extent of protein tyrosine phosphorylation of many proteins including ERK was higher in TrxR2DN-induced cells than in uninduced cells when stimulated with fetal bovine serum or EGF. Induction of TrxR2DN also resulted in the increased rate of progression of G_1 to S phase in cell cycle and cell proliferation and affected the expression of many proteins involved in cell cycle. These results suggest that TrxR2 participates in the regulation of protein tyrosine phosphorylation and cell growth as a component of the mitochondria specific H_2O_2-eliminating system that includes peroxiredoxin III and thioredoxin 2.

Keywords: Mitochondrial thioredoxin reductase; Hydrogen peroxide; Proliferation; Signal transduction

Thioredoxin reductase (TrxR), which catalyzes the reduction of the active site disulfide of thioredoxin (Trx), is an NADPH-dependent homodimeric oxidoreductase with one FAD molecule per subunit [1,2]. Mammalian TrxR is a selenoprotein that contains a penultimate selenocysteine (secys) residue [3] in the sequence –Gly–Cys–Secys–Gly–, which serves as a critical redox center [4,5]. The codon for Secys is UGA, which encodes a stop codon in general. To translate the UGA codon to a Secys residue, Secys insertion (SECIS) element should be present in the 3′-UTR [6].

The reduced Trx acts as a general disulfide reductant and is known to be involved in cell growth and apoptosis through overall regulation in cellular redox states as well as the redox regulation of enzymes and transcription factors, such as ribonucleotide reductase, peroxiredoxins, NF-κB, AP-1, etc. [7]. One of the important targets for reduction by Trx is peroxiredoxin (Prx) that reduces peroxides with the use of electron derived from Trx [8].

Mammalian TrxR exists at least in two distinct forms: cytosolic TrxR1 and mitochondrial TrxR2 [9,10]. Trx and Prx also exist in multiple isoforms. Among these isoforms, Trx2 and Prx III are expressed with a mitochondrial leading sequence (MLS) and transported specifically into the mitochondria [11,12]. Recently, Trx2 was shown to play essential roles in cell survival and mitochondria-mediated apoptosis [13,14].

In order to investigate the cellular function of TrxR2, we established TrxR2DN-inducible HeLa cell lines in which TrxR2 transcription was regulated by a Tet-off expression system. We observed that induction of TrxR2DN resulted in higher concentration of intracellular H_2O_2, elevated protein tyrosine phosphorylation, and increased cell growth.

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Materials and methods

Materials. Human placenta RNA, cDNA synthesis kit, HeLa Tet-off cell lines (HeLa-TO), pTRE, pHyg vectors, hygromycin, and tet-approved fetal bovine serum (FBS) were purchased from BD Biosciences (Palo Alto, CA). Epidermal growth factor (EGF), Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin–fungizone, and G418 were from Life technologies (Gaithersburg, MD). Primers for cloning of TrxR2 and TrxR2DN were from Biobasic (Canada). The rabbit polyclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was kindly gifted from Dr. K.S. Kwon (KRIBB, Daegeon, Korea). A rabbit polyclonal antibody against TrxR2 was kindly donated by S.G. Rhee (LCS, NHLBI, NIH, USA). Antibodies against cytochrome *c* oxidase subunit VII, cyclins, cyclin dependent kinases (CDKs), and p27 were from Santacruz (Santa Cruz, CA).

Cloning of human TrxR2. Human TrxR2 cDNA was amplified from human placental RNA by reverse transcription polymerase chain reaction (RT-PCR). All primers used are summarized in Table 1. RT reaction for synthesis of the first strand cDNA from human placental RNA was performed. PCR for the amplification of TrxR2 was performed in a 20 μl reaction volume containing 10 mM Tris–HCl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 2 U *Taq* polymerase, 5% DMSO, 1 μl first-strand cDNA, and 200 nM 170F (or 190F) and 2090R primers. The reactions were initial denaturation for 2 min at 94 °C, 5 cycles of 94 °C 5 s, 69 °C 10 s, 72 °C 2 min 30 s, 5 cycles of 94 °C 5 s, 67 °C 10 s, 72 °C 2 min 30 s, 25 cycles of 94 °C 5 s, 65 °C 10 s, 72 °C 2 min 30 s, and final extension at 72 °C for 10 min. The amplified PCR products were separated in a 1.5% agarose gel containing ethidium bromide. The amplified DNA was eluted from the gel using a Qiagen gel extraction kit. The TrxR2 DNA was ligated into a pCR3.1 vector and the resulting construct was verified by dideoxy sequencing.

Plasmid constructs. Dominant-negative mutant of TrxR2 (TrxR2DN) without a SECIS element in the 3′-UTR was prepared from the cloned TrxR2 cDNA as a template by PCR. Four primers (190F, 297-FgR, 298-FgF, and 1768R) were used to insert a FLAG epitope just behind the mitochondrial leading sequence (MLS) of TrxR2. PCR was performed using two separate sets of primers (one is 190F and 297-FgR; the other 298-FgF and 1768R) and TrxR2 cDNA as a template. The amplified DNAs were purified and mixed together, and then TrxR2DN was amplified using 190F and 1768R. The FLAG epitope-tagged TrxR2DN was cloned into a pCR3.1 vector (Invitrogen, Carlsbad, CA) and sequenced to verify its identity. In order to subclone TrxR2DN into a pTRE vector, TrxR2DN was isolated from pCR/TrxR2DN plasmids by *Eco*RI digestion and ligated into a *Eco*RI site of a pTRE vector using a Ready-to-go T4 DNA ligase mix (Amersham–Pharmacia Biotech., Piscataway, NJ). After transforming, pTRE/TrxR2DN plasmids were selected and amplified.

Transfection and selection of HeLa-TO/TrxR2DN. pTRE/TrxR2DN and pHyg plasmids were cotransfected into HeLa-TO cells using Lipofectamine reagent according to the protocol provided by the company. Cells were grown in media containing 100 μg/ml hygromycin and 100 μg/ml G418 for 2 weeks and drug-resistant colonies were picked to select clones that contained both plasmids. Approximately 100 hygromycin-resistant colonies were isolated and propagated as individual cell lines. Tetracycline-repressible TrxR2DN expression in hygromycin-resistant cells was screened by Western blot analysis with a rabbit polyclonal antibody against rat TrxR2.

Cell treatments. HeLa-TO/TrxR2DN cells were maintained in DMEM containing 10% FBS + 1 μg/ml tetracycline. For all subsequent experiments, cells were washed 3 times with DMEM and incubated in media containing 10% tet-approved FBS without tetracycline for 72 h to induce TrxR2DN expression. The cells were starved in DMEM for 48 h and then treated with 10% FBS and EGF (100 ng/ml) for the indicated times.

Measurement of TrxR activity. Specific activities of TrxR enzymes in TrxR2DN-induced cells were measured by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) reduction [3,4].

Detection of intracellular H_2O_2 by flow cytometric analysis. Intracellular levels of H_2O_2 were analyzed by flow cytometry using dihydrorhodamine (DHR) 123 (Molecular Probes, Eugene, OR) as a specific fluorescent dye probe [15,16]. TrxR2DN-induced or -uninduced and serum-deprived HeLa cells were incubated in DMEM containing 10 μM DHR123 for 30 min and washed two times with DMEM. Cells were treated with 100 ng/ml EGF for 30 min followed by 2 μg/ml catalase to remove the extracellular H_2O_2. After harvesting by trypsin–EDTA treatment, cells were fixed in 1% paraformaldehyde. The intracellular rhodamine 123 fluorescence intensity of 10,000 cells was measured for each sample using a Becton–Dickinson FACS Caliber flow cytometer.

Western blot analysis. Cells were harvested and lysed with a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1 mM PMSF) or an SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 2.5% β-mercaptoethanol, and 0.01% bromophenol blue). The proteins were separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blotted with primary antibodies against TrxR2, pERK (Cell Signaling Technology, Beverly, MA), phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY), GAPDH, CDKs, or cyclins. Secondary antibodies conjugated with horseradish peroxidase were detected using the Renaissance ECL kit (NEN Life Science Products, Boston, MA).

Immunofluorescence staining of TrxR2 in HeLa-TO/TrxR2DN cells. TrxR2DN-induced or -uninduced cells (10,000 cells/well) were grown on glass slides for 1 day and then washed 3 times with ice-cold phosphate buffered saline (PBS). Following fixation of the cells in cold acetone for 10 min, 5% goat serum in PBS was applied for 30 min at 37 °C. A rabbit polyclonal antibody against TrxR2 (1:100 dilution in 2% goat serum) or a mouse monoclonal antibody against cytochrome *c* oxidase subunit VII (1:50 dilution in 2% goat serum) was applied for 1.5 h. The cells were washed 5 times with ice-cold PBS and then blocked for 30 min. FITC-conjugated anti-rabbit or anti-mouse goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was applied for 45 min and washed 3 times with ice-cold PBS.

Fluorescence-activated cell sorting analysis for cell cycle progression. TrxR2DN-induced and -uninduced cells were starved for 2 days and incubated with 10% FBS for the indicated times. The cells were harvested, fixed, and stained with propidium iodide (PI), and the intracellular PI fluorescence intensity of 10,000 cells was measured for each sample using a flow cytometer [17].

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. TrxR2DN-induced or -uninduced cells were seeded in 96-well plates (500 or 1000 cells/well) and starved in DMEM for 24 h. The starvation media were replaced with DMEM containing 10% tet-approved FBS with or without tetracycline. At the indicated times, cell number was estimated by the MTT assay [18]. Cell growth was expressed as a percentage of TrxR2DN-uninduced control cells.

Table 1
Primers for amplification and cloning of wild type and dominant-negative TrxR2s

Primers	Sequences
170F	cctagctgcccagaagcccca
190F	atggcggcaatggcggtggcg
1768R	tgcagggatggcgcttaccctca
2090R	gcattgcagaaatgccaggggc
297-FgR*	cttgtcgtcatcgtctttgtagtcacctgctgctgcgccccgcgc
298-FgF*	gactacaaagacgatgacgacaagcagcgggactatgatctcctg
531R	agccatagttgggggcatcttgg

* The underlined portion of primers is the FLAG sequence.

Results

Generation of TrxR2DN and tetracycline-repressible TrxR2DN HeLa cells

The full-length cDNA for TrxR2 was amplified from human placental RNA by RT-PCR and used to generate a construct encoding TrxR2DN, which contains a FLAG-tag behind the MLS of TrxR2. Because the TrxR2DN construct does not have a Secys insertion (SECIS) element in 3′-UTR, the UGA codon for Secys in TrxR2 is translated to a stop codon in TrxR2DN, producing a catalytically inactive truncated mutant lacking the two C-terminal residues Secys and Gly of Trx2R (Fig. 1A). HeLa cells were transfected with pTRE/TrxR2DN (the plasmid expressing FLAG-tagged TrxR2DN under the control of the tetracycline-off system) and pHyg vector, and hygromycin-resistant cells were selected. Ten out of one hundred clones showed FLAG-TrxR2DN expression when incubated in media without tetracycline: FLAG-TrxR2DN was induced as a single protein band as detected by immunoblot analyses with anti-TrxR2 and anti-FLAG antibodies, and the amount of FLAG-TrxR2DN was increased gradually to 4 days (Fig. 1B). The fact that FLAG-TrxR2DN was expressed as a single protein band suggests that most of the induced proteins were transported to and cleaved in the mitochondria to generate the MLS-free enzyme. The upper 122 kDa protein band might be a dimeric form of TrxR2DN. Dominant negative effect of the mutant TrxR2 was confirmed by the measurement of TrxR activity in the whole cell lysates. Tetracycline-repressible TrxR2DN expression resulted in a decrease in the specific activity of TrxR enzyme (Fig. 1C). The TrxR activity was gradually decreased to 4 days of induction ($P < 0.01$) and was not decreased further to 9 days (data not shown). The mitochondrial localization of FLAG-TrxR2DN was also evident from immunofluorescence stainings, which exhibit a granular pattern similar to that of cytochrome *c* oxidase subunit VII, a well-known

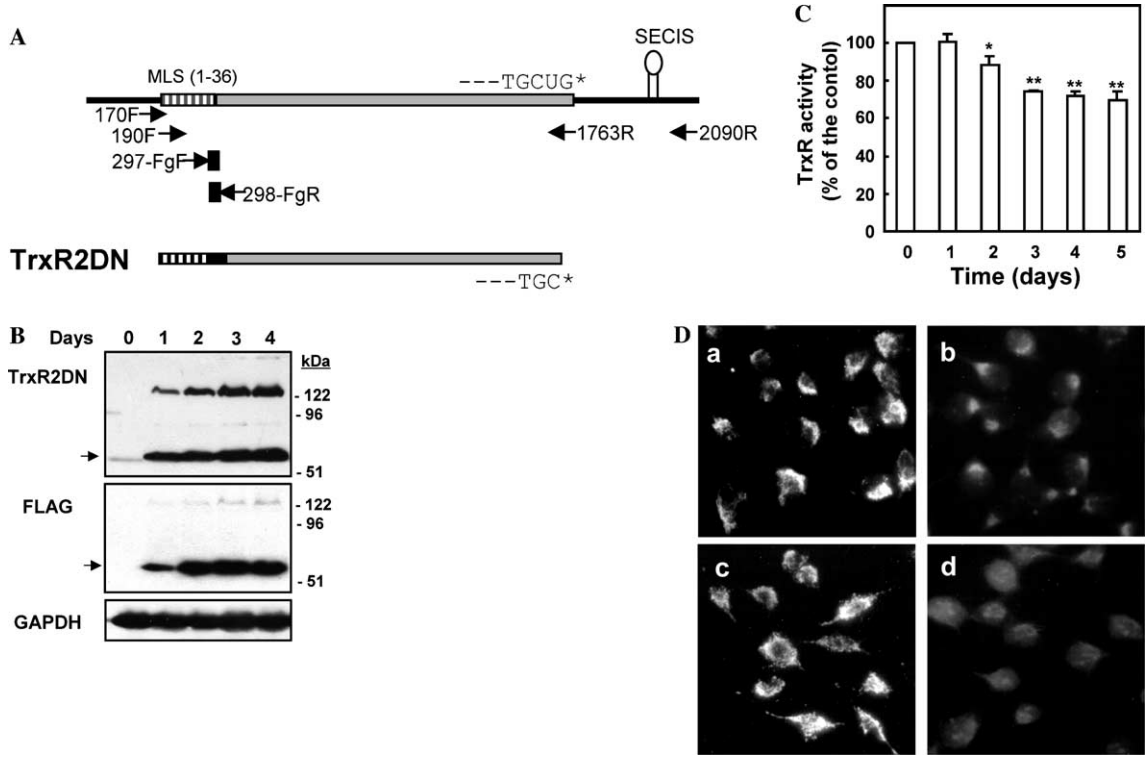

Fig. 1. Construction of a FLAG-tagged TrxR2DN and characterization of HeLa-TO/TrxR2DN cells. (A) To insert a FLAG epitope tag behind MLS (1–36) of TrxR2, 297-FgF, and 298-FgR primers were used. Since TrxR2DN does not have a SECIS element in 3′-UTR, the UGA codon for selenocysteine (U) in TrxR2 is translated to a stop codon in TrxR2DN. Black box indicates the FLAG sequence. (B) Tetracycline-repressible TrxR2DN induction. The HeLa-TO/TrxR2DN cells were incubated in the media with or without tetracycline for the indicated times and harvested. TrxR2DN expression was measured by Western blot analysis using a rabbit polyclonal antibody against TrxR2 and a mouse monoclonal antibody against FLAG. Equal loading of proteins was identified by using a rabbit polyclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (C) Measurement of TrxR activity. TrxR activities in TrxR2DN-induced cells were measured with increasing time by DTNB reduction, which was monitored spectrophotometrically at 412 nm. Values are means ± SD of two independent experiments using two different cell clones. Statistic analysis (Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$). (D) Immunofluorescence staining of TrxR2DN. TrxR2DN-induced (a,c,d) or uninduced cells (b) were immunostained with a TrxR2 antibody (a,b), a mouse monoclonal antibody against cytochrome *c* oxidase subunit VII (c), and preimmune serum (d). Fluorescence was visualized by a fluorescence microscope (200×).

mitochondrial protein (Fig. 1D). Mitochondria containing endogenous TrxR2 were stained in the uninduced cells (Fig. 1D, panel b).

Effect of Trx2DN expression on the intracellular concentration of H_2O_2 in EGF-stimulated cells

Stimulation of various cells with growth factor receptors results in a transient increase in the intracellular concentration of H_2O_2. We monitored the intracellular H_2O_2 production using an oxidation-sensitive fluorescence probe dihydrorhodamine 123 and flow cytometry. As shown in Fig. 2, induction of TrxR2DN alone caused a slight elevation of fluorescent intensity before stimulation with EGF ($P < 0.05$). Exposure of both TrxR2DN-induced and -uninduced cells to EGF resulted in an increase in the relative fluorescent intensity. The relative fluorescent intensity of induced cells was higher than that of uninduced cells ($P < 0.01$). These results suggest that the interference of TrxR2 function by the expression of TrxR2DN might cause accumulation of H_2O_2 by compromising the activity of the mitochondrial Trx2-dependent peroxidase Prx III.

Effect of TrxR2DN expression on the serum- or EGF-induced protein tyrosine phosphorylation

It was shown previously that H_2O_2 production is required for growth factor-mediated tyrosine phosphorylation of many proteins including MAP kinases. We tested whether TrxR2DN expression affects protein tyrosine phosphorylation. When TrxR2DN-induced or -uninduced cells were exposed to serum or EGF for the indicated times, TrxR2DN-induced cells showed more extensive tyrosine phosphorylation compared to uninduced cells at all time points (Figs. 3A and 3B). Similarly stronger activation of ERK was observed with TrxR2DN-induced cells (Figs. 3A and 3B). Furthermore, the EGF-induced tyrosine phosphorylation and ERK activation in both TrxR2DN-induced and -uninduced cells were inhibited by prior treatments of the cells

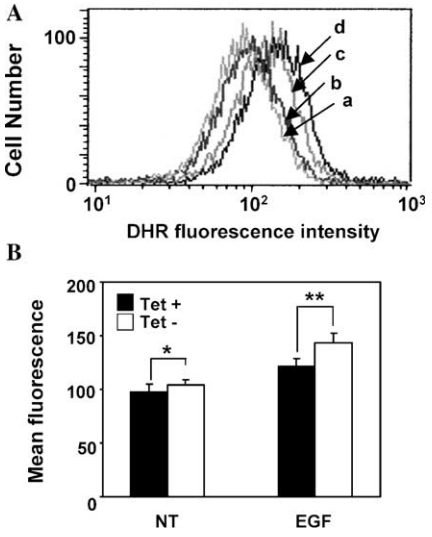

Fig. 2. Intracellular H_2O_2 in TrxR2DN-induced and -uninduced cells treated with EGF. TrxR2DN-induced or -uninduced cells were serum-deprived for 40 h, loaded with 10 μM of dihydrorhodamine 123, and then treated with 100 ng/ml EGF for 30 min. (A) The intracellular rhodamine 123 fluorescence intensity in cells; a, TrxR2DN-uninduced control cells; b, TrxR2DN-induced control cells; c, TrxR2DN-uninduced and EGF-treated cells; and d, TrxR2DN-induced and EGF-treated cells. (B) Mean fluorescence of the cells. Values are means ± SD of duplicates of three independent experiments using two different cell clones. NT, not treated; statistic analysis (Student's t test, $^*P < 0.05$, $^{**}P < 0.01$).

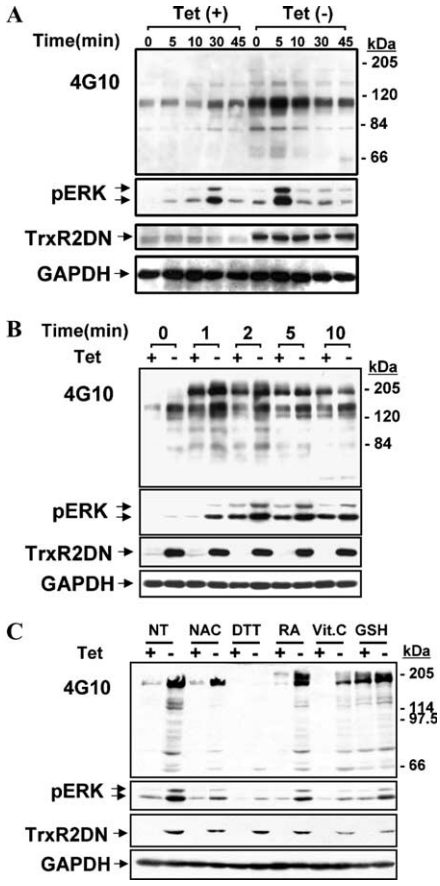

Fig. 3. Effect of TrxR2DN induction on serum or EGF-mediated tyrosine and ERK phosphorylations. The level of serum (A) or EGF (B)-induced tyrosine and ERK phosphorylation was higher in the TrxR2DN-induced cells than in the uninduced cells. Proteins (20 μg) were separated on a 10% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Tyrosine and ERK phosphorylations were measured by Western blotting with mouse monoclonal antibodies against phosphotyrosine (4G10) and phospho-ERK. TrxR2DN induction and equal loading of proteins were confirmed by Western blot analysis with TrxR2 and GAPDH antibodies. (C) Pretreatment of antioxidants decreased serum or EGF-mediated tyrosine and ERK phosphorylation in TrxR2DN induced cells. TrxR2DN-induced and -uninduced cells were pretreated with 2 mM of antioxidants for 1 h and then treated with EGF for 10 min. NT, not treated; NAC, *N*-acetylcysteine; DTT, dithiothreitol; RA, retinoic acid; Vit. C, vitamin C; and GSH, reduced glutathione.

with antioxidants like *N*-acetylcysteine and DTT (Fig. 3C). These results suggest that TrxR2DN induction affects growth factor-mediated protein tyrosine phosphorylation by altering the cellular redox status.

Effect of TrxR2DN expression on cell cycle progression and proliferation

Protein tyrosine phosphorylation is an important signal for cell proliferation. After addition of 10% FBS to TrxR2DN-induced or -uninduced cells that had been starved for 24 h, cell cycle progression was monitored by FACS analysis, and the ratio of the sum of cells in S and G_2M phases to the number of cells in G_1 phase [the ratio of $(S + G_2/M)/G_1$] was plotted with time (Fig. 4A). The ratio peaked at 12 h for TrxR2DN-induced cells, whereas the maximum ratio was seen at 24 h for uninduced cells, suggesting the G_1 to S phase transition of TrxR2DN-induced cells was much faster than that of uninduced cells. We also used the MTT assay as a method to compare the rate of cell proliferation (Fig. 4B). TrxR2DN-induced cells proliferated faster than uninduced cells. The changes in the concentrations of proteins involved in cell cycles cell cycle were measured by immunoblot analysis. Cyclin A, cyclin B, cyclin D_3, and p27, which are known to be involved in G_1 and S phases of the cell cycle, showed markedly different expressions between TrxR2DN-induced and -uninduced cells (Fig. 4C).

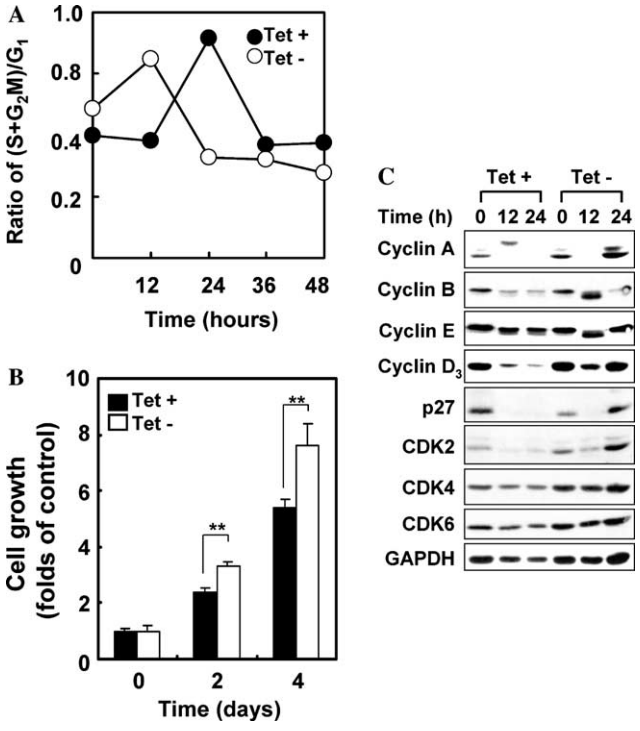

Fig. 4. Effect of TrxR2DN induction on cell cycle progression and proliferation. (A) Cell cycle progression. TrxR2DN-induced and -uninduced cells were starved for 2 days and incubated with 10% FBS for the indicated times. The cells were stained with propidium iodide. DNA contents were analyzed with a flow cytometer and the ratio of $(S + G_2M)/G_1$ was obtained. (B) Cell proliferation. Cell growth was measured by the MTT assay at the indicated times. Values are means ± SD of triplicates of three independent experiments (statistic analysis; Student's *t* test, $^{**}P < 0.01$). (C) Expression of cyclins, CDKs, and p27 were measured by Western blotting in the TrxR2DN-induced and -uninduced cells. Representative data from three independent experiments are shown.

Discussion

In this study, we generated a dominant negative form of TrxR2 by removing the SECIS element in the 3′-UTR of TrxR2 gene and thus blocking the translation of the UGA codon to Secys. The resulting TrxR2DN without the essential Secys is catalytically inactive but is expected to form dimers with the catalytically active endogenous TrxR2 in the mitochondria. Dominant-negative effect of the mutant TrxR2 was verified by the decrease in specific activities of TrxR enzymes in TrxR2DN-induced cells. The mitochondrial localization of TrxR2DN is evidenced by the absence of the MLS in mature TrxR2DN molecules and by the colocalization of TrxR2DN with cytochrome *c* oxidase. We attempted to detect the expected heterodimers of TrxR2DN and TrxR2 by immunoblot analysis after immunoprecipitation but failed because the size of TrxR2DN and TrxR2 is similar to that of IgG heavy chains (not shown). The dominant-negative effect of TrxR2DN is likely attributable to the formation of TrxR2DN–TrxR2 heterodimers or the non-productive complex between TrxR2DN and Trx2.

The mitochondria are the major source of intracellular superoxide anions, which are readily converted to H_2O_2 by the action of MnSOD [19]. Given that the mitochondria do not contain catalase, glutathione peroxidase, and Prx III are expected to be primarily responsible for the elimination of H_2O_2 [20,21]. Overexpression of TrxR2DN is likely to impede the function of TrxR2 to mediate the electron flow from NADPH to Prx III through Trx2, inhibiting the peroxidase activity of Prx III. Inefficient H_2O_2 elimination in the mitochondria would result in free diffusion of H_2O_2 across the mitochondrial membrane and its accumulation in the cytosol.

Many cell types produce H_2O_2 in response to a variety of extracellular stimuli including peptide growth factors [22]. Strong evidence suggests that NADPH oxidase, lipoxygenases, and cyclooxygenases are also involved in receptor-mediated H_2O_2 generation [23]. Specific inhibition of H_2O_2 generation resulted in a complete blockage of growth factor-induced signaling events such as protein tyrosine phosphorylation, MAP kinase activation, and cell proliferation [24,25]. Consistent with these observations, our results suggest that H_2O_2 accumulated as a result of inhibition of mitochondrial TrxR2 leads to the activation of cell proliferation machinery.

Acknowledgments

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