Reductase activity of human thioredoxin-like protein and its effects on cell apoptosis*

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Abstract Thioredoxin is a ubiquitous dithiol oxidoreductase found in many organisms and involved in numerous biochemical processes. Human thioredoxin-like protein (hTRXL) is differentially expressed at different development stages of human fetal cerebrum and belongs to an expanding family of thioredoxins. Recombinant hTRXL and truncated hTRXLs corresponding to the N-terminal (hTRXL-N) and C-terminal (hTRXL-C) domains are expressed and purified. In insulin disulfide reduction assay, both full-length hTRXL and hTRXL-N show reducing activity for the insulin disulfide bonds. As expected, the hTRXL-C failed to reduce insulin. MCF-7 cell stably transfected with hTRXL cDNA exhibits increased sensitivity to apoptosis induced by phorbol myristic acetate (PMA) and ionomysin.

Keywords: **human thioredoxin-like gene (*hTRXL*), fetal cerebrum, reduction assay, apoptosis.**

Thioredoxin is initially discovered in *Escherichia coli* as an electron donor for the ribonucleotide reductase, and since then, it has been proved to be associated with redox-mediated processes in *E. coli*. For example, thioredoxin-$(SH)_2$ can act as an essential subunit of T7 DNA polymerase[1] and is known to function in the maturation of filamentous bacteriophages M13 and f1[2,3]. In eukaryotic cells, thioredoxin can also serve as a reducing agent in sulfate reduction[4]. Moreover, it can facilitate refolding of disulfide-containing proteins[5] and modulate the activity of some transcription factors such as NF-kB and AP-1[6,7]. Other functions include action as antioxidant, the ability to reduce hydrogen peroxide, and protection of cells against oxidative stress[8]. A recent area of interest is the role of thioredoxin as a cell growth stimulator and an apoptosis inhibitor, both *in vitro* and *in vivo*. Recombinant human thioredoxin, when added to minimal culture medium in the absence of serum, stimulates the proliferation of a number of human solid tumor cell lines as measured over several days[9]. WEHI7.2 cells stably transfected with human thioredoxin cDNA and displaying increased levels of cytoplasmic thioredoxin, show increased growth and are resistant to drug-induced apoptosis both *in vitro* and *in vivo*[10]. In contrast, redox-inactive mutant thioredoxin reduces growth and enhances drug-induced apoptosis when transfected into WEHI 7.2 cells. Since the molecular studies have provided the proof-of-principle that the thioredoxin system is a rational target for anticancer drug development, the initial approach is to develop agents that might selectively inhibit the thioredoxin system and hence inhibit thioredoxin-dependent cell proliferation[11].

Human thioredoxin-like protein (hTRXL) belongs to the expanding thioredoxin family of proteins and is differentially expressed at different developmental stages of human fetal cerebrum. The full-length cDNA of hTRXL (GenBank Acc. No. AF051896) isolated and cloned by the method of DDRT-PCR and cDNA library screening is 1230 bp in length, which consists of 132 bp of 5′-untranslated sequence (including an in frame stop codon), an open reading frame of 870 bp and a 231 bp of 3′-untranslated sequence. This cDNA encoding for a 32 kD protein of 289 amino acids has two distinct domains. The 105 residues of N-terminal domain share 42% identity and 55% similarity to human thioredoxin and contain the conserved active site sequence CGPC (Cys-Gly-Pro-Cys). The C-terminal 184 amino acids of hTRXL, which is rich in acidic amino acids, has no similarity to any proteins in the public databases. The full-

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length cDNA isolated and cloned by the method of DDRT-PCR and cDNA library screening is identical to the previously published Txl/TRP32 sequence[12,13]. Northern blot analysis confirms that it has different expression patterns in human cerebrum of different developmental stages[14]. Both the Insulin disulfide reduction assay of the expressed recombinant protein encoded by *hTRXL* cDNA and the crystal structure of its N-terminal domain (unpublished data) have directly confirmed the hypothesis that hTRXL belongs to the expanding thioredoxin family.

Since the previous results suggest strongly that *hTRXL* is a gene related to the development of fatal brain, it is necessary to make further study on elucidating its role in cell growth and apoptosis.

1 Materials and methods

1.1 Expression and purification of target proteins

The full-length *hTRXL* cDNA in pDR2 vector (ClonTech) was used as a template for PCR to create in-frame constructs for further cloning[14]. Human thioredoxin full-length cDNA was also isolated by PCR-amplification using human fetal brain library (ClonTech) as a template[14]. The pGEX-4t-3 vector (Amersham Pharmacia Biotech) was used to create (GST)-fused proteins for bacterial expression. The expression of recombinant proteins was induced by 0.2 mmol/L isopropyl-1-b-D-galactopyranoside (IPTG) for 5 h at 37 ℃ with a cell density of $A_{600\,nm} = 0.8$ in *E. coli* strain BL21. GST fusion proteins were bound to glutathione-sepharose beads (Amersham Pharmacia Biotech), and were cleaved by incubation with thrombin protease (Sigma) at 4℃ for 14 h. The purified proteins were analyzed by SDS-PAGE.

1.2 Insulin disulfide reduction assay

E. coli thioredoxin (Sigma), human thioredoxin (hTRX), full-length hTRXL, hTRXL-N (residues 1 ~ 105) and hTRXL-C (residues 106 ~ 289) were compared for the reducing activity of insulin disulfide bonds as described previously[15]. The 600 μL reaction mixture contained 100mmol/L PBS (pH 7.0), 2 mmol/L EDTA, 0.13 mmol/L bovine insulin (Sigma) and 5 mmol/L proteins. The reaction was initiated by adding 1mmol/L DTT, and the absorbance at 650 nm was immediately recorded at room temperature. Measurements were performed at 1 min interval and the non-enzymatic reduction of insulin by DTT was recorded in a control cuvette without thioredoxin.

1.3 Transfection of MCF-7 cells

The *hTRXL* cDNA prepared as described previously was cloned into the *Eco*R I site of the pcDNA3.1/Zeo mammalian transfection vector and transfected into the breast cancer-derived MCF-7 cells. The transfected cells were maintained at culture densities up to 10^6 cells/mL in DMEM containing 10% fetal bovine serum, supplemented with 200 μg/mL Zeocin (GIBCO, BRL), and the clones were isolated in soft agarose and maintained in the culture medium with the same concentration of Zeocin.

1.4 Southern blot analysis

Southern blot hybridization was performed as described previously[16] using a full-length [α-^{32}P] dCTP-labeled *hTRXL* cDNA probe. Blots were quantified using a Molecular Dynamics Phosphorimager (Amersham Pharmacia Biotech).

1.5 Cell apoptosis analysis

Both the *hTRXL* transfected and the control MCF-7 cells (untransfected) were maintained in DMEM at a density of 3×10^5 cell/100mL at 37℃ and in 5% CO_2 for 24 h. Then the medium was replaced with DMEM containing 5 ng/mL phorbol myristic acetate (PMA) and 1.5 μg/mL ionomysin, and the cells were cultured for another 48 h. Totally 10^6 cells were harvested by centrifugation, fixed with 70% ethanol for 10 h at room temperature and washed with PBS. The cells were resuspended in 0.5 mL PBS with 20 μg/mL PI and 0.25 μg/mL RnaseA at 37℃ for 0.5 h, and were analyzed by flow cytometry.

2 Results and discussion

2.1 Thioredoxin-like reductase activity of hTRXL

To investigate the thioredoxin-like reducing activity of hTRXL, we expressed the full-length *hTRXL* along with truncated hTRXLs corresponding to the N-terminal (hTRXL-N, residues 1~105) and C-terminal (hTRXL-C, residues 106~289) domains in *E. coli*. The human thioredoxin (hTRX) was also prepared. The expressed proteins were purified by glutathione-sepharose column chromatography. The analysis of these proteins by SDS-PAGE is shown in Fig. 1.

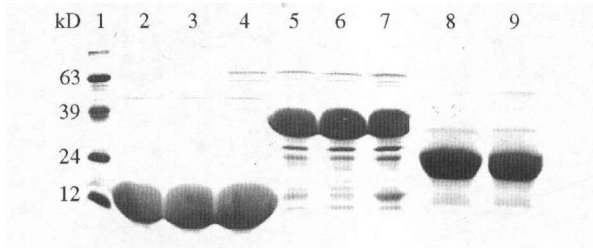

Fig. 1. Analysis of purified proteins by SDS-PAGE. 1. Protein marker; 2 ~ 4, hTRXL-N; 5 ~ 7, full-length *hTRXL*; 8 ~ 9, hTRXL-C.

In contrast to previously published work on Txl/TRP32[12,13], our experiments showed that both full-length *hTRXL* and hTRXL-N possessed similar reducing activity for the insulin disulfide bonds, with the kinetics faster than TRX but slower than *E. coli* thioredoxin (Sigma) (Fig. 2). As expected, the hTRXL-C failed to reduce insulin, demonstrating that the N-terminal region is responsible for the dithio-reducing enzymatic activity and the C-terminal region has little effect on the activity of the enzyme. The function of this unique C-terminal domain remains unknown.

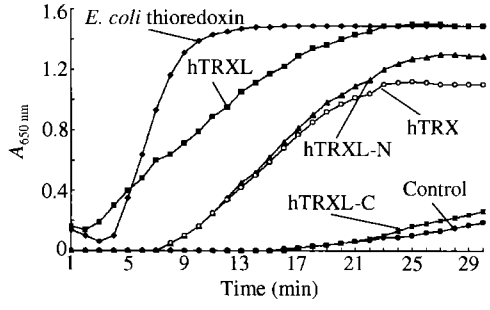

Fig. 2. Reductase activity of thioredoxin proteins.

2.2 Screening of stably transfected clones and Southern blot analysis

The pcDNA3.1/Zeo eukaryotic transfection vector has a Zeocin resistant tag which is for screening of the stably transfected clones. The MCF-7 cells transfected with pcDNA3.1/Zeo-*hTRXL* were screened by the character of Zeocin resistant. Three positive clones numbered B2, C2 and D2 were obtained by the screening. Genomic DNA of the 3 positive cell clones was hybridized with a full-length [α-^{32}P] dCTP-labeled *hTRXL* cDNA probe, the hybridization signals are shown in Fig 3.

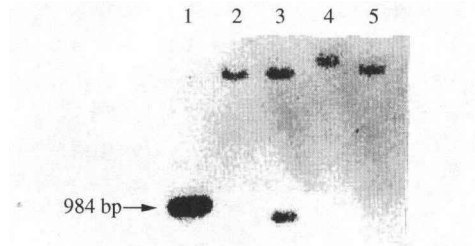

Fig. 3. Southern blot analysis of pcDNA3.1-*hTRXL* transfected clones. 1, *hTRXL* linear cDNA segment; 2, genomic DNA of MCF-7 cell; 3, genomic DNA of clone B2; 4, genomic DNA of clone C2; 5, genomic DNA of clone D2.

2.3 Apoptosis study

The MCF-7 control cells and *hTRXL* stably-transfected cells were incubated with PMA and ionomysin to induce apoptosis. The criteria used for the morphological identification of apoptotic cells included condensation and margination of the chromatin with the formation of crescents, cell shrinkage, nuclear fragmentation, cytoplasmic vacuolization and apoptot-

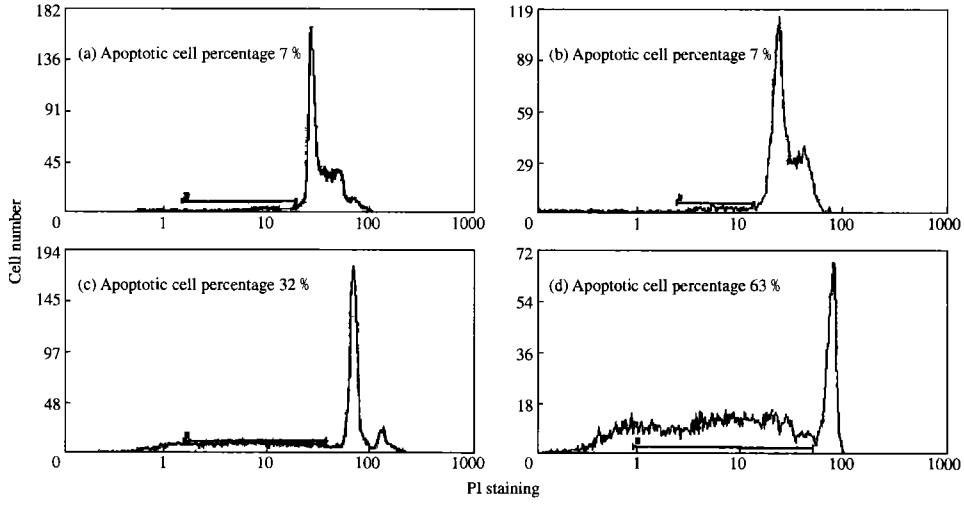

Fig. 4. Analysis of drug-induced apoptosis of MCF-7 cells transfected with pcDNA3.1-*hTRXL*. a. untransfected MCF-7 cells free from any inducers; b. untransfected MCF-7 cells treated with PMA and ionomysin after 48 h; c. pcDNA3.1-*hTRXL* transfected MCF-7 cells free from any inducers; d. pcDNA3.1-*hTRXL* transfected MCF-7 cells treated with PMA and ionomysim after 48 h.

ic body formation. Compared to untransfected cells, the *hTRXL*-transfected MCF-7 cells were subjected to apoptosis (Fig. 4) after induced by PMA and ionomysin, as measured by flow cytometry. The result suggested that *hTRXL* can stimulate apoptosis in MCF-7 cells.

Although hTRXL molecules have similar reducing activity for the insulin disulfide bonds, the cellular function of hTRXL we identified is different from that of the human thioredoxin (hTRX) reported previously[9,10]. It is possibly that the unique hTRXL C-terminal region may function as a recruiting factor or signal sequence leading the N-terminal thioredoxin-like domain to approach different substrates, and hence, resulting in the divergence in cellular function. A study to seek the possible substrates in the electron transport chain is currently underway.

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