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Characterization of human thioredoxin-like-1: Potential involvement in the cellular response against glucose deprivation

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Abstract **The thioredoxin system, composed of thioredoxin (Trx) and thioredoxin reductase (TrxR), emerges as one of the most important thiol-based systems involved in the maintenance of the cellular redox balance. Thioredoxin-like-1 (TXL-1) is a highly conserved protein comprising an N-terminal Trx domain and a C-terminal domain of unknown function. Here we show that TXL-1 is a substrate for the cytosolic selenoprotein TrxR-1. In situ hybridization experiments demonstrates high expression of *Txl-1* mRNA in various areas of central nervous system and also in some reproductive organs. Glucose deprivation, but not hydrogen peroxide treatment, reduced the levels of endogenous TXL-1 protein in HEK-293 cell line. Conversely, overexpression of TXL-1 protects against glucose deprivation-induced cytotoxicity. Taken together, the finding that *Txl-1* mRNA is highly expressed in tissues which use glucose as a primary energy source and the modulation of TXL-1 levels upon glucose deprivation indicate that TXL-1 might be involved in the cellular response to sugar starvation stress.**

Keywords: Thioredoxin; Glucose deprivation; Cell death; Stress

1. Introduction

The thioredoxin (Trx) family encompasses a group of redox proteins that function as general protein disulfide reductases [8] participating in many physiological and pathophysiological processes [1,5]. The redox activity of Trx is directly linked to their highly conserved active site (Cys-Gly-Pro-Cys) where the cysteine residues can undergo a reversible oxidation from a dithiol to a disulfide form. Oxidized inactive forms are reduced by the selenoprotein thioredoxin reductase (TrxR), which uses the reducing power of NADPH [9]. Trxs share a common globular structure consisting of a central core of β-sheets surrounded by α-helixes with the active site situated in a protrusion of the protein surface [12].

Different forms of Trxs have been reported in all organisms from prokaryotes to humans. In eukaryotic organisms Trxs are located in different subcellular compartments and are found either ubiquitously expressed or specifically localized in particular tissues [2,8,11,17,20,21,23]. Some of the previously described Trxs comprise additional domains with known or unknown counterparts in the databases [2,17,21,22,26,27].

Thioredoxin-like 1 (TXL-1), also known as TRP32 [17,21] is a two-domain protein of 32 kDa composed of a N-terminal Trx domain followed by a C-terminal domain of unknown function with no homology with any other protein in the databases. *TXL-1* mRNA is ubiquitously localized in all human tissues examined so far, although it is present at highest levels in tissues with an elevated metabolic rate [17,21]. The crystal structure of the TXL-1 N-terminal domain has been solved [14] and shows a monomeric structure, in contrast to the ubiquitous cytosolic TRX-1 which is dimeric in all crystal structures reported [37].

We report here novel features of human TXL-1 concerning its ability to serve as substrate for TrxR-1, new insights into its tissue localization and, more importantly, that TXL-1 is involved in the cellular response to glucose deprivation.

2. Materials and methods

2.1. Materials

All media and supplements used for cell culture including glucose-free DMEM were purchased from Life Technologies. Bovine TrxR-1 was from IMCO (Sweden). pET-15b vector was obtained from Novagen and pGEX-4T-1 vector was from Amersham Biosciences. pCDNA-myc empty vector was a gift from Dr. Eckardt Treuter. Anti-myc monoclonal antibody was purchased from Invitrogen. H_2O_2, Trypan Blue and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagents were obtained from Sigma–Aldrich.

2.2. Human TXL-1 protein expression and purification

The ORF encoding human TXL-1 was cloned into the *Bam*HI–*Eco*RI sites of the pGEX-4T-1 expression vector and used to transform *Escherichia coli* HMS174(DE3). Induction and purification of the recombinant protein was achieved as previously reported [21]. Thrombin (5 U/mg of fusion protein) was used to remove the glutathione-*S*-transferase (GST) domain by incubation overnight at 4 °C. The resulting protein preparation was then subjected to ion exchange chromatography using a Resource Q column (Amersham Biosciences), and human TXL-1 was eluted as a single peak using a gradient of NaCl. Protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad) using BSA as a standard.

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Abbreviations: DTT, dithiothretitol; GST, glutathione-*S*-transferase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Trx, thioredoxin; TrxR, thioredoxin reductase; Txl, thioredoxin-like; HEK, human embryonic kidney

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2.3. Enzymatic activity assays

Enzymatic activity of recombinant human TXL-1 was performed using two different assays. In the *DTT assay*, dithiothretitol (DTT) is used as reducing agent and the assay was carried out as previously described [38]. In the *TrxR assay*, recombinant TXL-1 activity was determined by its capability to reduce insulin disulfide bonds using NADPH as electron donor in the presence of calf thymus TrxR-1. The activity assay was performed essentially as described elsewhere [33] but monitoring insulin precipitation at 600 nm. In both cases, human TRX-1 was used as control.

2.4. Antibody production

Purified His-hTXL-1 [21] was used to immunize rabbits (Zeneca Research Biochemicals). After six immunizations, serum from rabbits was purified by ammonium sulfate precipitation. Affinity-purified antibodies were prepared as described [24] using the TXL-1 fragment from the GST-TXL-1 recombinant protein. The specificity of the antibodies was tested by western blot. Immunodetection was performed with horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1/5000 following the ECL protocol (Amersham Biosciences). Additionally, affinity purified polyclonal rabbit anti-TXL-1 and the respective blocking peptide were purchased from Abgent (San Diego, USA) for immunocytochemistry.

2.5. In situ hybridization

Adult (NMRI, weighting 20–25 g) and embryonic (embryonic days E9–E18; E1 = the day of copulation plug) mice were used. Adult animals were killed with carbon dioxide, the tissues and embryos were rapidly excised, and frozen on dry ice. The frozen tissues were sectioned with Microm HM-500 cryostat at 14 μm and mounted on Polylysine glass slides (Menzel, Braunschweig, Germany). The sections were stored at −20 °C until use. Three oligonucleotide probes directed against mouse and rat *Txl-1* mRNA (mouse nucleotides 189–222, 263–294 and 361–394, GeneBank Accession No. NM_016792) were used for in situ hybridization. The sequences exhibited less than 60% homology with other known genes in the GeneBank database. Several probes against non-related mRNAs with known expression patterns and with similar length and GC-content were used as controls. Addition of 100-fold excess of non-labeled probes quenched all signal. The in situ hybridization was carried out as described in detail previously [28].

2.6. Immunocytochemistry

For immunocytochemistry adult NMRI mice were perfused first with 20 ml of physiological saline followed with 50 ml of 4% paraformaldehyde in phosphate buffered saline (PBS, 0.1 M, pH 7.3). The brains were excised and further immersed in the same fixative for 1 h. After cryoprotection with 20% sucrose the tissues were sectioned at 10 μm in Microm 500 HM cryostat. Sections were incubated with the antibody (dil. 1:100) overnight in PBS containing 1% BSA and 0.1% Triton X-100. Immunoreactivity was visualized with Vectastain Elite-kit (Vector Laboratories, Burlingame, CA, USA) using nickel-intensified diaminobenzidine as a chromogen. Presaturation of the antibody with 40 μg of the blocking peptide and omission of the primary or secondary antibodies abolished all staining. Sections were examined under a Nikon FXA microscope equipped with a PCO Sensicam digital camera (PCO, Kelheim, Germany) and the images were processed using Corel Draw software (Corel Corporation Ltd., Ont., Canada).

2.7. Cell culture and transient transfections

Human embryonic kidney (HEK)-293 cells were cultured at 37 °C in an atmosphere of 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Transfection experiments were carried out as described elsewhere [13].

2.8. Stable transfection of HEK-293 cells

Plasmids pCDNA-myc and pCDNAmyc/TXL-1 were transfected into HEK-293 cells. Selection for G418 resistance (1 mg/ml) was initiated 48 h after transfection and individual clones were isolated to obtain three different stable cell lines: control clone transfected with empty pCDNAmyc vector and two clones transfected with pCDNA-myc/TXL-1 showing different TXL-1 expression levels. TXL-1 overexpression was confirmed by western-blot analysis using anti-myc (not shown) and anti-TXL-1 antibodies.

2.9. MTT cell viability assay

Cell viability was assessed by MTT assay [25] that detects the cellular ability to transform MTT tetrazolium salt into formazan. MTT (0.3 mg/ml in DMEM without phenol red) was added to the cells. After 1 h incubation at 37 °C, the medium was removed and the formazan crystals were dissolved in the same volume of isopropanol. Aliquots were transferred to 96-well plates and the absorbance was measured at 540 nm. Results were expressed as a percentage of viable cells.

2.10. Statistical analysis

Analyses of differences were carried out by ANOVA followed by the Student–Newman–Keuls post hoc test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Txl-1 is a two-domain protein conserved from fission yeast to humans

A protein–protein BLASTp search was performed to identify human TXL-1 orthologues in the public databases (http://www.ncbi.nlm.nih.gov/BLAST/), which resulted in several entries from the fission yeast to mammals. All these sequences were run on a W-Clustal alignment using the MegAlign program included in the DNAstar software package (Supplemental Data Fig. 1). Txl-1 protein is well conserved from lower eukaryotes to humans and the high identity is not only confined to the Trx domain, but being comparable all along the protein sequence. All the Txl-1 proteins analyzed are about the same length (290 residues) and contain many of the amino acid residues identified as essential for catalysis, maintenance of three-dimensional structure or protein–protein interactions in previously characterized Trxs [3].

As mentioned above, Txl-1 is a two-domain protein with an N-terminal Trx domain and a C-terminal domain with unknown function [17,21]. Nonetheless, the examination of the Conserved Domain Database at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) showed that two other classes of proteins different than Txl-1 contain a homologous domain, which is named Domain of Unknown Function

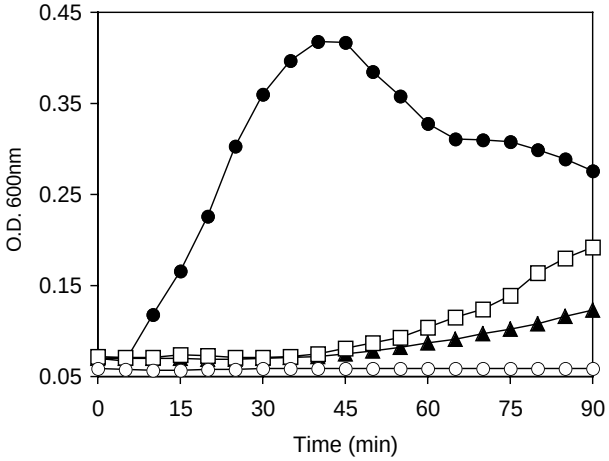

Fig. 1. Reducing activity of human TXL-1. TRX-1 (●) and purified TXL-1 (▲, 10 μg; □, 20 μg) were assayed for their ability to reduce insulin disulfide bonds in the presence of NADPH and calf thymus TrxR-1. Reactions with TrxR-1 alone (○) served as controls. A total of 1 μg of TrxR-1 was used in each reaction. Identical results were obtained from three independent experiments.