Cloning and Expression of a Novel Mammalian Thioredoxin*  

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We have isolated a 1276-base pair cDNA from a rat heart cDNA library that encodes a novel thioredoxin (Trx2) of 166 amino acid residues with a calculated molecular mass of 18.2 kDa. Trx2 possesses the conserved thioredoxin-active site, Trp-Cys-Gly-Pro-Cys, but lacks structural cysteines present in all mammalian thioredoxins. Trx2 also differs from the previously described rat thioredoxin (Trx1) by the presence of a 60-amino acid extension at the N terminus. This extension has properties characteristic for a mitochondrial translocation signal, and the cleavage at a putative mitochondrial peptide cleavage site would give a mature protein of 12.2 kDa. Western blot analysis from cytosolic, peroxisomal, and mitochondrial rat liver cell fractions confirmed mitochondrial localization of Trx2. Northern blot and reverse transcriptase-polymerase chain reaction analyses revealed that Trx2 hybridized to a 1.3-kilobase message, and it was expressed in several tissues with the highest expression levels in heart, muscle, kidney, and adrenal gland. N-terminally truncated recombinant protein was expressed in bacteria and characterized biochemically. Trx2 possessed a dithiol-reducing enzymatic activity and, with mammalian thioredoxin reductase and NADPH, was able to reduce the interchain disulfide bridges of insulin. Furthermore, Trx2 was more resistant to oxidation than Trx1.

Thioredoxin (Trx) is a 12-kDa protein, known to be present in many prokaryotes and eukaryotes and appears to be truly ubiquitous in all living cells (1, 2). It is characterized by an active site sequence -Trp-Cys-Gly-Pro-Cys-Lys-, conserved throughout evolution. The active site of thioredoxin is localized in a protrusion of its three-dimensional structure (3), and the two cysteine residues provide the sulfhydryl groups involved in Trx-dependent reducing activity. Oxidized thioredoxin, Trx-S2, is reduced to Trx-(SH)2 by the flavoenzyme thioredoxin reductase and NADPH (the thioredoxin system) (2).

Mammalian thioredoxin has been implicated in a wide variety of biochemical functions. It can act as hydrogen donor for ribonucleotide reductase (4) and methionine sulfoxide reductase (2), facilitate refolding of disulfide-containing proteins (5, 6), and activate the glucocorticoid or interleukin-2 receptors (7, 8). It can also modulate the DNA binding activity of some transcription factors either directly (TFIIC (9), BZLF1 (10), and NF-kB (11)) or indirectly (AP-1) through the nuclear factor Ref-1, which in turn is reduced by thioredoxin (12). The importance of the redox regulation of transcription factors by thioredoxin is exemplified with the v-fos oncogene where a point mutation of Cys154 → Ser results in constitutive activation of the AP-1 complex (13). Thioredoxin can be secreted by cells using a leaderless pathway (14–16) and stimulate the proliferation of lymphoid cells, fibroblasts, and a variety of human solid tumor cell lines (17–20). Furthermore, Trx is an essential component of the early pregnancy factor (21), it inhibits human immunodeficiency virus expression in macrophages (22), can reduce H2O2 (23), scavenge free radicals (24), and protect cells against oxidative stress (25).

Mammalian thioredoxins isolated from several sources (e.g. rat and calf liver, rabbit bone marrow, and human placenta) have certain structural differences with respect to those from prokaryotes. In addition to the active site cysteine residues, two or three (depending on the Trx source) additional structural cysteine residues exist in the C-terminal half of the molecule. Oxidation of these residues leads to a loss of its enzymatic activity (26).

More than one thioredoxin exists in many eukaryotes, e.g. yeast (27). However, only one thioredoxin has thus far been cloned from mammalian cells.

We report here the cloning of a full-length cDNA coding for a novel rat thioredoxin (Trx2) based upon protein homology and biochemical activity data. The N-terminal sequence contains a mitochondrial translocation signal, and mRNA analysis by RT-PCR and Northern blot reveals a unique expression pattern for Trx2 mRNA.

EXPERIMENTAL PROCEDURES

Cloning of the Rat Trx2 cDNA—A rat heart cDNA library (Clontech) was screened with 32P-labeled, degenerated oligonucleotide probes deduced from the amino acid sequence VVVDPSATWCGPC. Approximately 1 x 106 plaques were screened according the instructions of the manufacturer (Amersham Corp.), and a positive bacteriophage was isolated. The insert was cloned into the TA vector (Invitrogen) and sequenced. A 392-bp portion of the above clone was amplified by PCR (30 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min) with specific primers (trx2f1, 5′-AACCTTTATGTCACAGATGC-3′; and trx2r1, 5′-GCTGGAGTTCTCTAGTGTC-3′). The PCR product was 32P-labeled by random priming and used to rescreen the same library under high stringency conditions. Hybridization was performed at 60 °C in ExpressHyb hybridization solution (Clontech), followed by five 10-min washes in 2 × SSC, 0.1% SDS (1 × SSC is 0.15 μM NaCl plus 0.15 μM sodium citrate) at room temperature and finally two 40-min washes in 0.1% SSC, 0.1% SDS at 60 °C. More than 600,000 clones were screened, and five clones were isolated, cloned into the TA vector, and sequenced. 5′ rapid amplification of cDNA ends (RACE) with nested PCR using oligonucleotide-anchored heart cDNA template (Clontech) in the presence of an anchor-specific primer and an antisense primer complementary to the 3′-untranslated region of Trx2 (trx2r2, 5′-GCT-
GIGAGGTTCTAAGTGTTCC-3') was performed as described in the Clontech protocol. PCR products were cloned into the TA vector and sequenced.

**Northern Hybridization and RT-PCR Analysis of Rat Tissue Total RNA**—A rat multiple tissue Northern blot with 2 µg/lane of highly pure polyA+ RNA from liver (20) and further hybridized with different rat tissue total RNA. Rat Trx1 and Trx2 open reading frame probes were labeled with [32P]dCTP by a random priming procedure and hybridized in Ex-PressHyb solution (Clontech). For RT-PCR analysis male and female rats (6–8 weeks old) were killed by cervical dislocation, tissues were collected, and samples were immediately processed for total RNA isolation according to the acid guanidinium thiocyanate-phenol/chloroform single step extraction protocol (28). The integrity and quality of the purified RNA was controlled by formaldehyde denaturing agarose-gel electrophoresis and by measuring the A260/280 ratio. For first strand synthesis total RNA (1 µg) was dissolved in 10 µl of water, heated to 70 °C for 5 min, and then chilled on ice. The volume was increased to 20 µl, giving a final concentration of 1 mU each of dATP, dGTP, dCTP, dTTTP, 10 mM DTT, 5 pmol of random hexamers/µl (Promega), 1 unit of RNA-sin/µl, 200 units of Superscript RT (Life Technologies, Inc.), and the incubation buffer recommended by the supplier. For PCR amplification 1 µl of cDNA (total 20 µl) was subjected to PCR and amplified for 24 cycles by incubation at 94 °C for 10 s, 54 °C for 30 s, and 72 °C for 60 s in a PCR8600 thermoster (Perkin-Elmer). The oligonucleotides trx2f1 and trx2r1 were used for the amplification of a 392-bp fragment of the Trx2 mRNA. The oligonucleotides trx1f1, 5'-CTAAAAATGGTTAGAGCTGATGCAGAGGAG-3' and trx1r1, 5'-TGATTAGGCAAACTCCGTAAT-3' were used for the amplification of a 360-bp fragment of the Trx1 mRNA. The oligonucleotides Act5', 5'-CTGACACCAACACCTTCCA, and Act3', 5'-GGGACAGTGTTGTTGAG, were used for the amplification of a 238-bp fragment from β-actin mRNA. After agarose gel electrophoresis and blotting to nitrocellulose filters the PCR products were hybridized to [32P]-labeled internal oligonucleotides: trx2r2, 5'-CACA-CTTCCTCGTCTTTGCTACATCCTCTCTACAACGGAGTC-3', for Trx2; trx1r2, 5'-CTGAAATGTTGGCTGTGCTTACACAGCTTCTCGAGCA-AGGTGAT-3' for Trx1 and actin primer 5'-GAT- TACGCCAGATCGTTGTTAA-3'. Hybridization was performed at 50 °C in ExpressHyb hybridization solution followed by five 10-min washes in 2 × SSC, 0.1% SDS at room temperature and finally two 40-min washes in 0.1% SSC, 0.1%, SDS at 50 °C.

**In Vitro Transcription and Translation**—The TA-Trx2 clone (0.5 µg) was translated using the TNT-coupled reticulocyte lysate system (Promega) and SP6 RNA polymerase with incorporation of [35S]methionine for 60 min at 30°C. The translation products were analyzed in a 15% SDS-PAGE gel and visualized by autoradiography.

**Protein Expression and Purification**—The CDNA encoding a part of rat Trx2 (amino acids 60–166, ΔTrx2) was amplified by PCR from the TA-Trx2 plasmid by using two mutagenic primers that introduce a NdeI (trx2f1, 5'-ACACCAAGGTGTTGTTGCTACATCCTCTCTACAACGGAGTC-3', and a BamHI (trx2r2, 5'-CTGCGCGAGTCACAGTTACACCAGAC-3') and a BamHI site and C terminus of the protein, respectively. The amplified DNA was cloned into the NdeI-BamHI sites of the pET-15b expression vector (AMS Biotechnology), and Escherichia coli strain BL21(DE3) was transformed with pET-ΔTrx2. A single positive colony was inoculated in 1 liter of LB broth with 50 µg/ml ampicillin and grown at 37 °C until A600 = 0.5. Then, fusion protein expression was induced by addition of 0.5 mM IPTG, and growth was continued for another 3.5 h. The cells were harvested by centrifugation at 10,000 × g for 10 min, the pellet was resuspended in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. Lysozyme was added to a final concentration of 0.5 mg/ml with stirring for 30 min on ice. Subsequently, MgCl2 (10 mM), MnCl2 (1 mM), DNase I (10 µg/ml), and RNase A (10 µg/ml) were added, and the incubation was continued for another 45 min on ice. The cells were disrupted by sonication for 8 min, and the supernatant was cleared by centrifugation at 15,000 × g for 30 min and loaded onto a Talon resin column (Clontech), and the protein was eluted with 20 mM imidazole. The size and purity of the eluted protein was determined by SDS-PAGE.

**Antibodies and Immunoblotting Analysis**—Purified ΔTrx2 was used to immunize rabbits (Zapata Research Biochemicals, UK). Immunoreactive proteins from the rabbits was purified by ammonium sulfate precipitation. Affinity-purified antibodies were prepared using a cyagenon bromide-activated Sepharose 4B column, onto which 0.5 mg of ΔTrx2 had been coupled using the procedure recommended by the manufacturer (Pharmacia). Specificity of the antibodies was tested by Western blotting using recombinant Trx2 and total cell extracts.

Mitochondrial, peroxisomal, and cytosolic fractions were prepared from rat liver as previously described (29). For immunoblotting analysis samples were subjected to 15% SDS-PAGE, and the separated proteins were electrophoretically transferred to nitrocellulose membranes (Hy- bond-C Super, Amersham Corp.). The membranes were blocked with phosphate buffered saline containing 5% dry fat-free milk powder and 0.05% Tween-20. The membranes were further incubated with affinity-purified anti-Trx2 antibodies. Immunodetection was performed with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Corp.) diluted 1:5000 following the ECL protocol (Amersham Corp.) in a hydrogen peroxide catalyzed oxidation of luminol.

**Insulin Disulfide Reduction Assay of Thioredoxin**—The insulin disulfide reduction assay was essentially performed as described elsewhere (30) with a slight modification to activate Trx1 and Trx2 by reduction. Aliquots of Trx1 and Trx2 were preincubated at 37 °C for 20 min with 2 µl of 50 mM Hepes, pH 7.6, 100 µg/ml bovine serum albumin, and 2 µl of DTT in a total volume of 70 µl. Then, 40 µl of a reaction mixture composed of 200 µl of Hepes (1 M), pH 7.6, 40 µl of EDTA (0.2 M), 40 µl of NADPH (40 µg/ml), and 500 µl of insulin (110 mg/ml) were added. The reaction started with the addition of 10 µl of thioredoxin reductase from calf thymus (3.0 A260 unit), and incubation was continued for 20 min at 37 °C. The reaction was stopped by the addition of 0.5 ml of 6 M guanidine-HCl, 1 mM DTNB, and the absorbance at 412 nm was measured. Calf thymus TR and human thioredoxin were kind gifts from Prof. A. Holmgren, Karolinska Institute, Sweden.

**RESULTS**

**Cloning of Rat Thioredoxin 2**—The primary structure of the active site of thioredoxin, which is conserved throughout evolution, was used to design degenerate primers as probes for screening for novel thioredoxin genes. A partial clone was isolated from a rat cdna library and a DNA fragment of this clone was used to screen the same library under stringent conditions. All five clones were isolated and sequenced. All clones were overlapping and the longest one, Trx2, possessed an open reading frame of 501 bp beginning with an ATG initiation codon and ending with a TGA termination codon. To obtain the full-length cDNA a RACE-PCR technique was applied, utilizing antisense oligonucleotide primers specific for internal sequence within the Trx2 cDNA. A cDNA fragment was amplified that overlapped Trx2 and encoded 46 bp of a novel 5' sequence including an in-frame TGA stop codon upstream of the ATG initiation codon. The overall composite sequence consists of 1276 bp, including a stretch of 20 adenosines corresponding to the poly(A) tail and an AATAAAA motif, 18 bp upstream from the poly(A) tail. The open reading frame encodes a protein of 166 amino acids with a predicted mass of 18.2 kDa and a pI of 7.9 (Fig. 1, A and B).

In order to confirm that the open reading frame present in the Trx2 clone is functional and codes for translatable protein, the cDNA was transcribed from the SP6 promoter of the TA-Trx2 clone and translated in a rabbit reticulocyte in vitro translation system with incorporation of [35S]methionine. The translation product was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The result showed a 20-kDa translation product, indicating the presence of translatable, functional coding sequence (Fig. 2).

**Analysis of the Deduced Amino Acid Sequence**—The N-terminal region of the protein has high content of positively charged residues and a secondary structure prediction indicated a potential α-helix followed by β-sheets (Fig. 1). These features are common to most mitochondrial targeting signal peptides (31), and an algorithm analysis of the partial amino acid composition indicated mitochondrial intracellular localization (22). A motif for mitochondrial presequence proteases (32) was also found with the cleavage site between Ser-58 and Thr-59 (Fig. 3A). This putative cleavage results in a 12.2-kDa mature protein, which is similar in size to previously reported thioredoxins.

The C-terminal half of the protein contained the active site found in all thioredoxins with the characteristic amino acid
sequence, Trp-Cys-Gly-Pro-Cys-Lys. The molecule showed a 35% homology with other mammalian thioredoxins and many of the structural amino acids that are conserved in mammalian thioredoxins, i.e., Phe-12, Pro-40, Asp-59, Lys-82, were also conserved in Trx2 (Fig. 3B). However, amino acids participating in protein-protein interactions such as Ala-93 and Glu-57 are changed to Ile and Lys, respectively. One major difference between Trx2 and mammalian thioredoxins is the absence of structural cysteines, residues which are present in all mammalian thioredoxins.

Trx2 has higher homology with the *E. coli* thioredoxin than with the known mammalian proteins and a phylogenetic analysis places Trx2 in a different branch of the tree, distant from the mammalian proteins and closer to the prokaryotic and lower eukaryotic ones. Sequence relatedness is summarized in Fig. 4.

**Expression of Recombinant Thioredoxin 2 and Subcellular Localization**—The C-terminal part of Trx2, amino acids 60–166 (**Trx2**), which is homologous to thioredoxins, was cloned into the pET-15b expression vector under the control of a T7 promoter. This portion may also correspond to the mature protein when the protein is translocated in mitochondria and cleaved. The resulting plasmid pET-**Trx2** was transformed to *E. coli* BL21 (DE3) and the expression of **Trx2** was induced by the addition of IPTG for 3.5 h. The recombinant protein was purified to almost homogeneity by affinity chromatography with a Talon column. Samples from different steps in the purification were analyzed by SDS-PAGE on a 15% gel. A single band of 15 kDa was detected after the Talon chromatography (data not shown). We next analyzed the subcellular localization of Trx2 using affinity purified polyclonal antibodies and cytosolic, mitochondrial, and peroxisome cell fractions. As shown in Fig. 7, Trx2 is only present in mitochondrial fractions as neither cytosolic nor peroxisome enriched fractions displayed any signal. Rat Trx1 did not cross-react with the affinity purified antibodies against **Trx2**. By densitometric analyses Trx2 content in total cell-free extracts from rat liver was estimated to be around 0.1 μg/mg protein (data not shown). The transient preprotein with the mitochondrial translocation peptide was not detected, indicating that the translocation process is very fast. The recombinant ΔTrx2 in lane 1 has a higher molecular weight because the His tag was not removed by thrombin.

**Thioredoxin Catalyzed Insulin Reduction**—In order to con-

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**Fig. 1. cDNA, deduced amino acid sequence, and predicted secondary structure of Trx2.** A, the deduced amino acid sequence in the single-letter code is shown under the nucleotide sequence. The active site is boxed and the possible polyadenylation signal is underlined. B, secondary structure was predicted using the DNASTAR program and the Garnier-Robson algorithm. The Kyte-Doolittle algorithm was applied for the hydrophathy plot of Trx2 amino acid sequence.
firm the specificity of our recombinant ΔTrx2, we examined the reduction of insulin, a classical assay in which thioredoxin catalyzes disulfide reduction of insulin with NADPH in the presence of mammalian thioredoxin reductase. We compared the activities of human thioredoxin with the recombinant ΔTrx2. As shown in Fig. 8, when the samples were preincubated with DTT, ΔTrx2 and human Trx were equally good substrates for thioredoxin reductase. However, oxidized human Trx showed a decreased capacity to reduce insulin with a pronounced lag phase. The activity of ΔTrx2 was not affected upon oxidation. Although ΔTrx2 is homologous to the prokaryotic thioredoxins it could not function as a substrate for *E. coli* thioredoxin reductase (data not shown).

**DISCUSSION**

A Southern hybridization analysis of the human genome suggested several thioredoxin genes, including at least one inactive pseudogene (33). We report here the complete amino acid sequence deduced from a cDNA clone of a novel rat thioredoxin (Trx2).

The encoded protein sequence showed an interesting two-domain structure consisting of an N-terminal part of a 60-amino acid region rich in basic amino acids with a theoretical pI of 12.1 and a C-terminal part homologous to thioredoxin with a pI of 4.8. The N-terminal of Trx2 has characteristic properties of a mitochondrial translocation peptide and a proposed protease cleavage site which may give a mature protein of 12.2 kDa. In fact, a slightly larger mitochondrial form of Trx, compared to the cytosolic Trx, has been reported to be present.
in pig heart, based on electrophoretic mobility (34). In vitro coupled transcription/translation confirmed the presence of the putative open reading frame in the Trx2 clone. The size of the translation products in the SDS-polyacrylamide gel electrophoresis analysis was somewhat larger than the calculated sizes (20 kDa versus 18.2 kDa), but it may be due to the primary characteristic of the protein (e.g. charge). Of course, the native protein may still have a different size due to post-translational modification.

Although Trx2 is phylogenetically closer to prokaryotic than mammalian thioredoxin, some amino acids conserved in all prokaryotes like Trp-28 are not conserved in Trx2. Also the differences in amino acids involved in protein interaction such as Ala-93 and Glu-57 will probably confer a different specificity for Trx2 compared to Trx1. All previously described mammalian thioredoxins have 2–3 additional cysteine residues to the 2 localized in the active site. These structural or noncatalytic cysteine residues can undergo oxidation, a process which leads to inactivation. From the structure of reduced human thioredoxin Cys-72 is located in a loop in proximity to the active site. Ren et al. (26) showed that Cys-72 is responsible for dimer formation and subsequent loss of activity. The absence of corresponding structural cysteines in Trx2 confers a resistance to oxidation. This property might have important physiological implications for the role of Trx2.

Mammalian Trx can be found in many different cellular compartments including nucleus, endoplasmic reticulum, mitochondria, and plasma membranes (35, 36). Also Trx is differentially regulated and has separate functions in the promotion of cell growth to transcription factor activation and radical scavenging activities. Trx2 is highly expressed in tissues such as heart and skeletal muscle where Trx1 protein is not detectable (37). Reactive oxygen intermediates (ROI), which comprise hydrogen peroxide, hydroxyl radicals, and superoxide anions, are essential compounds of oxidative metabolism (38). An important source of ROI are mitochondria (39). Generally, ROI are regarded as toxic and harmful metabolites, and when their formation occurs in an uncontrolled fashion, they may be implicated in several diseases by inducing lipid peroxidation and disruption of structural proteins, enzymes, and nucleic acids. Recently, ROI in addition to being cytotoxic, have been reported to function as signal transducers of tumor necrosis factor-induced gene expression (40). Thioredoxin can reduce hydrogen peroxide and scavenge free radicals (23–25). Using affinity-purified antibodies against Trx2 we showed that it is localized in the mitochondria and that the mature protein has an apparent molecular mass of 13 kDa. A mitochondrial localized Trx2, which is more resistant to oxidation than is Trx1, may explain its high expression in heart and skeletal muscle, tissues with high metabolic activity, and confer an important regulatory and/or protective function.

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