Overexpressed Human Mitochondrial Thioredoxin Confers Resistance to Oxidant-induced Apoptosis in Human Osteosarcoma Cells*

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Oxidative damage to mitochondria is a central mechanism of apoptosis induced by many toxic chemicals. Thioredoxin family proteins share a conserved Cys-X-X-Cys motif at their active center and play important roles in control of cellular redox state and protection against oxidative damage. In addition to the well studied cytosolic and extracellular form (Trx1), rat and avian mitochondrial forms of thioredoxin (mtTrx) have been reported. In this study, we cloned the full-length human mtTrx cDNA and performed localization and functional studies in 143B human osteosarcoma cells. The coding sequence of human mtTrx consists of a region with homology to Trx1 as well as a putative mitochondrial localization signal (MLS) at its N terminus. In stably transfected cell lines, mtTrx had a mitochondrial localization as measured by subcellular fractionation studies and by confocal fluorescence microscopy. Deletion of the MLS rendered mtTrx to be solely expressed in the cytosolic fraction. On SDS-PAGE, transfected mtTrx had the same apparent molecular weight as the MLS truncated form, indicating that the leader sequence is cleaved during or after mitochondrial import. Treatment with the oxidant tert-butylhydroperoxide induced apoptosis in 143B cells. This oxidant-induced apoptosis was inhibited by overexpressing the full-length mtTrx in 143B cells. Thus, human mtTrx is a member of the thioredoxin family of proteins localized to mitochondria and may play important roles in protection against oxidant-induced apoptosis.

Thioredoxins (Trx) are a family of small proteins that contain a conserved redox active center, Trp-Cys-Gly-Pro-Cys-Lys (1). The reversible oxidation/reduction in the active center enables Trx to transfer electrons to protein disulfide substrates. Two other components, thioredoxin reductase (TR) and NADPH, reduce the oxidized Trx and complete Trx cycling (1). In the presence of TR and NADPH, Trx mediates rapid and reversible disulfide-thiol exchange of dithiol motifs in protein substrates. Although Trx was originally identified as an electron donor of ribonucleotide reductase, a wide range of other biological functions have been characterized based on its redox capability (1). Interestingly, Trx is implicated in apoptosis regulation through two different mechanisms: 1) scavenging reactive oxygen species (ROS) to protect against oxidative stress (1) and 2) direct binding and inhibiting the activity of the proapoptotic protein apoptosis signal-regulating kinase 1 (ASK1) (2, 3). Regulating the activities of transcription factors, such as NF-xB and AP-1, is another important aspect of its biological functions (1).

Multiple isoforms of Trx have been identified in different organisms, ranging from Escherichia coli to mammalian cells (4–10). In higher organisms, Trx has different subcellular localizations, including cytoplasm, nuclei, and mitochondria (5–10). The mitochondrial thioredoxin gene was cloned from rat and chicken (9, 10), and the protein has been purified from bovine and swine (5, 6). Like its cytosolic homolog, mtTrx can reduce insulin in vitro, suggesting that proteins share similar biological properties (10). The cloning of mitochondrial TR (11, 12) indicates that a complete system of thioredoxin exists in mitochondria, which may play potentially important physiological roles, particularly in protection against oxidants and other forms of chemical-induced toxicities. This is further supported by the recent finding that chicken cells lacking mtTrx showed increased spontaneous apoptosis and were more sensitive to serum deprivation-induced apoptosis (9).

In this study, we cloned the human mtTrx and determined that it is specifically localized in mitochondria. Northern blot analysis revealed the existence of two forms of human mtTrx mRNA and demonstrated that it is widely expressed in human tissues. The two forms of the mtTrx transcript differ in the 3'-untranslated region. Finally, stable overexpression of human mtTrx in 143B osteosarcoma cells protected cells from cell death induced by the chemical oxidant tert-butylhydroperoxide (tBH). These data suggest that the mtTrx system may represent an important defense mechanism that functions in mitochondria to protect against oxidative stress and apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and RNA Isolation—Human 143B osteosarcoma cells, human embryonic kidney 293 cells, and HT29 colon adenocarcinoma cells were from American Type Culture Collection (ATCC, Manassas, VA). 143B and 293 cells were cultured in Dulbecco’s modified Eagle’s medium, and HT29 cells were cultured in McCoy’s 5A medium, each with 10% fetal bovine serum in a humidified CO2 incubator at 37 °C.
Phoenix amphotropic retroviral packaging cells (derived from 293 cells) were obtained from ATCC upon approval by Dr. Gary Nolan (Stanford University, Stanford, CA) and were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Total RNA was isolated from 143B cells and 293 cells with TRIzol LS reagent (Invitrogen, Carlsbad, CA) and treated with a DNA-free kit (Ambion, Austin, TX) to remove any trace of contaminating DNA.

5’ and 3’ RACE—Total RNA isolated from 143B cells was used as template for cDNA synthesis. Primer 5′RACE 5′-CAG GCC ACC AGG ACT GCA TTT TGG GCC C-3′ corresponding to base pairs 99–126 (GenBank™ accession number NM-012473) and primer 3′RACE 5′-GCC CAG TCG GCA CGG CTC AGC ACA CC-3′, which corresponds to base pairs 394–413, were used to amplify 5′ and 3′ ends, separately, with SMART™ RACE cDNA amplification kit (CLONTECH, Palo Alto, CA). PCR products were ligated into TOPO TA cloning vector (Invitrogen), and multiple clones were picked for sequencing.

Northern Blot Analysis—Antisense RNA for the human mtTrx open reading frame (base pairs 168–519; GenBank™ accession number NM-012473) was synthesized and labeled with [α-32P]UTP using Strip-EZ™ RNA kit (Ambion). Human multiple tissue Northern (MTN™) Blots (CLONTECH) containing 1 μg of poly(A)+ RNA from 12 different human tissues were hybridized in ULTRAhyb solution (Ambion) according to the manufacturer’s guide. After stringent washes, the membrane was developed using either a PhosphorImager (Amersham Biosciences) or BioMax x-ray films (Eastman Kodak Co., Rochester, NY) in the presence of an intensifying screen.

Construction and Expression of Epitope-tagged Recombinant mtTrx Protein—The open reading frame was amplified using PCR with the primer pairs 5′-GGG ATC CGC CAT CAC GGC TCA GGC GCT AC-3′ and 5′-GCA CTC GAG CCA ATC AGC TTC TTC AGG A-3′ for the truncated form that lacks the putative mitochondrial localization signal, another 5′ primer, 5′-TGG ATC CGC CAT GAG CAT GTC CTT GAC-3′, was used according to the information obtained from PSORTII analysis. PCR products were ligated in frame into pcDNA3.1/V5-His (Invitrogen) in BamHIL/Xhol sites to produce two constructs, pcDNA3.1/V5-mtTrx and pcDNA3.1/V5-TmtTrx. Both plasmids contained a V5 epitope at their C terminus. All plasmids and constructs, pcDNA3.1/V5-His without an insert. After 24 h, cells were trypsinized and plated with different dilutions on 100-mm dishes. After 10 days in selection medium containing 800 μg/ml neomycin for 2 weeks. The expression level in these experiments was 10 μl of 4 mg/ml digitonin followed by incubation at room temperature for 2 min. After centrifugation at 600 × g for 2 min at room temperature, the supernatant was collected and analyzed using flow cytometry (BD Immunocytometry Systems, San Jose, CA).

Measurement of tBH—The concentration of tBH in the medium was measured spectrophotometrically with an enzyme-coupled assay utilizing glutathione peroxidase and glutathione disulfide reductase (19). The tBH concentration was measured as the decrease in absorbance at 340 nm (ε = 6.22 mm molar−1 cm−1).

RESULTS

Cloning of Human mtTrx cDNA and Expression of Recombinant mtTrx—A search of GenBank™ acquired several entries from NCBI Annotation Project (XM-056182) and another source (NM-012473), suggesting that a mitochondrial form of thioredoxin may be present in human cells. To confirm the existence of the mtTrx transcript in cultured human cell lines, we designed RT-PCR primers, 5′-TTC CTG GCC TCT GTC ATC TC-3′ and 5′-CTC ATA CTC AAT GCC GAG GTC-3′, which corresponded to putative human mtTrx sequence 28–47 and 367–387 bp, respectively (NM-012473). Using cDNA synthesized from total RNA obtained from human 143B osteosarcoma or embryonic kidney 293 cells as the template, RT-PCR experiments generated a single band on agarose gel electrophoresis (Fig. 1).
To obtain 5′/H11032 and 3′/H11032 ends of mtTrx cDNA, 5′/H11032 and 3′/H11032 RACE were applied. 5′ RACE yielded a cDNA fragment that contained the mtTrx coding sequence with an extra 77 bp sequence 5′ upstream of the translation initiation codon ATG (Fig. 2). Two cDNA fragments were produced from 3′ RACE, one with a 428-bp sequence 3′ downstream of the predicted TGA stop codon consistent with the longest mtTrx sequence in GeneBank™ (XMp-056182) and a higher molecular weight cDNA with an additional 330 bp of downstream sequence (Fig. 2). A BLAST search revealed that the 428- and 330-bp fragments were continuous on human chromosome 22 without any intervening sequence. The sizes of the predicted two forms of full-length mtTrx cDNA in human cells are 1006 bp and 1336 bp (GenBank™ accession number AF480262), respectively, without considering the poly(A)/H11001 tail length.

Tissue Distribution of mtTrx—Northern blot analyses with a mtTrx probe lacking the putative mitochondrial localization signal were carried out with 12-lane human MTN blots, which the conserved amino acid sequence of the active center is indicated as a box. The end of the shorter cDNA is pinpointed by an arrow. A possible polyadenylation recognition site is underlined.

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Fig. 2. cDNA and deduced amino acid sequence of human mtTrx. The amino acid sequence is represented as the single letter under the nucleotide sequence. The conserved amino acid sequence of the active center is indicated as a box. The end of the shorter cDNA is pinpointed by an arrow. A possible polyadenylation recognition site is underlined.
localization of mtTrx, plasmids containing either the full-length coding sequence or a truncated form (with N-terminal 55 amino acids deleted) of mtTrx with a C-terminal V5 epitope tag were stably transfected into 143B cells. The V5 epitope contained in the C terminus of the recombinant protein enabled the detection of the fusion protein. Both a full-length form and a truncated form yielded the same molecular weight band on the gel (Fig. 4A), suggesting that the MLS of mtTrx is cleaved in association with transport into mitochondria. Mitochondrial and cytosolic fractions from stably transfected cells were subjected to SDS-PAGE followed by Western blot analysis with anti-V5 antibody. As anticipated, the full-length mtTrx fusion protein, which has the complete MLS, appeared to be mainly localized in the mitochondria fraction (Fig. 4B), whereas the

Fig. 3. Northern blot analysis of the expression of mtTrx. A, MTN blots were hybridized with mtTrx probe as described under “Experimental Procedures.” The two bands were pointed out by arrows. The data presented are representative of three separate experiments with different membranes. B, quantitation of Northern blot data. The relative expression levels of mtTrx mRNA(s) were normalized to the levels of β-actin mRNA. The data presented are the averages of three separate experiments (mean ± S.E.). Significant differences from peripheral blood leukocyte expression level, determined by analysis of variance and Dunnett’s multiple comparison test are indicated as: **, p < 0.01 and *, p < 0.05.
truncated form of mtTrx lacking the putative MLS was observed only in the cytosolic fraction (Fig. 4B).

To further verify the subcellular localization of mtTrx, a retrovirus vector TJ84 was used to construct a GFP fusion protein of mtTrx. TJ84 itself encodes a fusion protein consisting of a nuclear localization signal fused to GFP (13). The nuclear localization signal was replaced by the full-length mtTrx coding sequence in frame with GFP. While the original TJ84 accumulated in the nuclei of transduced 143B cells (Fig. 5), replacement by MLS-containing mtTrx at the N terminus of GFP altered the distribution of GFP into the mitochondria, as confirmed by co-localization with TMRM (Fig. 5). Thus, the cloned human mtTrx is a nuclear DNA-encoded protein whose distribution into mitochondria is determined by its N-terminal MLS sequence.

Overexpression of mtTrx Protects Cells from tBH-induced Cell Death—mtTrx is a redox-active protein, and the Trx system plays critical roles in protecting against oxidative stress and mediating signal transduction (1). Overexpression of the cytosolic Trx has been shown to protect against oxidative stress-induced cell death (20, 21). tBH induces oxidative stress in different cell systems (17, 22). The activity of the important component of the Trx system, TR, appears related to the sensitivity of cells to tBH-induced cell death (22). Similar to the cytosolic homolog, overexpression of the truncated form of mtTrx in 143B cells, which specifically localized to the cytosol, showed protection against tBH-induced cytotoxicity (data not shown). These results confirm that the mtTrx protein has biological activities and a function similar to the cytosolic form.

To determine whether mtTrx, when expressed in the mitochondria, can protect against oxidant-induced cell death, we generated 143B cells that stably overexpress mtTrx and tested their sensitivity to tBH. Preliminary studies showed that substantial cell killing occurred in control cells with 300 μM tBH. Morphological changes of apoptosis were apparent as early as 6–8 h, but a longer time point (14 h) was selected to make sure that any observed difference in protection was not due only to a delay in the onset or progression of apoptosis. Cells (control, vector control, and mtTrx) were treated with 200 or 300 μM tBH for 14 h, and the viability of cells was evaluated by using a LIVE/DEAD cell viability assay. In this assay, two dyes are used to determine the live cells: ethidium homodimer, which stains dead cells that have a compromised plasma membrane, and calcein-AM, which detects live cells that have intracellular esterase activity to convert cell permeable, non-fluorescent calcein-AM into fluorescent calcein. Increased permeability of plasma membrane is a consequence of secondary necrosis in cultured cells and occurs late.

As shown in Fig. 6, 80% of cells transfected with mtTrx were still alive compared with 36% in non-transfected cells and 8% in empty vector-transfected cells after 14 h of exposure. To determine whether overexpression of mtTrx increased metabolism of tBH, we measured the concentrations of tBH in the medium as a function of time. As shown in Fig. 7, the kinetics of tBH degradation were comparable between vector control and mtTrx-overexpressing cells. Thus, mtTrx protects against oxidative damage caused by the oxidant rather than by enhancing the metabolism and elimination of the peroxide.

DISCUSSION

Trx exists in more than one form in diverse species and shows compartmentalized distribution in higher organisms (4–10, 23). Overexpression of the cytoplasmic Trx has been found to protect against oxidative stress. This information along with the conserved expression of mtTrx (5, 6, 9, 10) indicate that mtTrx may have similarly important biological functions in protection against oxidative stress in the mitochondria.

The RACE analysis indicates that there are two mRNA sequences that are different in the length of the 3′-untranslated region. The shorter one is in good agreement with the recently updated mtTrx sequence (GenBank™ accession no.
**Human Mitochondrial Thioredoxin**

33247

**A**

143B  Vector  MtTrx

- tBH  - tBH  - tBH

FL-3 (Ethidium Homodimer)  FL-1 (Calcein)

**B.**

Viability (%)

ctrl  200 uM  300 uM

143B  Vector  MtTrx

**FIG. 6.** Overexpression of mtTrx in 143B cells protected against tBH-induced cytotoxicity. A, flow cytometry analysis of cell viability with double staining of calcein (x-axis) and ethidium homodimer (y-axis). Cells in the lower right quadrant are viable. Non-viable cells had decreased calcein staining and increased ethidium homodimer staining. B, quantitation of flow cytometry data. Although 200 µM tBH did not cause significant cytotoxicity, 300 µM tBH caused dramatic cell death, which was protected by overexpression of mtTrx. Duplicate samples were used for each condition. The data presented are the averages of four separate experiments (mean ± S.E.). Significant differences from control, determined by one way analysis of variance and Dunnett’s multiple comparison test are indicated as **, p < 0.01.

NM-056182), except that it lacks the last 12 nucleotides. The longer one has an additional 330 bp 3' downstream of the previous sequence and contains the consensus polyadenylation signal “AAUAAA” and “CA” at the very 3' end. No such signal exists in the shorter cDNA fragments, raising a question concerning which is the correct in vivo transcript. One possibility is that both transcripts could exist and that an alternative polyadenylation signal is used for the short cDNA sequence. Northern blot analysis showed that two bands were present in some tissues and that the molecular weights were close to those of the two cDNA fragments achieved from RACE, 1.34 and 1.01 kb respectively. Taken together, these data suggest that the two mRNA species are both likely to exist in vivo.

Tissue distribution studies show a very generalized distribution in all tissues with a high expression in heart, skeletal muscle, kidney, and liver. These results are consistent with Northern blot analyses of mtTrx distribution in rat (10) in which high expression occurs in tissues with high energy demand. Because of the association of ROS production with mitochondrial respiration, increased expression of mtTrx could function along with GSH-dependent mechanisms to protect against ROS, regulate the protein redox state, and perhaps control the cellular responses to apoptosis.

In summary, the present study demonstrates that the nuclear-encoded human mtTrx is expressed as two mRNA species that differ in length of the 3'-non-coding region. mRNA expression was greater in heart, skeletal muscle, kidney, and liver than in other major tissues. An epitope-tagged mtTrx protein was targeted to mitochondria, and stable overexpression of this protein protected against oxidant-induced apoptosis. These results demonstrate that in addition to the well studied superoxide dismutase and GSH-dependent protein systems, human mitochondria contain a functional Trx-dependent system for protection against oxidative damage and oxidant-induced apoptosis.

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