Cloning, sequencing and functional expression of a novel human thioredoxin reductase

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Abstract The DNA sequence encoding a novel human thioredoxin reductase has been determined. The protein is predicted to have 524 amino acids including a conserved -Cys-Val-Asn-Val-Gly-Cys catalytic site and a selenocysteine containing C-terminal -Gly-Cys-SeCys-Gly. The predicted molecular mass is 56.5. The newly identified TR sequence exhibits 54% identity to a previously reported human thioredoxin reductase and 37% identity to human glutathione reductase. Transient transfection of human embryonal kidney cells results in a 5-fold increase in thioredoxin reductase activity but no increase in glutathione reductase activity.

Key words: Thioredoxin reductase; Human

1. Introduction

Thioredoxin reductase (TR) is a dimeric NADPH-dependent FAD containing enzyme that catalyzes the reduction of the active site disulfide of thioredoxin and other substrates [1]. TR is a member of a larger family of pyridine nucleotide-disulfide oxidoreductases, which includes lipoamide dehydrogenase, trypanothione reductase and glutathione reductase [2]. Thioredoxin is a low molecular weight (10–12 kDa) redox protein found in both prokaryotic and eukaryotic cells [1]. The cysteine residues at the conserved -Cys-Gly-Pro-Cys-Lys active site of thioredoxin undergo reversible oxidation-reduction catalyzed by TR [3]. Thioredoxin is a reducing cofactor for ribonucleotide reductase, which catalyzes the first unique step in DNA synthesis [4]. Through thiol-disulfide exchange, TR exerts redox control over transcription factors NF-κB [5] and glucocorticoid receptor [6], and indirectly through a nuclear redox factor Ref-1/HAPE, AP-1 (Fos/Jun heterodimer) [7] to modulate their binding to DNA. A number of human primary cancers such as lung, colon, cervical and liver cancers have been shown to over-express thioredoxin [8–11]. Transfection of cancer cells with thioredoxin can increase tumor growth and inhibits anti-cancer drug-induced apoptosis [12]. Redox activity is necessary for this effect as shown by the potentiation of apoptosis and a dominant-negative inhibition of tumor growth by transfection with a redox inactive mutant of thioredoxin ([13] and unpublished observations). Thus, the thioredoxin redox system plays an important role in cell growth and death.

Bacterial TR is highly specific for thioredoxin as a substrate [1]. In contrast, mammalian TR catalyzes the reduction of a variety of substrates including low molecular weight disulfides such as dithionitrobenzoate (DTNB) [3] and lipoic acid [14], and non-disulfides including selenite [15], alloxan [16] and lipid hydroperoxides [17]. Rat liver TR has also been reported to reduce dehydroascorbic acid to ascorbic acid and may be required for the maintenance of liver ascorbate content [18].

Mammalian TRs have been purified and have estimated subunit molecular weights of 58 kDa for the bovine enzyme [3], 58 kDa for the rat liver enzyme [3], and 65 kDa for the human placental enzyme [19]. The sequence of a human TR cDNA from a placental library predicts a protein with a mass of 54 kDa and high similarity (44% identity) to glutathione reductases, with a conserved -Cys-Val-Asn-Val-Gly-Cys- redox active site [20]. Subsequent work identified two human proteins with TR activity, one with a subunit molecular weight of 55 kDa from Jurkat T cells [21] and the other 57 kDa from lung adenocarcinoma cells [22]. The carboxy-terminal peptide sequences were identical to that predicted for human placental TR but contained a terminal selenocysteine (SeCys) residue, giving -Gly-Cys-SeCy^{496}-Gly^{497} instead of the predicted terminal Gly-Cys^{495}. This finding indicated that the human TR mRNA TGA encodes SeCys rather than acting as a stop codon [23]. Previous studies have shown that specific stem loop structures, termed SECIS (SeCys incorporation sequence) elements present in the 3′ untranslated regions of mRNAs are required to signal the cell to insert a SeCys at a TGA codon rather than terminating protein synthesis [24,25]. A putative SECIS element was identified between base pairs 2181 and 2226 of human TR mRNA [26]. The carboxy-terminal SeCys is required for the thioredoxin reductase activity of the enzyme. Expression in baculovirus of recombinant human TR lacking SeCys, either as a truncated protein or with Cys instead of SeCys, results in abolition of enzyme activity with thioredoxin as a substrate but the enzyme still retains some DTNB reducing activity [26]. Enzymatic cleavage of the carboxy-terminus SeCys of bovine TR with carboxypeptidase also results in a loss of enzyme activity [27].

Since the cloning of human placental TR [20], a rat neuroblastoma derived TR cDNA has been cloned [27] and much of the bovine liver TR protein sequenced, both showing high homology to the human placental TR [27]. Here, we report the cloning of a novel human TR with high homology to the previously cloned human TR. A putative SECIS element exhibiting all the conserved features required for function is present in the 3′ untranslated region of the sequence. We also report functional expression of this novel TR in human cells and analysis of expression in a number of human tissues and cancer cell lines.

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2. Materials and methods

2.1. Cloning

A λgt11 human placenta 5′ stretch cDNA library (Clontech, Palo Alto, CA) was screened using the *Bgl*II-*Sma*I fragment of human TR (bp 171–723) which contains the coding sequence for the -Cys-Val-Asn-Val-Gly-Cys- active site [20] as a probe. The *Bgl*II-*Sma*I fragment was labeled with [α-^{32}P]dCTP using RadPrime (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Approximately 10^5 plaques were hybridized in 0.5 M sodium phosphate, pH 7.2, 10 mM EDTA, 7% sodium dodecyl sulfate and 1% bovine serum albumin at 50°C for 72 h. The filters were washed with 2×SSC (0.3 M sodium chloride, 0.33 M sodium citrate), 0.05% sodium dodecyl sulfate at 50°C. The cDNA inserts of six positive plaques were screened using the polymerase chain reaction (PCR) with the cDNA insert screening amplimers for λgt11 (Clontech, Palo Alto, CA). 5′ insert screening amplimer: 5′-GAC TCC TGG AGC CCG-3′, 3′ insert screening amplimer: 3′-CGC GGC CAG CGA TGG-5′. PCR was performed with 25 cycles of 94°C for 1 min, 60°C for 1 min, 27°C for 1 min, and 1 cycle of 72°C for 10 min. The PCR fragments were separated on a 1% agarose gel and gel purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA). The purified fragments were digested with *Eco*RI and cloned into pBluescript, digested with *Eco*RI (Stratagene, La Jolla, CA) and the cDNA inserts sequenced on an Applied Biosystems 377 DNA fluorescent automated DNA sequencing system. cDNA clone number 5 which was determined to be 71% identical to TR was used to rescreen the human placenta cDNA library obtaining eight positive plaques. One cDNA (clone 30) was found upon sequencing to be similar but not identical to TR with a long open reading frame and missing both the 5′ and 3′ ends. Rescreening the placenta cDNA library with [α-^{32}P]dCTP-labeled cDNA 30 as a probe yielded no additional cDNA sequences. Next, a human heart cDNA library in lambda ZAP II (Stratagene, La Jolla, CA) was screened with cDNA 30 giving four positives plaques. The cDNA inserts were excised using the Exassist/SOLR in vivo excision protocol according to the manufacturer's instructions. Two clones, H15 and H19, contained additional cDNA sequence, but not the complete coding region or the polyA tail.

To obtain the 5′ and 3′ ends of cDNA 30, a human fetal heart cDNA library (Gibco BRL, Gaithersburg, MD) with average insert size of 2.2 kb was screened. cDNA clones were isolated using the Gene Trapper cDNA Positive Selection System (Gibco BRL, Gaithersburg, MD). Double stranded DNA was prepared from the human fetal heart cDNA library according to the manufacturer's instructions. Oligonucleotide F167-2 (GCC ACC GTG TCC AGC TTC AG) was polyacrylamide gel purified (Bio-synthesis, Lewisville, TX), phenol:chloroform:isoamyl alcohol (25:24:1) extracted, precipitated with 7.5 M ammonium acetate, resuspended in 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA and biotinylated using biotin-14-dCTP according to the manufacturer's instructions. Biotinylation reaction products were analyzed on a 16% polyacrylamide gel. The library DNA was made single stranded using Gene II and Exo III according to the manufacturer's instructions. The products were analyzed on a 0.8% agarose gel in 40 mM Tris-acetate, pH 7.2, 10 mM EDTA. Biotinylated F167-2 was hybridized with the single stranded DNA and this mixture was incubated with streptavidin paramagnetic beads. The captured cDNA was repaired to double stranded using non-biotinylated oligonucleotide F167-1 (GTG CCG CAT GAC TGG AGG AAG). The repaired cDNA was ethanol precipitated and Electromax DH10B cells (Gibco BRL, Gaithersburg, MD) were transformed with 2 μl of the cDNA by electroporation using the Cell-Porator system (Gibco BRL, Gaithersburg, MD). The conditions for electroporation were: 23 μl of cells, 0.15 gap chamber at settings of voltage: 400; capacitance 330 μF; impedance: low ohms; charge rate: fast; and resistance on voltage booster: 4000 Ω. Electroporated cells were plated on LB (Luria-Bertani medium) containing 100 μg/ml ampicillin and incubated overnight at 37°C. Positive cDNA clones were identified by colony PCR. Twenty-four colonies were screened. A master mix containing 22.5 μl of PCR Supermix (Gibco BRL, Gaithersburg, MD), 0.5 μg oligonucleotide F167-1, 0.5 μg oligonucleotide D905-4 (ATG TGA TCG GCT GAC AGC AG) and distilled water to 25 μl per reaction was made. One half of each colony was added to 25 μl of the master mix and amplified by PCR using the following program: 1 cycle, 94°C 1 min; 30 cycles of: 94°C, 52 s, 55°C, 1 min 9 s, 72°C, 1 min 17 s. Ten microliters of each reaction were analyzed on a 1% agarose gel. One cDNA product out of the 24 had the predicted size of 190 bp. The other half of this colony was grown at 37°C in LB with 100 μg/ml ampicillin overnight. DNA was prepared using Quantum Prep (Bio-Ras, Hercules, CA) and the construct digested with *Not*I and *Sal*I. The digest was analyzed on a 1% agarose gel in 40 mM Tris-acetate, pH 7.2, 10 mM EDTA and the cDNA insert was determined to be approximately 2.2 kb. The insert was sequenced and found to contain the complete coding sequence and additional sequences at the 5′ and 3′ ends of the cDNA 30. Sequence information was compiled and analyzed using the algorithms available through GCG (Genetics Computer Group Inc., Madison, WI). DNA and protein data bases were searched using the computer programs FASTA and BLASTP performed at the NCBI (National center for Biotechnology Information) using the BLAST network service (GCG). Gapped sequence alignments and identity/similarity comparisons were made using the PILEUP computer program (GCG).

2.2. Transfection

Transformed primary human 293 embryonal kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 nM sodium selenite for 4 days prior to transfection. Cells were seeded at approximately 50% confluence in 33 mm dishes and 24 h later 1 ml DMEM with 2% FBS, 100 nM sodium selenite, 120 μl of LipoTAXI transfection reagent (Stratagene, La Jolla, CA) and 40 μg cDNA of either TR-α/pCMV-Sport2 or TR-β/pCMX, together with 20 μg *Xenopus* SeCys-tRNA/pGEM3 [28] were added to each dish. The cells were incubated for 24 h at 37°C, the medium removed and replaced with DMEM with 10% FBS and 100 nM sodium selenite. The cells were incubated at 37°C for 24 h before assaying for TR and glutathione reductase activities.

2.3. Subcellular fractionation

MCF-7 human breast cancer cells were grown to 80% confluence in roller culture bottles. The cells were washed twice with phosphate buffered saline and 5×10^8 cells were scraped free and homogenized using 60 strokes of a tightly fitting Dounce homogenizer in 30 ml homogenization buffer consisting of 10 mM Tris buffer, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin at 4°C. The homogenate was centrifuged at $650\times g$ for 20 min and the resulting pellet re-homogenized in 2.4 M sucrose, 3.3 mM $CaCl_2$ and centrifuged at $650\times g$ for 30 min. The upper third layer was diluted 1:10 with 3.3 mM $CaCl_2$, layered over 1 M sucrose and centrifuged at $900\times g$ for 30 min to give a pellet which was resuspended, washed by twice by centrifugation in homogenization buffer and finally suspended in 15 ml homogenization buffer as the nuclear fraction. The supernatant resulting from the first homogenization step was centrifuged at $110\,000\times g$ for 60 min to give a pellet which was resuspended and washed by centrifugation, twice, in homogenization buffer and finally suspended in 15 ml homogenization buffer as the microsomal fraction. The post-microsomal supernatant was again centrifuged at $110\,00\times g$ to give the cytosol fraction. All fractions were stored frozen at −80°C

2.4. Enzyme assays

Cells were lysed by homogenizing in 50 mM Tris buffer, pH 7.3, 1 mM EDTA. TR activity [29] and glutathione reductase activity [30] in the total cell lysate were measured spectrophotometrically at 339 nm by the initial rate of oxidation of NADPH as previously described.

2.5. Northern blotting

To determine the distribution of TR-β cDNA in human tissues a full length probe was labeled with [α-^{32}P]dCTP and used to hybridize with Human Multiple Tissue Northern (MTN) Blot and Human Multiple Tissue Northern Blot IV (Clontech, Palo Alto, CA). The hybridization buffer was 0.5 M sodium phosphate, pH 7.2, 10 mM EDTA, 7% sodium dodecyl sulfate and 1% bovine serum albumin and hybridization was performed at 65°C for 18 h. The blots were washed with 0.04 M sodium phosphate pH 7.2, 1% sodium dodecyl sulfate, 1 mM EDTA at 65°C for 20 min. Blots were quantitated by densitometry using an Eagle Eye II (Stratagene, La Jolla, CA).

2.6. Western blotting

The distribution of TR-β protein was determined in a panel of

human tumor cell lines. The cell lines were obtained from the American Tissue Type Collection (Rockville, MD) or the National Cancer Institute (Bethesda, MD). The cells were grown and homogenized as previously described. Rabbit polyclonal antisera were raised against two unique internal peptide sequences of the TR-β (30-1, IKASFVDEHTVCGVAKG, amino acids 157–153; and 30-2, ARHGQEHVEVYHAHYK, amino acids 417–432) coupled to the multiple antigen peptide (MAP) conjugation system (Bio-Synthesis Inc., Lewisville, TX). Rabbits are injected at several sites with 1 mg of the AP-peptide in 0.5 ml TiterMax Gold adjuvant (CytRx Corp., Norcross, GA) and antisera collected 1 month later. Western blotting was conducted as previously described [9] with 40 μg total cell lysate per lane using antiserum 30-2, which showed no cross-reactivity with peptide 30-1 or with purified human placental TR, at a dilution of 1:4000. Detection was by the Renaissance chemiluminescence system (NEN Life Science Products, Boston, MA).

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(1)    attccccacaccctatcccagtgttccaccctaggtctgaaggcccccgccccgaatccg  (60)
(61)   gccgcattcggccccggtctagccagcgctctcacctctcccgcgacggcccgcccggga (120)
(121)  ctggacccgccccggtccggcgcaggcagcgcggcgggcagccctagctgccccagaagc (180)
(181)  cccacgacgatggcggcaatggcggtggcgctgcggggattaggagggcgcttccggtgg (240)
                M  A  A  M  A  V  A  L  R  G  L  G  G  R  F  R  W
(241)  cggacgcaggccgtggcgggcggggtgcggggcgcggcgcggggcgcagcagcaggtcag (300)
        R  T  Q  A  V  A  G  G  V  R  G  A  A  R  G  A  A  A  G  Q
(301)  cgggactatgatctcctggtggtcggcgggggatctggtggcctggcttgtgccaaggag (360)
        R  D  Y  D  L  L  V  V  G  G  G  S  G  G  L  A  C  A  K  E
(361)  gccgctcagctgggaaggaaggtgtccgtggtggactacgtggaaccttctccccaaggc (420)
        A  A  Q  L  G  R  K  V  S  V  V  D  Y  V  E  P  S  P  Q  G
(421)  acccggtggggccttggcggcacctgcgtcaacgtgggctgcatccccaagaagctgatg (480)
        T  R  W  G  L  G  G  T  C  V  N  V  G  C  I  P  K  K  L  M
(481)  caccaggcggcactgctgggaggcctgatccaagatgcccccaactatggctgggaggtg (540)
        H  Q  A  A  L  L  G  G  L  I  Q  D  A  P  N  Y  G  W  E  V
(541)  gcccagcccgtgccgcatgactggaggaagatggcagaagctgttcaaaatcacgtgaaa (600)
        A  Q  P  V  P  H  D  W  R  K  M  A  E  A  V  Q  N  H  V  K
(601)  tccttgaactggggccaccgtgtccagcttcaggacagaaaagtcaagtactttaacatc (660)
        S  L  N  W  G  H  R  V  Q  L  Q  D  R  K  V  K  Y  F  N  I
(661)  aaagccagctttgttgacgagcacacggtttgcggcgttgccaaaggtgggaaagagatt (720)
        K  A  S  F  V  D  E  H  T  V  C  G  V  A  K  G  G  K  E  I
(721)  ctgctgtcagccgatcacatcatcattgctactggagggcggccgagataccccacgcac (780)
        L  L  S  A  D  H  I  I  I  A  T  G  G  R  P  R  Y  P  T  H
(781)  atcgaaggtgccttggaatatggaatcacaagtgatgacatcttctggctgaaggaatcc (840)
        I  E  G  A  L  E  Y  G  I  T  S  D  D  I  F  W  L  K  E  S
(841)  cctggaaaaacgttggtggtcggggccagctatgtggccctggagtgtgctggcttcctc (900)
        P  G  K  T  L  V  V  G  A  S  Y  V  A  L  E  C  A  G  F  L
(901)  accgggattgggctggacaccaccatcatgatgcgcagcatccccctccgcggcttcgac (960)
        T  G  I  G  L  D  T  T  I  M  M  R  S  I  P  L  R  G  F  D
(961)  cagcaaatgtcctccatggtcatagagcacatggcatctcatggcacccggttcctgagg (1020)
        Q  Q  M  S  S  M  V  I  E  H  M  A  S  H  G  T  R  F  L  R
(1021) ggctgtgccccctcgcgggtcaggaggctccctgatggccagctgcaggtcacctgggag (1080)
        G  C  A  P  S  R  V  R  R  L  P  D  G  Q  L  Q  V  T  W  E
(1081) gacagcaccaccggcaaggaggacacgggcacctttgacaccgtcctgtgggccataggt (1140)
        D  S  T  T  G  K  E  D  T  G  T  F  D  T  V  L  W  A  I  G
(1141) cgagtcccagacaccagaagtctgaatttggagaaggctggggtagatactagccccgac (1200)
        R  V  P  D  T  R  S  L  N  L  E  K  A  G  V  D  T  S  P  D
(1201) actcagaagatcctggtggactcccgggaagccacctctgtgccccacatctacgccatt (1260)
        T  Q  K  I  L  V  D  S  R  E  A  T  S  V  P  H  I  Y  A  I
(1261) ggtgacgtggtggaggggcggcctgagctgacacccatagcgatcatggccgggaggctc (1320)
        G  D  V  V  E  G  R  P  E  L  T  P  I  A  I  M  A  G  R  L
(1321) ctggtgcagcggctcttcggcgggtcctcagatctgatggactacgacaatgttcccacg (1380)
        L  V  Q  R  L  F  G  G  S  S  D  L  M  D  Y  D  N  V  P  T
(1380) accgtcttcaccccgctggagtatggctgtgtggggctgtccgaggaggaggcagtggct (1440)
        T  V  F  T  P  L  E  Y  G  C  V  G  L  S  E  E  E  A  V  A
(1441) cgccacgggcaggagcatgttgaggtctatcacgcccattataaaccactggagttcacg (1500)
        R  H  G  Q  E  H  V  E  V  Y  H  A  H  Y  K  P  L  E  F  T
(1501) gtggctggacgagatgcatcccagtgttatgtaaagatggtgtgcctgagggagccccca (1560)
        V  A  G  R  D  A  S  Q  C  Y  V  K  M  V  C  L  R  E  P  P
(1561) cagctggtgctgggcctgcatttccttggccccaacgcaggcgaagttactcaaggattt (1620)
        Q  L  V  L  G  L  H  F  L  G  P  N  A  G  E  V  T  Q  G  F
(1621) gctctggggatcaagtgtggggcttcctatgcgcaggtgatgcggaccgtgggtatccat (1680)
        A  L  G  I  K  C  G  A  S  Y  A  Q  V  M  R  T  V  G  I  H
(1681) cccacatgctctgaggaggtagtcaagctgcgcatctccaagcgctcaggcctggacccc (1740)
        P  T  C  S  E  E  V  V  K  L  R  I  S  K  R  S  G  L  D  P
(1741) acggtgacaggctgctgagggtaagcgccatccctgcaggccagggcacacggtgcgccc (1800)
        T  V  T  G  C  C* G
(1801) gccgccagctcctcggaggccagacccaggatggctgcaggccaggtttgggggggcctca (1860)
(1861) accctctcctggagcgcctgtgagatggtcagcgtggagcgcaagtgctggacaggtggc (1920)
(1921) ccgtgtgccccacagggatggctcaggggactgtccacctcacccctgcacctctcagcc (1980)
(1981) tctgccgccgggcacccccccccaggctcctggtgccagatgatgacgacctgggtggaa (2040)
(2041) acctaccctgtgggcacccatgtccgagccccctggcatttctgcaatgcaaataaagag (2100)
(2101) ggtacttttttctgataaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa (2160)
(2161) aaaaaaaaaaaaaaaaaaaa                                          (2180)
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of human TR-β. The box shows the conserved active site. The penultimate predicted amino acid residue is selenocysteine designated C*.

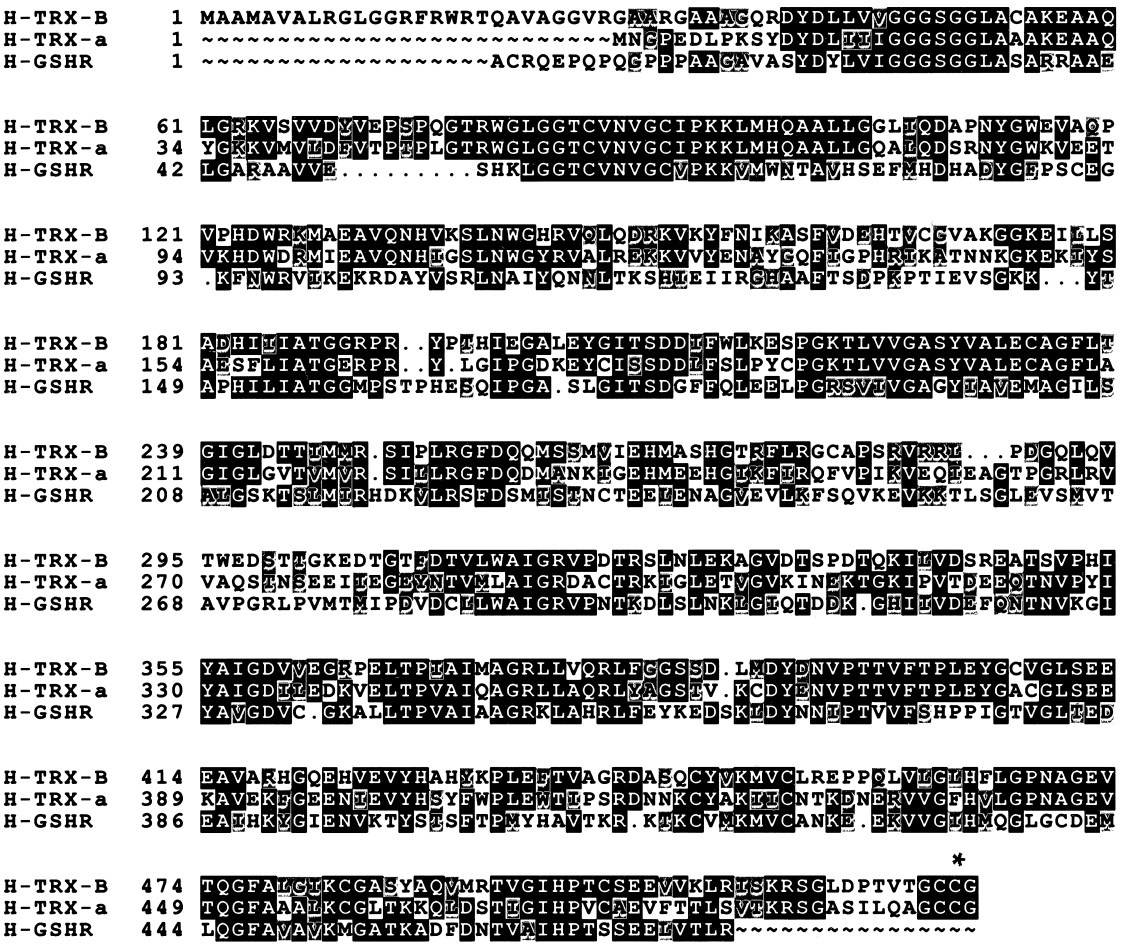

Fig. 2. Alignment of the predicted amino acid sequence of TR-β (H-TRX-β) with human TR-α (H-TRX-α) and human glutathione reductase (H-GSHR). Identical amino acid residues are shown in black boxes. The penultimate SeCys amino acid of both TR-β and TR-α is designated by C*.

3. Results

3.1. Cloning and sequence analysis

Fig. 1 shows the consensus sequence for the new human TR, designated here TR-β to distinguish it from the reported human TR designated TR-α. The predicted amino acid sequence of TR-β has two potential ATG start codons in close proximity. The longest open reading frame gives a protein with 524 amino acids and a molecular mass 56 540. TR-β has the conserved -Cys-Val-Asn-Val-Gly-Cys active site of TR-α and a selenocysteine (SeCys) residue in the carboxy-terminal -Gly-Cys-SeCys-Gly. The predicted amino acid sequence of TR-β showed highest identity to a rat TR (79%) (GenBank accession number AF0722865) and to human TR-α (54%) [20] (Fig. 2). TR-β exhibits 37% identity to human glutathione reductase. TR-β exhibits an N-terminal extension with a high content of positively charged amino acids compared to TR-α. The motif search program Prosite (GCG) did not identify a nuclear or mitochondrial localization sequence in TR-β.

A sequence search of the 3′ untranslated region for the conserved motifs present in SECIS elements, followed by computer and manual folding of the candidate regions, revealed a single region satisfying all of the consensus SECIS sequence and structure requirements [31–33]. Comparison of

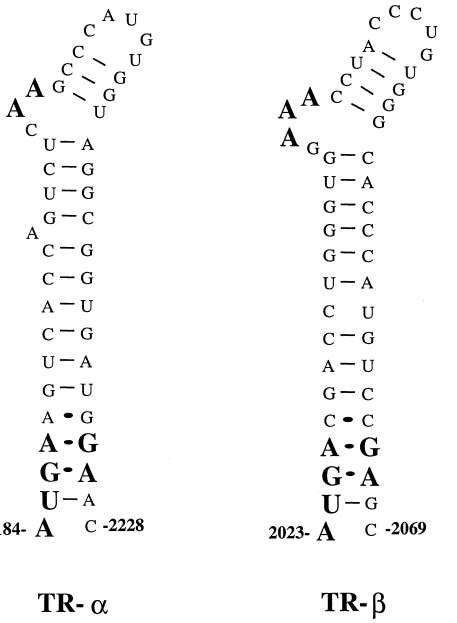

Fig. 3. Predicted TR-α and TR-β SECIS structures. Conserved nucleotides present in all SECIS elements are shown in bold.

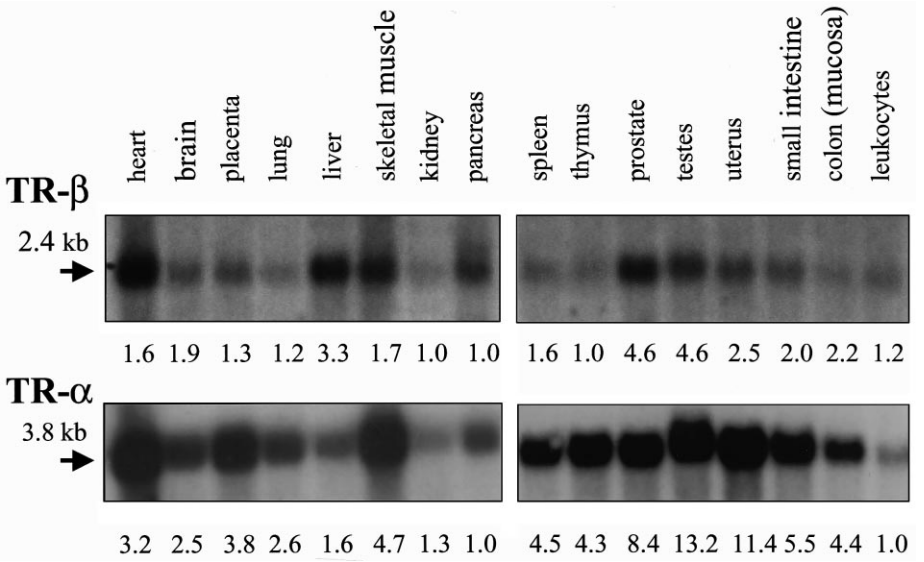

Fig. 4. Northern hybridization analysis of TR-β mRNA in human tissues. Also shown for comparison is TR-α mRNA. The values underneath are relative levels corrected for expression of histone mRNA (not shown).

the putative TR-β element with that of TR-α reveals little primary sequence conservation aside from the invariant nucleotides shown in bold, but the predicted structures are quite similar, though not identical (Fig. 3). The relative positions of the two SECIS elements in the 3′ untranslated regions are also quite similar: 253 and 272 nucleotides downstream of the UGA codon in TR-α and TR-β, respectively.

3.2. Northern hybridization

The relative expression patterns of TR-β mRNA and TR-α mRNA in human tissues were compared by Northern analysis (Fig. 4). The highest levels of TR-β mRNA were found in prostate, testis, liver, uterus and small intestine, with intermediate levels in brain, skeletal muscle, heart and spleen, and low levels in placenta, kidney, pancreas, thymus and peripheral blood leukocytes. This pattern of expression is distinct from that exhibited by TR-α.

3.3. Western blotting analysis

TR-β protein expression patterns were assessed in MCF-7 cell subcellular fractions by Western analysis with a polyclonal antiserum made against a synthetic TR-β peptide (Fig. 5).

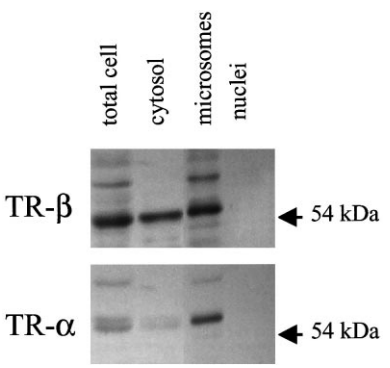

Fig. 5. TR-α and TR-β levels in different subcellular fractions of MCF-7 human breast cancer cells measured by Western blotting. The arrow shows the size of the protein.

A predominant band of approximately 54 kDa was found in the cytosol and associated with the microsomal fraction. A fainter band at approximately 58 kDa was also seen. In contrast, TR-α protein was predominantly associated with the microsomal fraction. Neither TR-α or TR-β are integral membrane proteins and their association with the microsomal fraction could be reversed by washing in 2 M NaCl (results not shown). Relative protein levels for TR-β were assessed in a panel of human tumor cell lines (Fig. 6). A protein band of 54 kDa was found at high levels in HT-29 colon cancer, MCF-7 breast cancer and Jurkat T cell leukemia lines. Weaker 56 kDa and 58 kDa protein bands were also apparent in some of the cell lines.

3.4. Expression of thioredoxin reductase activity

Transfection of human 293 kidney cells with TR-β cDNA resulted in a 5.5-fold increase in thioredoxin reductase activity of the cells compared to a 4.5-fold increase using TR-α cDNA (Fig. 7). Interestingly the background activity of reduction of insulin in the absence of thioredoxin was also increased about 4-fold by TR-β cDNA, but not by TR-α cDNA, suggesting that TR-β can directly reduce proteins such as insulin. The inclusion of 100 nM selenium in the growth medium increased the expression of TR activity from TR-β (results not shown). The TR-β cDNA transfected cells showed no increase in glutathione reductase activity with a value ($\pm$S.E.M., $n=4$) of 6.7 ± 1.5 nmol/min/mg protein in the TR-β transfected cells, compared to 8.5 ± 0.5 nmol/min/mg in mock-transfected cells and 8.1 ± 0.6 nmol/min/mg in the *Xenopus* SeCys-tRNA transfected cells ($P < 0.05$ in both cases).

4. Discussion

The possibility of multiple forms of human TR has been raised by the identification of two distinct proteins of 55 and 57 kDa exhibiting TR activity and penultimate carboxy-terminal SeCys in human tumor cells [21,22]. The 57 kDa form of TR from HeLa and human lung adenocarcinoma cells has been reported to further consist of two forms, a heparin bind-

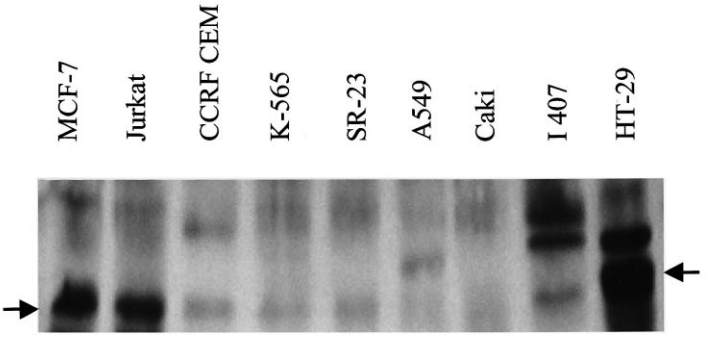

Fig. 6. TR-β levels in different human cancer cell lines measured by Western blotting. 40 μg of total cell lysate protein was applied to each lane. The cell lines used were MCF-7 breast, Jurkat T cell leukemia, CCRF CEM acute lymphoblastic T cell leukemia, K-565 leukemia, SR-23 leukemia, A549 lung, Caki renal, I 407 embryonic intestine. The arrow on each side shows the position of the protein band at 54 kDa determined by molecular weight markers (not shown).

ing and a non-heparin binding form with only the non-heparin binding form showing cross-reactivity with anti-rat liver polyclonal antibody [34]. This was suggested to be due to a post-translational modification of the TR protein although mammalian TR is not a glycoprotein. Two distinct rat TRs, exhibiting 82% amino acid sequence identity have been reported ([27] and GenBank accession number AF072865).

We report the identification and initial characterization of a novel human TR, differing in molecular mass and exhibiting a distinct pattern of human tissue expression from that previously described for TR-α. The predicted mass of TR-β of 56.5 kDa agrees well with the apparent mass of 54 kDa measured by SDS polyacrylamide gel electrophoresis and Western blotting. TR-β has 54% identity to the previously described TR-α with the same catalytic site but an N-terminal extension.

We predict TR-β to be a selenoprotein with a carboxy-terminal -Gly-Cys-SeCys-Gly, rather than ending in -Gly-Cys, as would be predicted by TGA functioning as a stop codon. Several pieces of evidence support this. First, the predicted SeCys-containing carboxy-terminus of TR-β is homologous with that of TR-α, a known SeCys containing protein [22]. Second, the 3′ untranslated region contains a consensus SECIS element. Third, co-transfection of 293 cells with *Xenopus* SeCys-tRNA cDNA, encoding the tRNA which delivers SeCys to the growing polypeptide [35], markedly increases the expression of TR activity. Fourth, supplementation of the growth medium with selenium further increases the expression of TR activity from TR-β, as reported for TR-α [36].

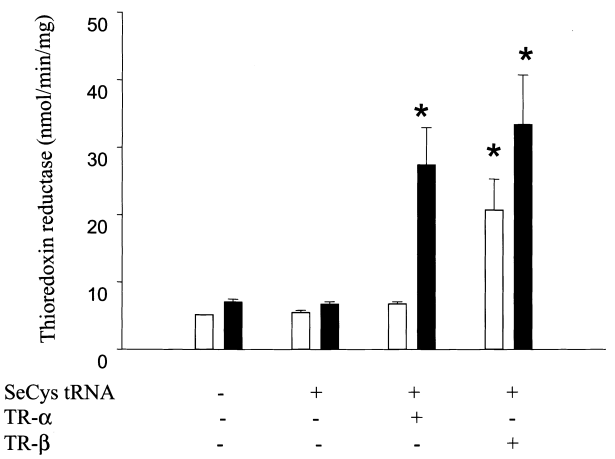

Fig. 7. Thioredoxin reductase activity of human 293 embryonal kidney cells transfected with TR-α and TR-β together with *Xenopus* SeCys-tRNA, measured 48 h after transfection. Cells were grown in medium with 2% serum for the first 24 h then 10% serum for the final 24 h, together with 0.1 μM sodium selenite. The open bars show activity in the absence of added human thioredoxin and the closed bars activity in the presence of 2 μM human thioredoxin. Values are the means of four determinations and bars are S.E.M. $^{*}P < 0.05$ compared to the appropriate non-transfected value.

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