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# Identification and Characterization of a New Mammalian Glutaredoxin (Thioltransferase), Grx2

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# Identification and Characterization of a New Mammalian Glutaredoxin (Thioltransferase), Grx2

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**Abstract:** A thiol/disulfide oxidoreductase component of the GSH system, glutaredoxin (Grx), is involved in the reduction of GSH-based mixed disulfides and participates in a variety of cellular redox pathways. A single cytosolic Grx (Grx1) was previously described in mammals. We now report identification and characterization of a second mammalian Grx, designated Grx2. Grx2 exhibited 36% identity with Grx1 and had a disulfide active center containing the Cys-Ser-Tyr-Cys motif. Grx2 was encoded in the genomes of mammals and birds and expressed in a variety of cell types. The gene for human Grx2 consisted of four exons and three introns, spanned 10 kilobase pairs, and localized to chromosome 1q31.2–31.3. The coding sequence was present in all exons, with the first exon encoding a mitochondrial signal peptide. The mitochondrial leader sequence was also present in mouse and rat Grx2 sequences and was shown to direct either Grx2 or green fluorescent protein to mitochondria. Alternative splicing forms of mammalian Grx2 mRNAs were identified that differed in sequences upstream of exon 2. To functionally characterize the new protein, human and mouse Grx2 proteins were expressed in *Escherichia coli*, and the purified proteins were shown to reduce mixed disulfides formed between GSH and *S*-sulfocysteine, hydroxyethyldisulfide, or cystine. Grx1 and Grx2 were sensitive to inactivation by iodoacetamide and $H_2O_2$ and exhibited similar pH dependence of catalytic activity. However, $H_2O_2$-inactivated Grx2 could only be reactivated with 5 mM GSH, whereas Grx1 could also be reactivated with dithiothreitol or thioredoxin/thioredoxin reductase. The Grx2 structural model suggested a common reaction mechanism for this class of proteins. The data provide the first example of a mitochondrial Grx and also indicate the occurrence of a second functional Grx in mammals.

**Abbreviations:** The abbreviations used are: Trx, thioredoxin; Grx, glutaredoxin; EST, expressed sequence tag; GFP, green fluorescent protein; HEDS, hydroxyethyldisulfide; DTT, dithiothreitol.

## INTRODUCTION

In animal cells, GSH and thioredoxin (Trx) systems are thought to be two major redox systems. They participate in a variety of cellular functions, such as providing reducing equivalents for ribonucleotide reductase, antioxidant defense, control of cellular redox state, and the redox control of transcription and signal transduction (1–4). Trx and GSH systems exhibit partially overlapping functions but also display unique properties (2, 4).

The Trx system, consisting of Trx, Trx reductase, and Trx peroxidase (5, 6), was found in both cytosolic and mitochondrial fractions (7–9), and additional homologs of these proteins were also detected (6, 10, 11). The proteins of mitochondrial and cytosolic Trx systems were shown to be encoded by distinct genes. The components of the mitochondrial Trx system were identified by the presence of signal peptides and by experiments that directly tested intracellular localization of these proteins.

The GSH system is composed of GSH, GSSG reductase, glutaredoxin (thiol/disulfide oxidoreductase (Grx), also called thioltransferase), and GSH peroxidase (1, 3). The composition of Trx and GSH systems is similar in that Trx and Grx are thiol/disulfide oxidoreductases characterized by the Trx fold; GSH peroxidase and Trx peroxidase are thiol-dependent peroxidases also containing the Trx fold; and Trx reductase and GSSG reductase are NADPH-dependent FAD-containing homodimeric pyridine nucleotide disulfide oxidoreductases.

In contrast to the Trx system, the GSH system was only found and characterized in the cytosol. Moreover, single GSSG reductase and Grx genes were described in mammals, and only one GSH form exists in mammalian cells. However, GSSG reductase was shown to possess a mitochondrial signal peptide, allowing it to be directed to mitochondria (12). Likewise, GSH was detected in mitochondrial fractions (mitochondrial GSH accounts for ~10% of the cellular GSH pool), and two GSH peroxidases, GPx1 and GPx4, can also be targeted to mitochondria (13, 14). Thus, Grx is the only component of the GSH system that was not found to reside in mitochondria.

Grx is a small, 10–14-kDa GSH-dependent protein (1–4). Its function is to maintain reduced state of cysteines in cellular proteins. Like Trx, Grx contains the N-terminal redox center, the so-called C*XX*C motif (two cysteines separated by two other amino acid residues). The Cys-Pro-Tyr-Cys version of this sequence is typically present in Grx. The two thiol groups in this motif are the source of reducing equivalents for substrate reduction. These residues form reversible disulfide bond during catalysis and were shown to be the enzyme active site. Grx also contains conserved C-terminal sequences involved in GSH binding (15).

It appears that the absence of Grx in mitochondria contrasts with the expected importance of this protein in providing reducing equivalents for DNA synthesis and maintaining reduced status of thiols in proteins in this cellular compartment. In this report, we describe identification and functional characterization of a mitochondrial Grx.

## EXPERIMENTAL PROCEDURES

*Expression Constructs* — Expression constructs were generated using EST clones corresponding to mouse and human Grx2 mRNAs. The mouse Grx2 expression construct was generated by polymerase chain reaction amplification using the mouse Grx2 cDNA as a template and primers containing *Nde*I (5'-GACACGTCATATGGGAAACAGCACATCGTC-3') and *Xho*I (5'-CCACTCGAGATGTCTTTCCTCTTGTTTTTTTTTTAAATAACAC-3') sites. The human Grx2 sequence was amplified by polymerase chain reaction using a plasmid containing the human Grx2 insert as a template and primers containing *Nco*I (5'-ACAGCCATGGAGAGCAATACATCATC-3') and *Xho*I (5'-TAACTCGAGCTGAAATTCTTTCCTCTTAC-3') sites. The amplified products and the pET21d(+) vector (Novagen) were digested with *Nco*I and *Xho*I, and the polymerase chain reaction fragments encompassing the mouse or human Grx2 were ligated into the restricted pET21d(+) using T4 DNA ligase. Once subcloned, the amplified regions were sequenced in their entirety.

*Purification of Recombinant Proteins* — Human and mouse Grx2 were expressed in *Escherichia coli*, and proteins were isolated as follows. Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside in 200 ml of cell culture media. Cells were harvested by centrifugation at $10,000 \times g$ for 10 min and resuspended in 8 ml of ice-cold 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 (buffer A). After sonication, the lysate was centrifuged at $18,000 \times g$ for 20 min. The supernatant was applied onto a 5-ml His-bind column preequilibrated with buffer A. The column was washed with 30 ml of buffer A and, in addition, with 15 ml of 60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. Grx2 was eluted with 10 ml of 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. Human Grx1 was expressed in *E. coli* and purified as described previously (16).

*Constructs with Green Fluorescent Protein (GFP)* — For the N-Grx2-GFP construct, the mouse Grx2 cDNA was amplified with primers Grx2-F1 (5'-CGAGCTCACCATGTCCTGGCGCCG-3') and Grx2-R2 (5'-GTG-GATCCCATGTCTTTCCTCTTGTT-3') and cloned into the *Sac*I/*Bam*HI sites of pEGFP-N2 vector (CLONTECH). The Grx2-GFP construct was obtained by amplification of the Grx2 cDNA with primers Grx2-F2 (5'-CGAGCT-CACCATGGGAAACAGCACAT-3') and Grx2-R2 and cloning the product into the *Sac*I/*Bam*HI sites of pEGFP-N2. The construct encoding the N-terminal sequence of Grx2 (33 amino acids) fused to GFP (the N-GFP construct) was obtained by amplification of the mouse cDNA with primers Grx2-F1 and Grx2-R1 (5'-TGGATCCCGAGCCCGCAGCT-3') and cloning the product into the *Sac*I/*Bam*HI sites of pEGFP-N2. The GFP-N-Grx2 construct was obtained by amplification of the mouse Grx2 cDNA with Grx2-F1 and Grx2-R2C (5'-TGGATCCATGTCTTTCCTCTTGTT-3') and cloning the product into the *Sac*I/*Bam*HI sites of pEGFP-C3 vector (CLONTECH). All plasmids were transformed into *E. coli* strain NovaBlue (Novagen), and the plasmids were isolated using Plasmid Maxi Kit (Qiagen).

*Dual Fluorescence Confocal Microscopy* — NIH-3T3 cells cultured in 60-mm dishes were transfected with appropriate constructs using the calcium phosphate method of transfection. Cells were transfected with 8.8 μg of plasmid as described (17) and incubated for 24 h in a CO2 incubator. A Mito-Tracker (Molecular Probes, Inc., Eugene, OR) was used as a reference marker for mitochondria. The transfected cells were rinsed with serum-free Dulbecco's modified Eagle's medium containing 10 mM HEPES and then incubated for 20 min at room temperature in the same medium containing MitoTracker at a 1:2000 dilution. The cells were washed twice with serum-free Dulbecco's modified Eagle's medium-HEPES and were immediately used for image collection. Double-labeled images of life cells were collected with water immersion lens using a dual excitation/emission and dual channel mode on a Bio-Rad MRC1024ES laser-scanning microscope.

*Grx Activity Assay* — The standard assay for Grx2 activity was the procedure developed by Mieyal *et al.* (18, 19) and modified by Raghavachari and Lou (20). Briefly, the reaction mixture contained 0.2 mM NADPH, 0.5 mM GSH, 0.1 M potassium phosphate buffer (pH 7.4), 0.4 units of GSSG reductase, and an aliquot of purified Grx2 in a total volume of 1 ml. The reaction was carried out at 30 °C following a 5-min preincubation with 2 mM synthetic substrate, hydroxyethyldisulfide (HEDS). The decrease in absorbance of NADPH at 340 nm was monitored for 5 min using Beckman DU 640 Spectrophotometer (Beckman, Fullerton, CA). To determine the Grx2 activity, the slope of the linear portion of the time course for 340-nm absorption loss in a control (Grx2-free) sample was subtracted from the slope of the samples containing Grx2. One unit of Grx2 activity was defined as 1 μmol of NADPH oxidized per min under these standard assay conditions.

*Kinetic Studies and Temperature and pH Dependences of Catalytic Activity* — Temperature (20–45 °C) and pH (6.5–9.0) dependences of Grx2 activities were determined using the standard Grx2 assay. Studies on substrate specificities utilized *S*-sulfocysteine and cystine in place of HEDS. Substrate concentrations were in the range of 0.0125–1.6 mM and assayed with 5 milliunits of Grx2. The apparent $K_m$ and $k_{cat}$ values were calculated using the Lineweaver-Burk plot.

*Effect of Iodoacetamide on Grx2 Activity* — A 0.02-unit aliquot of reduced Grx2 was incubated with 30 μM iodoacetamide in 50 μl of 0.1 M potassium phosphate buffer, pH 7.4, for 30 min prior to the Grx2 assay.

*Effect of $H_2O_2$ on Grx2 Activity* — Purified Grx2 was initially incubated with 5 mM DTT at room temperature for 30 min and filtered through PD-10 column (Amersham Pharmacia Biotech) to remove unreacted DTT. An aliquot (0.02 units) of the resulting reduced Grx2 in 10 mM potassium phosphate buffer, pH 7.4, was preincubated with various concentrations of $H_2O_2$ (0.125–1.5 mM) in a total volume of 6 μl for 5 min at 25 °C. Ten μl of catalase (2 μg) was subsequently added, and the reaction mixture was incubated for additional 5 min at room temperature to degrade the remaining $H_2O_2$. The Grx2 activity was then determined using the standard Grx2 assay. To examine if $H_2O_2$-treated Grx2 could be reactivated by reducing agents, Grx2 (0.2 units) was incubated with 1 mM $H_2O_2$ in 120 μl of 10 mM potassium phosphate buffer, pH 7.4, for 5 min at 25 °C. The reaction was stopped by adding 2 μg of catalase. The sample was then divided into six fractions, and each fraction was mixed with 0.1 mM phosphate buffer, pH 7.4, 5 mM DTT, 0.5 mM GSH, 5 mM GSH, Trx/Trx reductase system, or Trx alone and incubated at 25 °C for 20 min. The final volume of each reactivation reaction was 40 μl. Ten μl of each sample was used for the Grx2 assay. Concentrations of NADPH, *E. coli* Trx, and *E. coli* Trx reductase in the Trx/Trx reductase system were 0.25 mM, 10 μM, and 0.8 μM, respectively. Trx/Trx reductase system without $H_2O_2$-inactivated Grx2 was used as a control. Human recombinant Grx1 was assayed in parallel with Grx2.

*Sequence Analyses* — BLAST programs were used to search for homologs of previously described glutaredoxins in EST and nonredundant data bases at the National Center for Biotechnology Information. These programs were also used to determine the structure of the human Grx2 gene and to identify alternative splicing forms of mammalian Grx2 mRNAs. Multiple sequence alignments were constructed using the ClustalW program.

*Molecular Modeling* — Homology between Grx2 proteins and Grx from other sources was sufficient to develop a molecular model of the core of the Grx2 structure. Mouse Grx2 tertiary structure was modeled using the SWISS-MODEL program (21–23) and the following known three-dimensional structures as templates: the NMR solution structure of human Grx1 in the fully reduced form (1JHB) (24), crystal structure of pig Grx1 (1KTE) (25), and NMR structure of human mutant Grx1 complexed with GSH (1B4QA) (26). The modeling was performed for sequences spanning residues 49–150 of mouse Grx2.

*Other Techniques* — EST clones were obtained from Research Genetics, and their nucleotide sequences were determined at the University of Nebraska–Lincoln DNA sequencing facility. SDS-polyacrylamide gel electrophoresis was carried out using polyacrylamide gels, standards, and other electrophoretic reagents from Novex.

## RESULTS

*Identification and Sequence Analyses of Grx2* — Homology search analyses using known Grx sequences revealed a previously uncharacterized sequence that was represented by several mammalian ESTs. Multiple sequence alignments of human ESTs for this sequence identified a cDNA that contained an open reading frame of 164 amino acid residues. Subsequent homology analyses revealed that this open reading frame was also encoded by mouse, rat, and swine ESTs, suggesting that it was conserved among mammals and birds.

Nucleotide sequences of representative human and mouse ESTs were experimentally verified (Figure 1). The new human open reading frame exhibited 36% identity to human Grx1 and was designated as Grx2. Multiple sequence alignments of human, mouse, and rat Grx2 with known Grx1 proteins (Figure 2*A*) indicated that Grx2 had a conserved disulfide active center, the C*XX*C motif, typical of Grx and Trx proteins. In addition, sequences that were previously demonstrated to be involved in GSH binding were conserved between Grx1 and Grx2. Grx2 exhibited highest homology to Grx from plants and

fungi (*e.g.* 48% identity to Grx from a higher plant *Vernicia fordii*; accession number AF047694). It was also highly homologous to the N-terminal sequence of human thioredoxin reductase isozyme TGR that consists of a natural fusion of N-terminal Grx and C-terminal Trx reductase domains (39% identity, accession number AF171055). Two additional unpublished sequences for Grx2 recently appeared in GenBank™ (accession numbers NM016066 for CGI-133 protein and AF132495 for glutaredoxin 2). These sequences were in a good agreement with our human Grx2 sequence.

*Structure and Localization of the Human Grx2 Gene* — Human Grx2 cDNA matched the internal sequences of human BAC clone RP11-101E13 (accession number AL136370), which allowed us to

```
      1                                           14
      m   s   w   r   r   a   a   s   v   g   r   r   l   v
CTGCTGCTCTCCCGGAGCTGCCGGGGACCTTTGGCTATGTCCTGGCGCCGCGCGCGCTTCGGTGGGGAGGCGGCTGGTG

     15                                          40
      a   s   g   r   i   l   a   g   r   r   g   a   a   g   a   a   g   s   g   m   g   n   s   t   s   s
GCGAGCGGGAGGATCTTGGCGGGGCGGCGTGGCGCGGCTGGAGCTGCGGGCTCGGGAATGGGAAACAGCACATCGTCG

     41                                          66
      f   w   g   k   s   t   t   t   p   v   n   q   i   q   e   t   i   s   n   n   c   v   v   i   f   s
TTTTGGGGGAAGTCTACAACTACTCCTGTGAACCAGATCCAAGAAACAATTTCTAACAATTGTGTGGTGATCTTCTCA

     67                                          92
      k   t   s   [c   s   y   c]  s   m   a   k   k   i   f   h   d   m   n   v   n   y   k   a   v   e   l
AAAACATCCTGCTCTTACTGTTCCATGGCCAAGAAGATTTTCCATGACATGAATGTCAACTACAAGGCTGTGGAGTTG

     93                                          118
      d   m   l   e   y   g   n   q   f   q   d   a   l   h   k   m   t   g   e   r   t   v   p   r   i   f
GATATGCTGGAATATGGCAACCAGTTTCAAGATGCGCTTCACAAGATGACTGGGGAAAGAACCGTTCCCAGGATATTT

     119                                         144
      v   n   g   r   f   i   g   g   a   a   d   t   h   r   l   h   k   e   g   k   l   l   p   l   v   h
GTCAATGGACGATTTATTGGAGGCGCAGCGGACACTCACAGGCTTCACAAAGAAGGGAAATTGCTGCCTCTGGTTCAT

     145                             156
      q   c   y   l   k   k   k   q   e   e   r   h
CAGTGTTATTTAAAAAAAAAAACAAGAGGAAAGACATTGAATGATGTGGATAGTCGCCATACCAGTAAATGCTAGTTAG

TGCAGTCGTACCTTTGACTTGAGGCTGTTTTCAGTGTGTGGATTGCCTTCATAAAGATGATTCAGAATAATGAACAAT
AAATTGCCCTGGACCCTTCAAAAAAAAAAAAAAAAAAAAA
```

**Figure 1. Amino acid and nucleotide sequences of mouse Grx2.** The open reading frame is shown *above* the cDNA sequence. The active site Cys-Ser-Tyr-Cys sequence is *boxed*. The termination signal is *underlined. Numbers* correspond to amino acid residues within mouse Grx2. The predicted mitochondrial signal peptide is *underlined*.
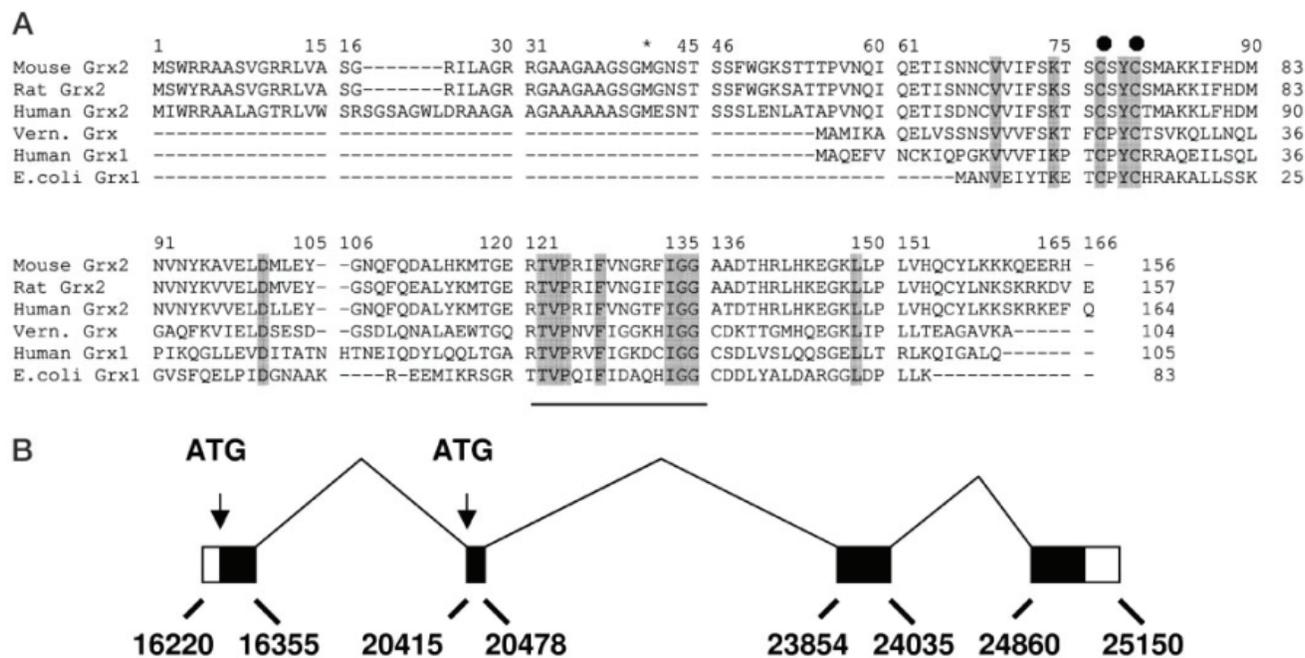


```
A
            1            15 16           30 31          * 45 46           60 61            75 ● ●         90
Mouse Grx2  MSWRRAASVGRRLVA SG-------RILAGR RGAAGAAGSGMGNST SSFWGKSTTTPVNQI QETISNNCVVIFSKT SCSYCSMAKKIFHDM  83
Rat Grx2    MSWYRAASVGRRLVA SG-------RILAGR RGAAGAAGSGMGNST SSFWGKSATTPVNQI QETISNNCVVIFSKS SCSYCSMAKKIFHDM  83
Human Grx2  MIWRRAALAGTRLVW SRSGSAGWLDRAAGA AGAAAAAASGMESNT SSSLENLATAPVNQI QETISDNCVVIFSKT SCSYCTMAKKLFHDM  90
Vern. Grx   --------------- --------------- --------------- ---------MAMIKA QELVSSNSVVVFSKT FCPYCTSVKQLLNQL  36
Human Grx1  --------------- --------------- --------------- ---------MAQEFV NCKIQPGKVVVFIKP TCPYCRRAQEILSQL  36
E.coli Grx1 --------------- --------------- --------------- --------------- -----MANVEIYTKE TCPYCHRAKALLSSK  25

            91          105 106         120 121         135 136         150 151         165 166
Mouse Grx2  NVNYKAVELDMLEY- -GNQFQDALHKMTGE RTVPRIFVNGRFIGG AADTHRLHKEGKLLP LVHQCYLKKKQEERH -         156
Rat Grx2    NVNYKVVELDMVEY- -GSQFQEALYKMTGE RTVPRIFVNGIFIGG AADTHRLHKEGKLLP LVHQCYLNKSKRKDV E         157
Human Grx2  NVNYKVVELDLLEY- -GNQFQDALYKMTGE RTVPRIFVNGTFIGG ATDTHRLHKEGKLLP LVHQCYLKKSKRKEF Q         164
Vern. Grx   GAQFKVIELDSESD- -GSDLQNALAEWTGQ RTVPNVFIGGKHIGG CDKTTGMHQEGKLIP LLTEAGAVKA----- -         104
Human Grx1  PIKQGLLEVDITATN HTNEIQDYLQQLTGA RTVPRVFIGKDCIGG CSDLVSLQQSGELLT RLKQIGALQ------ -         105
E.coli Grx1 GVSFQELPIDGNAAK ----R-EEMIKRSGR TTVPQIFIDAQHIGG CDDLYALDARGGLDP LLK------------ -         83
```



```
B
         ATG                  ATG
          ↓                    ↓
        ▯■■▯                  ■■                   ■■■■                 ■■■▯
       16220  16355        20415  20478        23854  24035        24860  25150
```

**Figure 2. Amino acid and nucleotide sequence analyses of mammalian Grx2 proteins.** *A*, multiple sequence alignment of human, mouse, and rat Grx2, human Grx1, *E. coli* Grx1, and *V. fordii* (higher plant) Grx. Sequences of human and mouse Grx2 proteins were determined in the present study. The rat Grx2 sequence was compiled from 10 unique ESTs. Two conserved cysteines that are located in the N-terminal portion of the protein and that form a redox active center are shown by *closed circles above* the sequence. Residues identical in all six sequences are *highlighted*. The *star above* the sequences indicates a methionine residue that is translated from the second in-frame ATG codon (see text for details). The *line below* the sequences indicates a conserved region that is involved in GSH binding. *B*, organization of the human Grx2 gene. *Boxes* indicate exons, and *lines connecting boxes* indicate introns. *Open boxes* indicate untranslated regions, and *filled boxes* indicate coding regions. The initiator and the second in-frame ATG codons are shown by *arrows. Numbers under* the *boxes* indicate nucleotides within the human genomic clone (accession number AL136370) derived from chromosome 1q31.2–31.3.

localize the Grx2 gene to human chromosome 1q31.2–31.3. Nucleotide sequence identities with several other partial genomic sequences (*e.g.* BAC clones RP11-556C11 and RP11-239J11) also indicated the location of the human Grx2 gene on chromosome 1. Organization of the human Grx2 gene is shown in Figure 2*B*. The 10-kb gene contained 4 exons and 3 introns with the coding regions present in exons 1–4. The initiator ATG codon, in a moderately favorable Kozak consensus sequence for initiation of translation (GCTATGC), was present in the first exon, while an additional in-frame ATG codon (in a more favorable Kozak sequence, GGAATGG) occupied nucleotide positions 2–4 within exon 2.

*Mitochondrial Signal Peptide* — Examination of the human Grx2 sequence with SignalP and PSORT programs indicated the presence of a predicted mitochondrial signal peptide (hydrophobic sequence with a high proportion of lysine or arginine residues that forms an amphiphilic α-helix). Mitochondrial leader sequences were also present in mouse and rat Grx2 sequences. As often observed in mammalian genes, the mitochondrial signal peptide was encoded by exon 1, whereas sequences conserved within the Grx family of enzymes were encoded by downstream exons.

*Localization of Grx2 to Mitochondria* — To test whether the first exon of Grx2 encodes a signal peptide that directs the protein to mitochondria, we expressed various fusion proteins of Grx2 and GFP, followed by the detection of GFP fluorescence by confocal microscopy (Figure 3). When the N-terminal signal peptide of Grx2 was fused to the N terminus of GFP, the latter protein was detected in mitochondria. Likewise, expression of a full-length Grx2 fused to the N terminus of GFP directed the fusion protein to mitochondria. However, GFP alone (data not shown) and GFP placed downstream of Grx2 that lacked the signal peptide were not directed to mitochondria and did not show any specific localization pattern. Thus, Grx2 has a functional mitochondrial signal peptide and should be considered a mitochondrial protein. Interestingly, when GFP was present upstream of the full-length Grx2 (*last row* in Figure 3), the fusion protein was significantly enriched in nuclei.

*Alternative Splicing Forms* — Examination of EST sequences for human, mouse, and rat Grx2 revealed alternative mRNA forms that differed in sequences upstream of exon 2. For example, a human cDNA sequence was detected (accession number AA421320) that contained sequences upstream of exon 1 on chromosome 1q31.2–31.3, which replaced sequences corresponding to exon 1, whereas downstream sequences (exons 2–4) for both forms were identical.

Alternative splicing forms of mouse (Figure 4*A*) and rat (Figure 4*B*) Grx2 mRNAs were also detected. These rodent forms either lacked sequences corresponding to a portion of exon 1 downstream of the translation initiation ATG codon or contained new sequences in place of sequences encoding mitochondrial signal peptides. In the rat, one alternative splicing form was represented by 12 ESTs, which is approximately half of all available rat Grx2 EST sequences. This alternative splicing form lacked 90 nucleotides within exon 1 that resulted in an in-frame stop signal between the initiation ATG and the second ATG codons (Figure 4*B*). Mouse EST sequences for Grx2 showed even more complex sequence variations. While sequences corresponding to exon 2 were identical in all mouse ESTs, we detected four distinct alternative sequences (accession numbers BE192757, AA466975, AA050108, and W61977) in place of sequences corresponding to exon 1 (Figure 4*A*). Overall, these data indicated extensive sequence variations in 5'-sequences of mammalian Grx2 mRNAs and suggested alternative first exon splicing as a mechanism to generate these mRNA forms.

*Expression of Grx2 mRNA* — Grx2 was represented by 24 human ESTs derived from nine cDNA libraries, including nine fetal brain, four testes, two fetal kidney, and two colon ESTs as well as several individual sequences derived from prostate tumor, corneal stroma, HL60 (promyelocyte) cell line, kidney, and pancreatic adenocarcinoma. Mouse Grx2 was represented by 36 ESTs from 12 cDNA libraries. Like the human protein, it was highly represented by ESTs derived from testes (seven sequences), fetal (two sequences), and embryonic (nine sequences) tissues. Mouse Grx2 was also represented by mammary gland, lymph node, kidney, thymus, placenta, myotubes, proximal colon, and hypothalamus ESTs. The data suggested that Grx2 mRNA is expressed in a variety of tissues with possible increased levels in testes and fetal and embryonic tissues. It should be noted that several cDNA libraries that contained Grx2 clones were normalized. This fact limits the assessment of absolute expression levels of Grx2 mRNA.

*Characterization of Recombinant Human and Mouse Grx2* — Human and mouse Grx2 were expressed in *E. coli* in the form of His-tagged proteins. Recombinant proteins were isolated using a nickel affinity column and analyzed by gel electrophoresis (Figure 5). To express mature human and mouse Grx2s (forms that lack mitochondrial leader peptides), constructs were developed in which translation was initiated from the second in-frame ATG codon that corresponded to nucleotides 2–4 within human exon 2. Examination of human and mouse recombinant proteins revealed little difference in their catalytic activities (data not shown).

*Catalytic Properties of Grx2* — Catalytic properties of human Grx2 were determined in parallel with and compared with recombinant human Grx1. The activity of Grx2 was limited to a narrow range of pH between 7.5 and 8.5, with the optimal pH of 8.0. This pH dependence of Grx2 activity as well as temperature dependence (data not shown) were similar to those of Grx1 (17–19, 27).
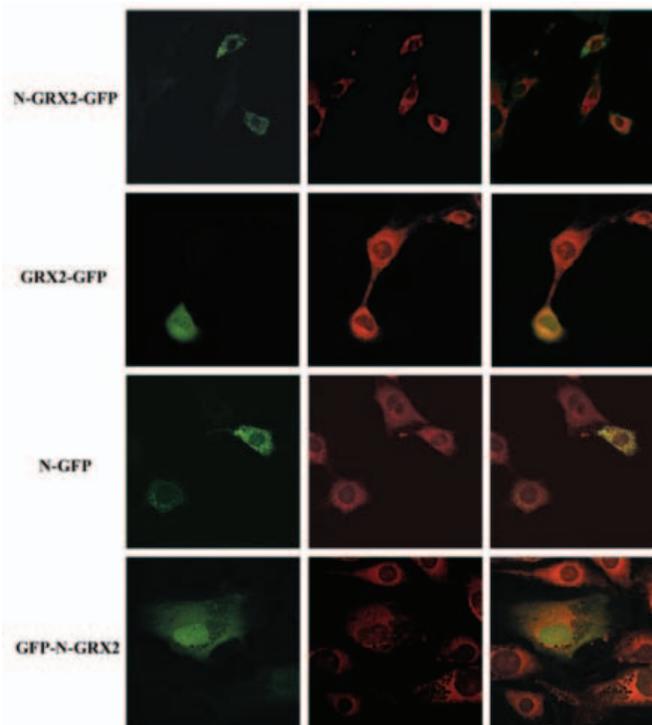


**Figure 3. Expression of the GFP–Grx2 fusion proteins.** Confocal images of NIH 3T3 cells expressing GFP-tagged Grx2 proteins are shown. A set of three images is shown for each construct. The *left panels* show green fluorescence protein corresponding to transiently expressed fusion proteins; the *center panels* show fluorescence of a mitochondrial marker, MitoTracker (Molecular Probes); and the *right panels* are obtained by merging the *left* and *center panels*. From *top* to *bottom*, the following are shown. *N-Grx2-GFP*, full-size Grx2 fused to the N terminus of GFP; *Grx2-GFP*, same construct in which the mitochondrial signal peptide was removed; *N-GFP*, signal peptide of Grx2 fused to the N terminus of GFP; *GFP-N-Grx2*, full-length Grx2 fused to the C terminus of GFP.

Recombinant Grx2 catalyzed reduction of mixed disulfides formed between GSH and HEDS, *S*-sulfocysteine, or L-cystine (Figure 6). The apparent $K_m$ values for HEDS, *S*-sulfocysteine, and L-cystine were 1.68, 1.77, and 0.30 mM, respectively (Table I). The catalytic efficiency ($k_{cat}/K_m$) of Grx2 was different for the three substrates. Grx2 displayed the highest efficiency with cystine, followed by HEDS and *S*-sulfocysteine with equal efficiency (Table I).

*Sensitivity of Grx2 to Iodoacetamide and Hydrogen Peroxide* — Grx2 activity was inhibited in the presence of the alkylating agent, iodoacetamide. Incubation of the protein with iodoacetamide for 15 min resulted in 95% inhibition, suggesting that an active site SH group(s) was involved in the reaction catalyzed by Grx2. Grx2 was also partially inactivated when treated with $H_2O_2$. As shown in Figure 7, the activity loss was 20% in the presence of 0.1 mM and 40% in the presence of 0.5 mM $H_2O_2$ and remained at 50–55% even after $H_2O_2$ was increased to 1.5 mM. The activity of Grx1 was also inhibited by $H_2O_2$ (Figure 7), as previously reported (28). Grx2 activity could be almost fully regenerated by treating the partially inactivated

protein with 5 mM GSH; however, no protein reactivation was observed in the presence of 5 mM DTT or the *E. coli* Trx/Trx reductase system (Table II). This observation contrasted with the finding that Grx1 could be reactivated by these reagents (data not shown).

*Molecular Modeling* — Amino acid sequence similarity between Grx1 (for which several three-dimensional structures are available) and Grx2 was sufficient to generate a molecular model of Grx2. We modeled mouse Grx2 structure using NMR structures of human Grx1 and x-ray structure of pig Grx1 as templates (Figure 8). The predicted Grx2 structure revealed conservation of all major structural features found in mammalian Grx1. Specifically, Grx2 had a Trx fold that can be recognized by the presence of a characteristic β-sheet core surrounded by α-helixes. The C*XX*C motif in Grx2 was located at the N-terminal end of a first α-helix, which is another typical structural feature of Trx fold proteins. The close structural similarity between Grx1 and Grx2 could explain similarity in their catalytic properties. The model, however, was not sufficient to explain differences in reactivation properties between Grx1 and Grx2.



**Figure 4. Alternative splicing forms of rat and mouse Grx2.** *A*, comparison of mouse Grx2 cDNA sequence (mitochondrial form) and alternative cDNA forms found by the analysis of mouse EST data base (accession numbers of EST sequences are indicated on the *left*). The second ATG codon (corresponds to ATG shown in Figure 2*B*, positions 2–4 within exon 2 of the human Grx2 gene) is shown in *boldface type*. The *vertical line* corresponds to the exon 1-exon 2 junction in the human Grx2 gene and separates alternative first exon sequences from common downstream sequences. *B*, two alternative forms of the rat Grx2 cDNA. N-terminal sequence of the mitochondrial form of rat Grx2 is shown at the *top*. This sequence is obtained by translating the nucleotide sequence shown *below* the amino acid sequence. Within this nucleotide sequence, *lowercase letters* indicate sequences that are lacking in the alternative form of rat Grx2. Translation of that cDNA form (shown *below* the nucleotide sequence) results in an in-frame stop signal followed by the second in-frame ATG codon. Two ATG codons that may potentially initiate translation are shown in *boldface type* and *underlined*. The corresponding Met residues are also shown in *boldface type*.
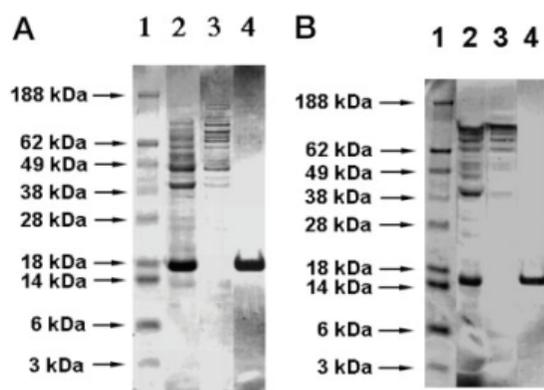


**Figure 5. Gel electrophoretic analysis of human and mouse recombinant Grx2.** His-tagged human and mouse Grx2 were expressed in *E. coli* as described under "Experimental Procedures." The proteins were isolated using the nickel affinity column and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Coomassie Blue staining of the protein is shown. *A*, fractions containing human Grx2. *B*, fractions containing mouse Grx2. In *both panels*, *lane 1* shows molecular weight standards (indicated by *arrows* on the *left*); *lane 2* shows crude extracts of Grx2-overexpressing cells; *lane 3* shows flow-through fraction obtained after crude extract was passed through the nickel affinity column; and *lane 4* shows Grx2 eluted from the nickel affinity column.

**Table I. Catalytic properties of Grx2**

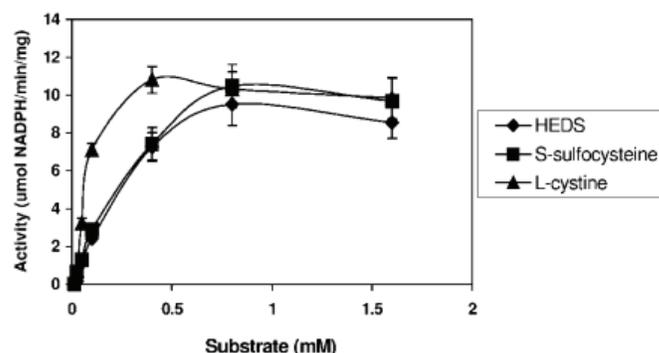| Substrate | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
|---|---|---|---|
| | *mM* | *min⁻¹* | *mM⁻¹ min⁻¹* |
| HEDS | 1.68 | 40.0 | 23.8 |
| *S*-Sulfocysteine | 1.77 | 46.9 | 26.5 |
| Cystine | 0.3 | 23.1 | 77.0 |



**Figure 6. Substrate specificity of human Grx2.** Catalytic activities of human Grx2 were determined in the presence of 0–1.5 mM HEDS (*diamonds*), *S*-sulfocysteine (*squares*), or L-cystine (*triangles*) and expressed as mM NADP⁺ produced/min/μg of protein.
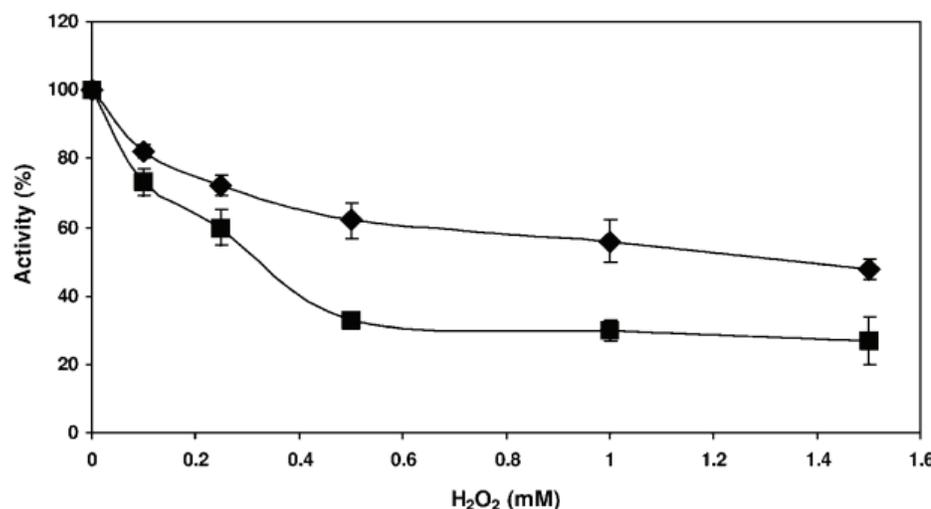
**Figure 7. Inactivation of human Grx1 and Grx2 by hydrogen peroxide.** Human Grx1 (*squares*) and Grx2 (*diamonds*) were treated with 0–1.6 mM hydrogen peroxide followed by analysis of their catalytic activities using the standard Grx assay. Enzyme activity is expressed as percentage of the initial activity.

## DISCUSSION

We described identification and characterization of a novel mammalian mitochondrial glutaredoxin (thioltransferase), Grx2. The new protein exhibited only 36% sequence identity with the previously characterized mammalian cytosolic glutaredoxin, Grx1. However, it conserved the disulfide active center C*XX*C sequence and a substrate binding motif and exhibited catalytic properties typical of previously characterized Grx proteins.

To characterize Grx2, we analyzed catalytic properties of bacterially expressed and purified mouse and human proteins in parallel with Grx1. Substrate specificity and substrate inhibition patterns were similar for two proteins. Likewise, Grx1 and Grx2 were both sensitive to inactivation by an alkylating agent, iodoacetamide, and an oxidant, hydrogen peroxide. However, the $H_2O_2$-inactivated Grx2 could only be reactivated fully in the presence of high concentrations of GSH, while other strong reducing agents, such as DTT and Trx were not effective in Grx2 reactivation. This property of Grx2 distinguished this protein from Grx1, whose activity could be restored effectively by DTT or the Trx/Trx reductase system.

The conserved sequences within mammalian Grx2 were preceded by a leader sequence indicative of a mitochondrial protein, and this signal was shown to direct either GFP or Grx2-GFP fusion protein to mitochondria. This finding that Grx2 resides in mitochondria is significant in view of the previously established location of other components of the GSH system in this cellular compartment. Although no mitochondrial Grx was previously reported in any eukaryotic organism, our data suggest that Grx2 is a functional mitochondrial protein.

The finding of a second Grx in mammals is similar to the fact that several Grx isoenzymes were previously described in bacteria, yeast, and plants. These proteins exhibit strong sequence homology, often have overlapping functions, and generally participate in the same or related metabolic pathways. However, recent evidence indicates that Grx isozymes may also play unique roles in cellular redox regulation (2, 4, 29).

The yeast *Saccharomyces cerevisiae* genome encodes two Grx isozymes containing the C*XX*C motif. Expression of both proteins is induced in response to oxidative, osmotic, and heat stress and in response to stationary phase growth and growth on nonfermentable carbon sources (30). In addition, yeast Grx1 and Grx2 are activated by the high osmolarity glycerol pathway and negatively regulated by the Ras-protein kinase A pathway. However, Grx1 is induced to significantly higher levels compared with Grx2 following heat and osmotic shock, whereas Grx2 is rapidly induced in response to reactive oxygen species and upon entry into stationary phase growth (30).

In addition, the *S. cerevisiae* genome encodes three Grx isozymes, which contain an active center motif with a single conserved Cys (29). The five yeast Grx isozymes show a complex functional relationship, such as the synthetic lethality of either Grx5 and Grx2

**Table II. Oxidation of Grx2 by $H_2O_2$ and its reactivation by reductants**
Data are expressed as mean ± S.D. *n* = 3.

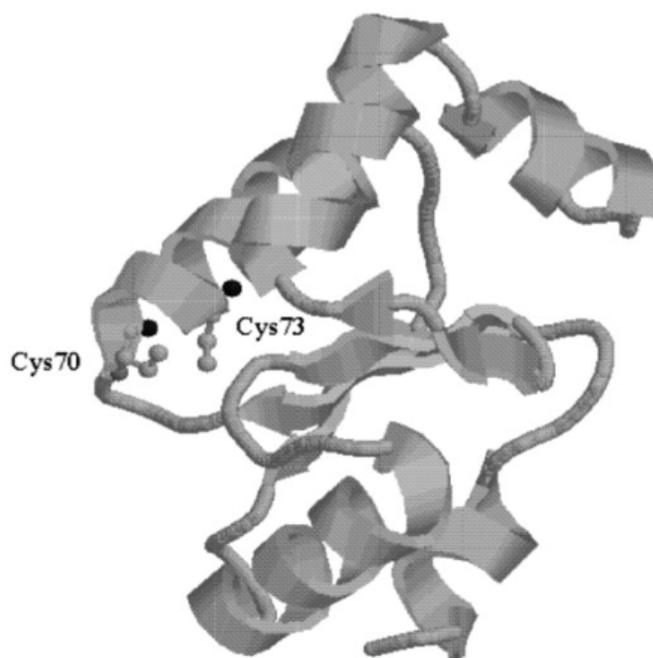| Treatment | Percentage of Grx2 activity |
|---|---|
| | % |
| Inactivated Grx2 (1 mM $H_2O_2$) | 47.3 ± 4.9 |
| Inactivated Grx2 + GSH (0.5 mM) | 52.2 ± 5.8 |
| Inactivated Grx2 + GSH (5 mM) | 96.4 ± 1.9 |
| Inactivated Grx2 + DTT (5 mM) | 50.4 ± 11.6 |
| Inactivated Grx2 + Trx/Trx reductase | 57.3 ± 3.8 |



**Figure 8. Predicted three-dimensional structure of mouse Grx2.** Molecular modeling of the Grx2 structure was performed with SWISS-MODEL program. Sequence spanning residues 49–150 of mouse Grx2 was modeled. The active site cysteine residues (Cys[70] and Cys[73]) are indicated.

mutations or inactivation of the Grx5 gene together with genes for Grx3 and Grx4. In addition, Grx5 plays a specific role in protection against oxidative and osmotic stresses (29).

The *E. coli* genome encodes three functional Grx isozymes characterized by common as well as unique functional properties (4). In contrast to *E. coli* and *S. cerevisiae*, where multiple Grx proteins occupy the same (cytosolic) compartment, the two mammalian Grx proteins appear to reside in different compartments. Thus, the finding of similar catalytic profiles for these enzymes is not surprising. Expression patterns of Grx2 mRNA, which appears to be prevalent in fetal and embryonic tissues and in testes, may provide an additional clue to functional distinction between mammalian Grx1 and Grx2.

In addition to mRNA forms encoding mitochondrial Grx2, we detected mRNA forms that were obtained by alternative splicing. Such forms were detected in the human, mouse, and rat sequences. Interestingly, some of the alternative forms contained in-frame stop signals. It is not clear how these forms could result in expression of functional proteins. One possibility is that the ATG codon that occupies positions 2–4 within exon 2 in the human sequence and the corresponding ATG codons in the mouse and rat sequences could function as an alternative translation initiation site. This ATG is conserved in all mammalian Grx2 sequences, and we showed that it could initiate translation of human and mouse Grx2 proteins when these proteins were expressed in *E. coli*. The use of this ATG codon to initiate protein synthesis may potentially result in a cytosolic form of Grx2. In addition, the fusion protein, in which GFP was upstream of Grx2, was enriched in nuclei, so we cannot exclude the nuclear location of some of the alternative splicing forms.

This situation would be similar to expression of phospholipid hydroperoxide glutathione peroxidase, whose gene contains two in-frame ATG codons. The use of the first ATG yields a mitochondrial protein, whereas the second ATG also can initiate glutathione peroxidase synthesis, resulting in a cytosolic enzyme. Moreover, the use of an alternative first exon instead of the mitochondrial exon appears to direct the protein to the nucleus (31).

The role of the GSH system in mitochondria is not fully understood. Nevertheless, one may envision the importance of the GSH system in protecting this organelle from reactive oxygen species that are generated as by-products of metabolic processes. Grx2 may be expected to serve an important role in this protection by maintaining reduced states of thiols in mitochondrial proteins and by reducing their glutathionylated cysteine residues with the concurrent oxidation of GSH to GSSG. This physiological function of Grx2 may also be extended to pathological and toxicological conditions that are characterized by elevated levels of reactive oxygen species generated in mitochondria and where the demand for the Grx function may be increased.

*Addendum* — During the revision of this paper, a paper was published online that also describes identification and characterization of Grx2; see ref. #32.

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