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Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene

(selenium/thioredoxin reductase/TGA/selenocysteine)

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ABSTRACT **The possible relationship of selenium to immunological function which has been suggested for decades was investigated in studies on selenuim metabolism in human T cells. One of the major ^{75}Se-labeled selenoproteins detected was purified to homogeneity and shown to be a homodimer of 55-kDa subunits. Each subunit contained about 1 FAD and at least 0.74 Se. This protein proved to be thioredoxin reductase (TR) on the basis of its catalytic activities, cross-reactivity with anti-rat liver TR antibodies, and sequence identities of several tryptic peptides with the published deduced sequence of human placental TR. Physicochemical characteristics of T-cell TR were similar to those of a selenocysteine (Secys)-containing TR recently isolated from human lung adenocarcinoma cells. The sequence of a 12-residue ^{75}Se-labeled tryptic peptide from T-cell TR was identical with a C-terminal-deduced sequence of human placental TR except that Secys was present in the position corresponding to TGA, previously thought to be the termination codon, and this was followed by Gly-499, the actual C-terminal amino acid. The presence of the unusual conserved Cys-Secys-Gly sequence at the C terminus of TR in addition to the redox active cysteines of the Cys-Val-Asn-Val-Gly-Cys motif in the FAD-binding region may account for the peroxidase activity and the relatively low substrate specificity of mammalian TRs. The finding that T-cell TR is a selenoenzyme that contains Se in a conserved C-terminal region provides another example of the role of selenium in a major antioxidant enzyme system (i.e., thioredoxin–thioredoxin reductase), in addition to the well-known glutathione peroxidase enzyme system.**

Selenium is a trace element that is important for a number of physiological processes and for human health. Its essentiality is based on the fact that it is a specific component of Se-dependent enzymes and tRNAs. In known Se-dependent enzymes Se occurs as an active center amino acid residue, selenocysteine (Secys) (1, 2) or as a metal-coordinated labile cofactor (3, 4). In both cases Se is essential for the catalytic activities exhibited by the specific selenoenzymes. The mechanism of incorporation of Secys is established for prokaryotes (5), and many aspects of its incorporation in eukaryotic proteins are well understood (6).

The UGA codon directs incorporation of this amino acid residue both in prokaryotes and eukaryotes. In addition, conserved stem-loop structures immediately following UGA in prokaryotes (5) and in 3′-untranslated regions in eukaryotes (7) function as determinants of Secys incorporation instead of termination of translation.

Among eukaryotic proteins that contain Se as a Secys residue, the most studied are members of the glutathione peroxidase family (8). These enzymes that catalyze the glutathione- or thioredoxin-dependent reduction of hydroperoxides are important intra- and extracellular antioxidants in mammals and birds. A different metabolic role of selenium was established by the discovery of the selenium-dependent 5′ deiodinases (9). These enzymes function in the synthesis and maintenance of optimal levels of essential thyroid hormones required for growth and development. Selenoproteins of unknown function include selenoprotein P, the major plasma selenoprotein (10) that contains 10 Secys residues and selenoprotein W, a small Secys-containing protein present in muscle (11).

Recently a new selenoprotein purified from human lung adenocarcinoma cells was shown to be a homodimer of 57 kDa subunits containing FAD and Secys (12). This enzyme exhibited thioredoxin reductase (TR) activities similar to those of human placenta (13) and rat liver (14) TRs. Although the lung enzyme did not react with anti-rat liver TR antibodies, which suggests it might be a novel type of TR, it clearly is related to another form of the enzyme present in the lung cells that is immunoreactive (S.-Y. Liu and T.C.S., unpublished data).

In view of the fact that selenium has been indirectly implicated in immunological function in numerous nutritional studies and that HIV-infected individuals are reported to have decreased levels of plasma Se and selenium-containing glutathione peroxidase (15), we initiated studies on selenium metabolism in human T cells. Identification of one of the selenoproteins detected in T cells as TR and demonstration that the location of Secys in this protein corresponds to a TGA codon in the cloned human placental gene (16) is reported in the present communication.

MATERIALS AND METHODS

Materials. The following materials were from the indicated sources: [^{75}Se]selenious acid from the Research Reactor Facility (University of Missouri, Columbia); precast polyacrylamide gels and molecular weight standards from NOVEX (San Diego); isoelectrofocusing standards from Pharmacia; ECL immunoblotting detection system from Amersham; trypsin and 2′,5′-ADP- Sepharose from Sigma; C_{18} (218TP54) reversed-phase HPLC column from Vydac; sulfopropyl (SP) HPLC column from TosoHaas (Montgomeryville, PA); and heat-inactivated fetal bovine serum and RPMI 1640 medium from Mediatech (Herndon, VA). Human thioredoxin, rat liver thioredoxin reductase, and rabbit polyclonal antibodies to rat liver thioredoxin reductase were kindly provided by Ho Zoon Chae (National Institutes of Health). Human lung adenocar-

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Abbreviations: Secys, selenocysteine; TR, thioredoxin reductase; SP, sulfopropyl; DTNB, 5,5′-dithiobis(2-nitrobenzoate); MALDI, matrix-assisted laser desorption ionization.

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cinoma thioredoxin reductase labeled with ^{75}Se was kindly provided by Song-Yuan Liu (National Institutes of Health). All other reagents were of the highest grade available.

Cell Growth. A human Jurkat T-cell line, JPX9 (29), was grown on RPMI 1640 medium in the presence of 10% fetal bovine serum and antibiotic–antimycotic solution in 5% CO_2. For preparation of ^{75}Se-labeled cells, 12 μl of [^{75}Se]selenious acid, 300 μCi (1 Ci = 37 GBq), in 30% nitric acid was added to 40 ml of medium and cells were grown for 3 days under the same conditions. Cells were collected by centrifugation, washed two times with phosphate-buffered saline, and stored at −80°C prior to use.

TR Purification. For purification of TR, unlabeled cells were mixed with ^{75}Se-labeled cells. The cells were suspended in 2 volumes of cold 25 mM Tris·HCl (pH 7.8), 1.5 mM EDTA, and 2 mM DTT (buffer A) containing 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF and were disrupted by sonication. The pellet, obtained after centrifugation of the disrupted cells, was washed with a minimal volume of buffer A and again collected by centrifugation. The supernatants were combined and applied to a DEAE-Sephadex anion-exchange column equilibrated with buffer A. The proteins were eluted with a linear 0–0.4 M gradient of sodium chloride in buffer A. The radioactivity of fractions containing ^{75}Se was determined in a Beckman 5500 γ counter and the peak Se-containing fractions were analyzed on SDS gels. Fractions containing a 55 kDa selenoprotein were combined, diluted four times with buffer A, and applied to a 2′,5′-ADP Sepharose affinity column equilibrated with buffer A. The column was washed sequentially with buffer A, 0.2 M sodium chloride in buffer A, 0.3 M sodium chloride in buffer A, and 1 M sodium chloride in buffer A. The TR detected as a ^{75}Se-containing protein was eluted with 1 M NaCl in buffer A. Ammonium sulfate (4 M, pH 8.0), was added to the enzyme solution to a final concentration of 0.5 M and the TR solution was applied to a phenyl-Sepharose column and eluted with a linear 0.5–0 M gradient of ammonium sulfate in 25 mM Tris·HCl (pH 7.8). The resulting ^{75}Se-labeled TR that eluted at 0.2 M ammonium sulfate was concentrated to about 1 mg/ml and stored at −20°C.

Carboxymethylation and Tryptic Digestion of TR. ^{75}Se-labeled TR (100 μg, 3×10^5 cpm) in 160 μl of 100 mM Tris·HCl (pH 7.7) and 6 mM EDTA was denatured by mixing with 160 mg of dry guanidine–hydrochloride in a 1.5 ml microcentrifuge tube. The solution was sparged with argon for 20 min and after the addition of 15 μl of 100 mM DTT the reaction mixture was incubated for 40 min at room temperature. For alkylation a 33 μl aliquot of 250 mM iodoacetate was added and the solution was incubated under argon at room temperature in the dark for 40 min. To quench the reaction, 15 μl of 1 M DTT was added. The solution was diluted to 650 μl with 4 M guanidine–hydrochloride and dialyzed with two changes against 2 liters of 20 mM Tris·HCl (pH 8.0). The resulting carboxymethylated TR was mixed in a 1.5 ml microcentrifuge tube with 2.5 μl of 0.66 mg/ml trypsin in 20 mM Tris·HCl (pH 8.0) and incubated at 37°C for 20 h. After 8 h of incubation another 2.5 μl of the same trypsin solution was added. Five microliter aliquots of the digestion mixture were collected at 1 min, 8 h, and 20 h of incubation and analyzed by SDS/PAGE. Based on the Coomassie blue staining of the protein and the PhosphorImager detection of ^{75}Se in the gel, most of the TR was digested at 8 h, and digestion was complete at 20 h. After 20 h of incubation a ^{75}Se-containing peptide appeared as a diffuse 4-kDa species in SDS gels.

Separation and Sequencing of Se-Containing Peptide. The tryptic digestion mixture of carboxymethylated TR was acidified with HCl to pH 2.0, mixed with 600 mg of dry guanidine-hydrochloride and 5 μl of 0.5 M DTT, and loaded on a C_{18} reversed-phase HPLC column, equilibrated in 0.05% trifluoroacetic acid. The 100-min gradient of acetonitrile from 0 to 50% in 0.05% trifluoroacetic acid was applied to elute peptides at 1 ml/min flow rate. A single ^{75}Se-containing peptide (1.53×10^5 cpm) was eluted with 23% acetonitrile. A 35% portion of the isolated peptide was dried on a SpeedVac SC110 (Savant), dissolved in 6 M guanidine–hydrochloride and 2 mM DTT, and subjected to automatic sequencing on a Hewlett–Packard Protein Sequencer G100A. At each cycle, all eluates were collected and analyzed for the presence of ^{75}Se by gamma counting. The remainder of the ^{75}Se-containing peptide was loaded on a sulfopropyl (SP) HPLC column and eluted with a gradient from 0 to 0.5 M NaCl in 0.05% trifluoroacetic acid over a 30-min time period. A ^{75}Se-containing peptide was eluted as a single peak, centered at 100 mM NaCl, that separated from the major impurities. This peptide was used directly for sequencing, and fractions corresponding to each cycle of the Edman degradation were collected and analyzed for the presence of ^{75}Se.

Other Methods. Isoelectrofocusing (pH 3–7 range), SDS, and native PAGE analyses were performed according to standard procedures using 5%, 12%, or gradient 4–20% polyacrylamide gels. Gels were stained with Coomassie blue R-250. For detection of ^{75}Se in gels, a PhosphorImager (Molecular Dynamics) was used. Thioredoxin- and 5,5′-dithiobis(2-nitrobenzoate) (DTNB)-dependent assays of catalytic activities were performed as described (12). The selenium content of TR was determined with a Perkin–Elmer model 4100 ZL atomic absorption spectrometer by using a palladium/magnesium nitrate modifier and temperature conditions as described (17). The molecular masses of purified TR and some of the tryptic peptides were determined by matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry with cinapinic acid as a matrix. Analytical HPLC gel-filtration for the determination of the molecular mass of the enzyme was performed as described (4). An immunoblotting procedure with an ECL detection system was used to study the cross-reactivity of human T-cell TR to polyclonal rabbit antibodies raised against rat liver TR. For this experiment, rat liver TR served as a control.

RESULTS

Analysis of extracts of ^{75}Se-labeled human T cells by SDS/PAGE followed by PhosphorImager detection of radioactivity revealed the presence of several selenium-containing proteins. One of the most abundant selenium-containing proteins in the extracts, detected as a prominent ^{75}Se-labeled 55-kDa protein band on the gels, was selected for preliminary characterization. About 40% enrichment of this protein was achieved by using DEAE Sephadex ion-exchange and phenyl Sepharose hydrophobic interaction chromatographic procedures. During these isolation steps it was observed that a flavin-containing species coeluted with the ^{75}Se-labeled 55-kDa protein. This suggested that the T-cell protein might be a TR similar to the Secys-containing flavoprotein recently isolated from a human lung adenocarcinoma cell line (12). The lung enzyme, a homodimer of 57-kDa subunits, contained FAD and exhibited catalytic activities typical of mammalian TRs. To determine whether the T-cell selenoprotein is also a TR, the partially purified enzyme preparation was tested in immunoblot assays for interaction with anti-rat liver TR polyclonal antibodies. A strong signal exhibited by a 55-kDa protein band indicated the presence of TR.

For further purification of the 55-kDa selenoprotein, a 2′,5′-ADP Sepharose affinity matrix that has been used routinely for efficient purification of mammalian TRs (18) was employed. As judged by SDS and native PAGE analyses (Fig. 1 *A* and *D*), an apparently homogeneous preparation of the enzyme was obtained by the modified isolation procedure described in *Materials and Methods*. The purified enzyme migrated as a 130-kDa species in gradient native PAGE gels

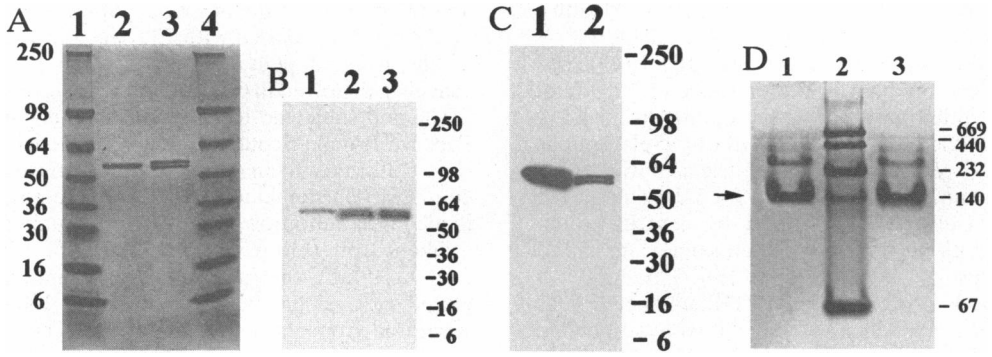

FIG. 1. Characterization of ^{75}Se-labeled 55-kDa T-cell selenoprotein (TR). (*A*) SDS/PAGE analysis of TR. Lanes 1 and 4, standards; lane 2, rat liver TR; lane 3, human T-cell TR. Molecular masses of standards (in kDa) are shown. Gradient 4–20% gel was used. (*B*) PhosphorImager detection of ^{75}Se in TRs on SDS gel. Lane 1, human lung TR; lane 2, human T-cell TR after DEAE step; lane 3, pure T-cell TR. Molecular masses of standards (in kDa) are shown. (*C*) Immunoblot detection of human T-cell TR with anti-rat liver TR antibodies. Lane 1, rat liver TR; lane 2, T-cell TR. Molecular masses of standards (in kDa) are shown. Gradient 4–20% gel was used. (*D*) PAGE analysis of native TR. Lane 1, T-cell TR; lane 2, standards; lane 3, rat liver TR. Molecular masses of standards (in kDa) are shown. Location of homodimeric TR is indicated by the arrow. Small portions of T cell and rat liver TR migrated also as higher molecular mass aggregates. Gradient 4–20% gel was used.

(Fig. 1*D*) and the elution position of the protein from a calibrated gel-filtration column corresponded to a 120-kDa species (data not shown). These properties indicate that the native selenoenzyme is a homodimer of 55-kDa subunits, a structure typical of mammalian TRs.

On SDS/PAGE gels the enzyme migrated as two barely separating protein bands that are approximately equally stained with Coomassie blue (Fig. 1*A*). Both bands showed about equal intensity of ^{75}Se radioactivity (Fig. 1*B*). To avoid generation of isoforms by proteolysis during purification, several protease inhibitors had been added to cells prior to sonic disruption. Size differences due to partial N-terminal proteolytic cleavage seemed unlikely since all protein molecules in the native enzyme preparation were N-blocked as judged by Edman degradation sequence analysis. Also, based on the fact that the human lung TR selenoprotein was shown to lack glycosyl groups (12), differences due to varying states of glycosylation of the T-cell enzyme appeared very unlikely. The isolated human lung TR also was N-blocked. In some cases the presence of isoforms of differing net charge can explain the separation of the protein bands in gels. Such differences can be reflections of the presence of covalent modifying groups or protein alteration due to oxidative damage. However, although the apparently homogeneous preparation of the lung TR contained two protein species of differing isoelectric points, pH 5.2 and pH 5.3 (12), it migrated in SDS gels as a single protein band. The purified T-cell TR showed isoforms ranging in pI values from 5.15 to 5.35 when analyzed using a pH 3–7 isoelectric focusing gel (not shown). As controls another preparation of the human lung TR that had been isolated by a different procedure and rat liver TR were run on the same gel. The pI values of the lung enzyme varied from 5.1 to 5.4, whereas a single isoform with a pI value of 5.85 was detected in the rat liver enzyme. The basis of these differing isoelectric points of protein species present in the various enzyme preparations is unknown.

MALDI mass spectrometric analysis of the native T-cell enzyme revealed the presence of a single polypeptide subunit with a molecular mass of a 56,018 Da. This mass is significantly larger that the predicted value of 54,625 Da for the mass of the 499-amino acid polypeptide. Although the identity of the group present at the blocked amino terminus is unknown, it is unlikely to be of a size sufficient to account for the difference in mass. Whether tightly bound cofactors such as the FAD present in the enzyme are resolved during MALDI analysis appears to be unpredictable and highly subject to precise conditions used.

The purified T-cell TR cross-reacted with the anti-rat liver TR antibodies in the immunoblot assay (Fig. 1*C*) and both of the protein bands detected on SDS gels appeared to be equally reactive. The TR isolated from human lung adenocarcinoma cells by a different procedure that involved absorption and elution from a heparin-agarose affinity matrix failed to react with the same antibody preparation (12). However, it now appears that a second form of the enzyme also is present in the lung cells, and this protein is recognized by the anti-rat liver TR antibodies in the immunoblot assay (S.-Y. Liu and T.C.S., unpublished).

The isolated T-cell enzyme exhibited catalytic activities typical of mammalian TRs. NADPH-dependent reduction of DTNB and human thioredoxin-dependent reduction of insulin with activities of 25 and 19 μmol NADPH oxidized/min/mg protein, respectively, were observed. These values are in the range of those reported for mammalian TRs from other sources (12–14, 19–21).

The electronic absorption spectrum of the T-cell TR revealed a characteristic flavin profile. The ratio of absorptivities at 280 nm and 450 nm was found to be 10 (ratio of absorptivities at maxima of 277 nm and 466 nm was 9), which is typical of mammalian TRs and indicative of an approximately full complement of FAD (two flavins per homodimer).

The amount of Se in T-cell TR determined by graphite furnace atomic absorption spectroscopy was 0.74 Se per 55-kDa subunit. The lower then stoichiometric amount of Se in T-cell TR presumably is due to the inaccuracies of determination of Se in proteins of similar molecular masses. Thus the T-cell TR probably contains 1 Se per enzyme subunit.

To determine the location of selenium in the human T-cell TR, the reduced ^{75}Se-labeled enzyme was carboxymethylated and cleaved with trypsin followed by separation of peptides on a reversed-phase HPLC column (Fig. 2). The ^{75}Se was eluted from the column in fractions of a single major radioactive peak. Further purification using a SP HPLC column separated the ^{75}Se-labeled peptide from the major impurities (data not shown). The overall high (about 50%) recovery of ^{75}Se in the resulting purified carboxymethylated peptide suggested the presence of a single stable selenium moiety, presumably carboxymethyl-selenocysteine.

The purified ^{75}Se-labeled peptide isolated from the SP HPLC column was subjected to automated Edman degradation N-terminal sequencing analysis. Fractions corresponding to each cycle of the degradation were collected and analyzed for radioactivity. The resulting unique amino acid sequence matched exactly the deduced C-terminal 10-residue sequence

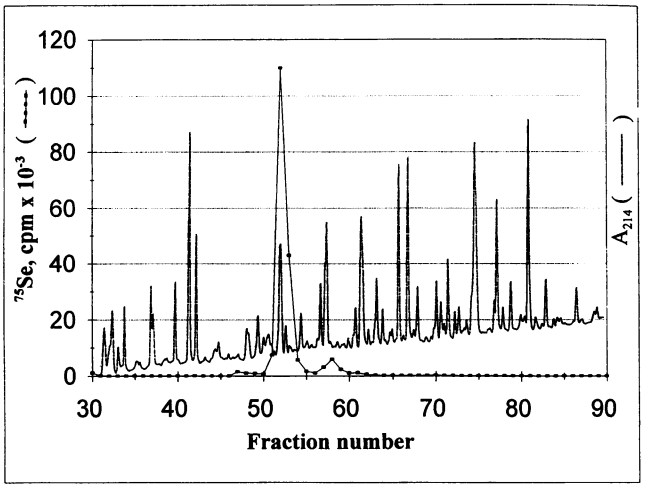

FIG. 2. Reversed-phase HPLC separation of a tryptic digest of carboxymethylated ^{75}Se-labeled TR. The tryptic digest of T-cell TR was chromatographed on a C_{18} column developed with a gradient of acetonitrile in 0.05% trifluoroacetic acid. Elution of peptides was followed by absorptivity at 214 nm (shown in arbitrary units) and by determination of ^{75}Se in collected fractions.

of placental TR derived from the cloned cDNA (16), except that the peptide contained two additional residues at the C terminus (Table 1). The ^{75}Se in the peptide was eluted in cycle 11 where no amino acid was identified and this corresponded to the TGA codon in the gene. Cycle 12 contained a modest amount of glycine, and no additional amino acids were detected in cycles 13 and 14. From these data it is apparent that the TGA codon corresponds to Secys in the protein rather than serving as a termination codon as was concluded previously (16). Thus the three final triplets of the gene, TGA GGT TAA, actually encode Secys and a C-terminal glycine with TAA serving as the actual termination codon (Table 1).

The sequence of an additional major peptide was determined by Edman degradation analysis of the impure ^{75}Se-labeled peptide fraction eluted from the reversed-phase HPLC column (Fig. 2). In addition to the sequence of the ^{75}Se-labeled 12-residue C-terminal peptide (Ser-488–Gly-499), the sequence of another peptide which matched residues Phe-157–Arg-166 of the predicted gene sequence was clearly discernable. Identity of the latter peptide (expected mass 1159 Da) was confirmed by MALDI mass spectrometry. Sequences of three additional minor peptides that could be determined from the sequence analysis matched the sites of the deduced placental TR cDNA spanning Lys-299–Gly-306, Ile-316–Val-326, and Thr-313–Val-326. These data show that peptides comprising a total of 44 amino acids from T-cell TR share identity with deduced peptide sequences in placental TR.

DISCUSSION

A 12-residue ^{75}Se-labeled tryptic peptide isolated from human T-cell TR was shown to contain Secys in the position corresponding to a TGA codon in the human placental TR gene. The entire amino acid sequence of the peptide was identical to that predicted from the gene sequence plus an additional two residues, Secys and glycine. In the reported placental gene sequence (16), TGA had been interpreted as a termination codon, but from our studies it is clear that this TGA encodes Secys. The corresponding locations of Secys in the peptide and the TGA codon in the gene clearly indicate that the selenoamino acid is specifically incorporated cotranslationally as directed by UGA.

The finding that T-cell TR contains a C-terminal amino acid sequence, Cys-Secys-Gly, is particularly interesting. This relatively rare occurrence of Secys adjacent to a cysteine residue, and in this case only one residue removed from the C terminus, has important implications concerning its accessibility for possible interaction with disulfide substrates during catalysis. In view of the fact that a sulfur analog of the enzyme with a deduced C-terminal sequence of Cys-Cys-Gly presumably exists in *Caenorhabditis elegans* (22), the catalytic activities of the two pure enzymes will be very interesting to compare. In contrast to the Secys-containing enzyme, overproduction of the naturally occurring cysteine mutant form should not be difficult.

Human placental TR shares a strong homology with other NADPH-dependent FAD-containing oxidoreductases including human and *Escherichia coli* glutathione reductases, *Trypanosoma cruzi* trypanothione reductase, *C. elegans* TR, and dihydrolipoamide dehydrogenase (16). The TRs from *E. coli* and other bacteria, in contrast, lack a strong homology with the human TR, indicating significant differences in structure. Based on detailed studies of glutathione reductases and the strong homology with human TR, it was suggested that the putative N-terminal FAD-binding domain of the latter contains the redox active disulfide moiety that is reduced by transfer of reducing equivalents from NADPH via FAD (16). Utilization of the resulting dithiol to reduce the disulfide of a thioredoxin substrate would by analogy involve a conserved histidine residue, H472 (Table 2), in the C-terminal region to facilitate the redox transfer. However, in contrast to glutathione reductase, the human TR contains an additional 16 amino acid C-terminal segment that includes the Secys-498 residue (Table 2). The precise role of this selenoamino acid, which previously was not known to be present in mammalian TRs, will be interesting to elucidate from a mechanistic point of view. A possible clue that it is a direct participant in the reduction of disulfide substrates comes from the observation that reaction of NADPH-reduced human and calf liver TRs with 1-chloro-2,4-dinitrobenzene caused rapid and complete inactivation of the enzymes with respect to their ability to

Table 1. Comparison of the deduced amino acid sequence of the C-terminal region of human placental TR with the Edman degradation sequence of a ^{75}Se-labeled tryptic peptide from human T-cell TR

	Sequence
Nucleotide sequence of C-terminal region of human placental TR	TCT-GGG-GCA-AGC-ATC-CTC-CAG-GCT-GGC-TGC-TGA-GGT-TAA
Deduced amino acid sequence of C-terminal region of human placental TR	S - G - A - S - I - L - Q - A - G - C -**end** (488 … 497)
Amino acid sequence of ^{75}Se-labeled tryptic peptide from human T-cell TR	S - G - A - S - I - L - Q - A - G - C*- U†- G -**end**
Yields of amino acid residues during Edman degradation sequence analysis (pmol)	39‡-119-130-25‡ -44‡-98 -83 -93 -75 -52 - 6

*Carboxymethylated Cys-497 was determined during Edman degradation based on the elution position of a carboxymethylated cysteine standard.
†U represents Secys. This residue is not detected during Edman degradation.
‡Yields for serine and isoleucine residues are usually lower than for other residues. The low yield of Gly-499 in cycle 12 is indicative of its poor binding to the sequencing column because this is the C-terminal residue in the peptide.

Table 2. Alignment of C-terminal regions of human glutathione reductase (GR), human TR, and *C. elegans* TR

		Sequence	
		* *** * ####	
Human TR	468	TIGIHPVCAEVFTTLSVTKRSGASILQAGCUG	499
C. elegans TR	468	TIAIHPCSSEEFVKLHITKRSGQDPRTQGCCG	499
Human GR	463	TVAIHPTSSEELVTLR	478

*, Conserved residues in NADPH-dependent disulfide oxidoreductase enzyme family. H467 and E472 of GR and corresponding residues of other enzymes of this family are involved in interaction with active center cysteines. #, Conserved C-terminal regions of human and *C. elegans* thioredoxin reductases. U represents Secys residue. Numbering in the sequence of human TR starts from the methionine residue that was suggested to initiate translation (16).

reduce thioredoxin or DTNB, whereas the alkylated enzymes exhibited greatly increased NADPH oxidase activities (23). In view of the known high reactivity of ionized selenol groups with alkylating agents, the Secys residue in the enzymes is the obvious target. Moreover, the slow rate of reaction of the alkylating agent with glutathione at pH 7.5 as compared with the almost 10^4 times faster rate of inactivation of the enzyme is consistent with the fact that a selenol is completely ionized at pH 7.5, whereas the cysteine thiol group of glutathione is only partially ionized. The ability of the alkylated enzyme to catalyze transfer of reducing equivalents from NADPH to oxygen suggests that a normal tightly coupled redox cycle involving the bound FAD moiety and Secys has been interrupted with the result that oxygen could then serve as direct electron acceptor for the reduced FAD.

In addition to disulfide-containing proteins such as thioredoxin and protein disulfide isomerase (24) that serve as substrates for mammalian TR, other unrelated substrates for the enzyme have been reported. These include DTNB, alloxan, menadione and oxidized lipoamide (14), Secys (25), and certain lipid hydroperoxides such as oxygenated forms of arachidonic acid (26). With the latter hydroperoxide substrates, a large rate enhancement of the reduction to the corresponding alcohols was observed when Secys (50-fold molar excess over enzyme) was included in the reaction mixtures. Similar large rate enhancements had been reported (27) for reduction of free and protein bound disulfides by selenols. The precise mechanism of the TR catalyzed peroxidase reaction, which presumably involves the Secys residues of the enzyme (two per protein dimer) together with the excess ionized free Secys formed enzymatically by reduction of selenocystine, is not established. In view of the fact that free Secys is present in biological systems in very low concentrations, the importance of TR as a peroxidase *in vivo* is unclear. However, based on these observations, one more type of antioxidant system with the potential of a protective role in the cell should be considered.

It is of interest that levels of TR in three different transformed cell lines (12, 19, 20) are approximately 10 times higher than the levels of enzyme reported in bovine liver (21), rat liver (14), human placenta (13), and human T cells. Whereas 3000- to 4000-fold enrichment was required to obtain homogeneous enzymes from the mammalian tissues, pure enzyme was obtained from transformed cells after only 150- to 500-fold enrichment (12, 19, 20). Depending on availability of NADPH, a ready supply of reducing equivalents can be maintained through the action of TR and this, together with the known high levels of glutathione in transformed cells, can be important factors in the rapid growth of tumors. Since selenium is a constituent of TR and other selenoproteins, the amount of this trace element in unsupplemented culture media may not be sufficient for the synthesis of the high levels of selenoenzymes found in transformed eukaryotic cells.

In view of the importance of the thioredoxin-TR system as a source of reducing equivalents for numerous essential cellular processes such as conversion of ribonucleotides to deoxyribonucleotides, reduction of protein disulfide groups and protein folding, reduction of methionine sulfoxide in proteins to methionine, reduction of thiol-specific peroxide reductase, redox regulation of DNA binding by transcription factors such as nuclear factor-κB and AP-1, and maintenance of the redox balance of cells, the recognition of Secys as a component of mammalian TR is especially significant (28). Previously the selenium-dependent glutathione peroxidases were the only Secys-containing enzymes known to have essential roles in antioxidant processes. The availability of the two NADPH-linked enzymes, TR and glutathione reductase, whose roles are to maintain levels of reduced thioredoxins and reduced glutathione in the cell, ensures the optimal function of numerous essential processes.

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1. Stadtman, T. C. (1996) *Annu. Rev. Biochem.*, in press.
2. Cone, J. E., Martin del Rio, R., Davis, J. N. & Stadtman, T. C. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2659–2663.
3. Gladyshev, V. N., Khangulov, S. V. & Stadtman, T. C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 232–236.
4. Gladyshev, V. N., Khangulov, S. V. & Stadtman, T. C. (1996) *Biochemistry* **35,** 212–223.
5. Böck, A., Forchhammer, K., Heider, J., Leinfelder, W., Sawers, G., Veprek, B. & Zinoni, F. (1991) *Mol. Microbiol.* **5,** 515–520.
6. Hatfield, D. L., Choi, I. S,. Ohama, T., Jung, J.-E. & Diamond, A. M. (1994) in *Selenium in Biology and Human Health*, ed. Burk, R. F. (Springer, New York), pp. 26–44.
7. Berry, M. J., Banu, L., Chen, Y., Mandel, S. J., Kieffer, J. D., Harney, J. W. & Larsen, P. R. (1991) *Nature (London)* **353,** 273–276.
8. Ursini, F., Maiorino, M., Brigelius-Flohe, R., Aumann, K. D., Roveri, A., Schomburg, D. & Flohe, L. (1995) *Methods Enzymol.* **252B,** 38–53.
9. Berry, M. J., Banu, L. & Larsen, P. R. (1991) *Nature (London)* **349,** 438–440.
10. Hill, K. E. & Burk, R. F (1994) in *Selenium in Biology and Human Health*, ed. Burk, R. F. (Springer, New York), pp. 117–132.
11. Vendeland, S. C., Beilstein, M. A., Chen, C. L., Jensen, O. N., Barofsky, E. & Whanger, P. D. (1993) *J. Biol. Chem.* **268,** 17103–17107.
12. Tamura, T. & Stadtman, T. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1006–1011.
13. Oblong, J. E., Gasdaska, P. Y., Sherrill, K. & Powis, G. (1993) *Biochemistry* **32,** 7271–7277.
14. Luthman, M. & Holmgren, A. (1982) *Biochemistry* **21,** 6628–6633.
15. Favier, A., Sappey, C., Leclerc, P., Faure, P. & Micoud, M. (1994) *Chem. Biol. Interact.* **91,** 165–180.
16. Gasdaska, P. Y., Gasdaska, J. R., Cochran, S. & Powis, G. (1995) *FEBS Lett.* **373,** 5–9.
17. Daher, R. & van Lente, F. (1994) *Clin. Chem.* **40,** 62–70.
18. Holmgren, A. & Bjornstedt, M. (1995) *Methods Enzymol.* **252B,** 199–208.
19. Chen, C.-C., McCall, B. L. B. & Moore, E. C. (1977) *Prep. Biochem.* **7,** 165–177.

20. Tsang, M. L.-S. & Weatherbee, J. A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7478–7482.
21. Martinez-Galisteo, E., Padilla, C. A., Garcia-Alfonso, C., Lopez-Barea, J. & Barcena, J. A. (1993) *Biochimie* **75,** 803–809.
22. Sulston, J., Du, Z., Thomas, K,. Wilson, R., Hillier, L., Staden, R., Halloran, N., Green, P., Thierry-Mieg, J,. Qiu, L., Dear, S., Coulson, A., Craxton, M., Durbin, R,. Berks, M., Metzstein, M., Hawkins, T., Ainscough, R. & Waterston, R. (1992) *Nature (London)* **356,** 37–41.
23. Arner, E. S. J., Bjornstedt, M. & Holmgren, A. (1995) *J. Biol. Chem.* **270,** 3479–3482.
24. Lundstrom, J. & Holmgren, A. (1990) *J. Biol. Chem.* **265,** 9114–9120.
25. Bjornstedt, M., Kumar, S. & Holmgren, A. (1992) *J. Biol. Chem.* **267,** 8030–8034.
26. Bjornstedt, M., Hamberg, M., Kumar, S., Xue, J. & Holmgren, A. (1995) *J. Biol. Chem.* **270,** 11761–11764.
27. Singh, R. & Kats, L. (1995) *Anal. Biochem.* **232,** 86–91.
28. Tamura, T., Gladyshev, V. N., Liu, S.-Y. & Stadtman, T. C. (1996) *BioFactors* **5,** 99–102.
29. Nagata, K., Ohtani, K., Nakamura, M. & Sugamura, K. (1989) *J. Virol.* **63,** 3220–3226.