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Redox Regulation of Cell Signaling by Selenocysteine in Mammalian Thioredoxin Reductases*

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The intracellular generation of reactive oxygen species, together with the thioredoxin and glutathione systems, is thought to participate in redox signaling in mammalian cells. The activity of thioredoxin is dependent on the redox status of thioredoxin reductase (TR), the activity of which in turn is dependent on a selenocysteine residue. Two mammalian TR isozymes (TR2 and TR3), in addition to that previously characterized (TR1), have now been identified in humans and mice. All three TR isozymes contain a selenocysteine residue that is located in the penultimate position at the carboxyl terminus and which is encoded by a UGA codon. The generation of reactive oxygen species in a human carcinoma cell line was shown to result in both the oxidation of the selenocysteine in TR1 and a subsequent increase in the expression of this enzyme. These observations identify the carboxyl-terminal selenocysteine of TR1 as a cellular redox sensor and support an essential role for mammalian TR isozymes in redox-regulated cell signaling.

Reactive oxygen species (ROS),[1] such as hydrogen peroxide, the superoxide anion radical, and the hydroxyl radical, have been thought of as toxic by-products of cellular oxygen metabolism. Excessive production of ROS or an insufficiency of antioxidant defenses has been implicated in apoptosis, aging, and cancer (1). However, recent evidence indicates that, at low concentrations, ROS mediate regulatory events and are essential participants in cell signaling (2). Redox signaling is thought to be achieved through the coupling of ROS with oxidation-reduction processes that involve essential thiol groups in proteins, resulting in the modulation of tyrosine or serine-threonine phosphorylation of target proteins (3, 4). The redox state of essential thiol groups is controlled by two cellular redox systems: the thioredoxin (thioredoxin, thioredoxin reductase (designated TR herein, but it should be noted that TrxR has also been used, *e.g.* see Ref. 5–8), and thioredoxin peroxidase) and glutathione (glutathione, glutathione reductase, glutaredoxin, and glutathione peroxidase (GPX)) systems (9). Although the role of ROS in intracellular signaling has been demonstrated (2, 10), the direct targets of these molecules remain poorly characterized, and the specific mechanisms through which ROS are sensed within mammalian cells are unclear (3, 4, 11).

Several proteins, including apoptosis signal-regulating kinase 1 (ASK1; this protein belongs to a family of mitogen-activated protein kinase kinase kinases) (12), protein-tyrosine phosphatase 1B (PTP1B) (13), and thioredoxin peroxidase (14), have been identified as components of redox signaling pathways that act downstream of the generation of intracellular ROS. The activities of these proteins are regulated by thioredoxin; reduced thioredoxin activates the phosphatase and peroxidase, and inactivates the kinase (12–14). Thioredoxin is a 12-kDa protein that catalyzes the reduction of protein disulfides and regulates a variety of processes including reduction of ribonucleotide reductase, protein-DNA interactions, and cellular growth (9). The redox state and activity of thioredoxin are in turn controlled by TR, a selenocysteine-containing flavoprotein composed of two identical 57-kDa subunits. The selenocysteine residue is located at the penultimate COOH-terminal position of TR, is encoded by a UGA codon (15–17), and is essential for enzyme activity (6, 18). TR is a member of the pyridine nucleotide-disulfide oxidoreductase family of enzymes, but other members of this family lack the COOH-terminal extension containing the selenocysteine residue (15, 16). The reactivity of TR with various alkylating agents, quinones, and gold-containing organic compounds is about 3 orders of magnitude greater than that of these other enzymes (for example, glutathione reductase and lipoamide dehydrogenase) (5, 7, 19). The inactivation of TR by such compounds has been attributed to the irreversible modification of the selenocysteine residue (6, 7, 18, 19).

Structurally, selenocysteine differs from cysteine by the single substitution of selenium for sulfur. Selenium is a better nucleophile than sulfur and, under physiological conditions, selenocysteine residues are ionized whereas cysteines are typically protonated (20, 21). These properties render selenocysteine a promising candidate for an ROS sensor in proteins. Indeed, several selenoproteins (including TR) and selenium compounds exhibit affinity for hydroperoxides and oxygen radicals (22–24).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF171053 (mTR3), AF171054 (hTR3), and AF171055 (hTR2).

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[1] The abbreviations used are: ROS, reactive oxygen species; TR, thioredoxin reductase; 5-IAF, 5-iodoacetamidofluorescein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PTP1B, protein-tyrosine phosphatase 1B; ASK1, apoptosis signal-regulating kinase 1; DNCB, 1-chloro-2,4-dinitrobenzene; EGF, epidermal growth factor; HPLC, high performance liquid chromatography; GPX1, glutathione peroxidase 1; GPX4, glutathione peroxidase 4; PAGE, polyacrylamide gel electrophoresis.

We have tested whether selenocysteine in mammalian thioredoxin reductase may be involved in redox regulation of cell signaling. We initially identified two new thioredoxin reductases, TR2 and TR3, and found that all three mammalian thioredoxin reductases conserve the COOH-terminal penultimate selenocysteine residue. We further found that, when cells generated ROS, selenocysteine in TR1 was oxidized while TR1 expression was elevated. The data suggest that the selenocysteine residue in thioredoxin reductases serves as a cellular redox sensor.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human thioredoxin was obtained from American Diagnostica; recombinant *Escherichia coli* thioredoxin, hydrogen peroxide, 1-chloro-2,4-dinitrobenzene (DNCB) and NADPH from Sigma; 5-IAF and anti-fluorescein antibodies from Molecular Probes; pre-cast NuPAGE polyacrylamide gels and immunoblot system from Novex; ADP-Sepharose, phenyl-Sepharose, and DEAE-Sepharose from Amersham Pharmacia Biotech; C_{18} and phenyl-high performance liquid chromatography (HPLC) columns from TosoHaas; mouse and human expressed sequence tag clones for TR1, TR2, and TR3 from Research Genetics, Genome Systems, and ATCC; mouse tissues from Pel-Freez; and mouse multiple tissue Northern blot from CLONTECH. Other reagents were of the highest grade available.

Isolation of TR1, TR2, and TR3—To obtain ^{75}Se-labeled tissues, we injected mice with 0.2 mCi of [^{75}Se]selenite (specific radioactivity 1000 Ci/mmol; University of Missouri Research Reactor) 2 days before tissue removal as described (25). Thioredoxin reductases were purified from ^{75}Se-labeled mouse liver and testis according to the previously published procedure (16), which consisted of the subsequent anion-exchange (DEAE-Sepharose), affinity (ADP-Sepharose), and hydrophobic-interaction (phenyl-Sepharose or phenyl-HPLC column) chromatographic procedures. To distinguish between TR isozymes, we assumed that, on the basis of the deduced protein sequences, TR3 is more basic than is either TR1 or TR2, and that the molecular mass of TR2 is higher than that of TR1 or TR3. Indeed, during purification, TR1, TR2, and TR3 had different affinities for DEAE-Sepharose and phenyl-Sepharose columns. Purification yielded homogenous preparations of ^{75}Se-labeled liver TR1, testis TR1, testis TR2, and liver TR3. The enzymes were detected during purification by their TR catalytic activities as described by Tamura and Stadtman (17), by the presence of ^{75}Se in fractions analyzed with a 5-detector Wallac γ-counter and by immunoblot assays. Rabbit antibodies specific for TR1 were generated against two keyhole limpet hemocyanin-conjugated peptides corresponding to residues 383–400 and 486–499 of human TR1, with the exception that selenocysteine 498 and cysteine 497 were replaced with serines. Rabbit antibodies specific for TR2 were generated in response to a keyhole limpet hemocyanin-conjugated peptide corresponding to residues 560–579 of human TR2, with the exception that selenocysteine was replaced with cysteine. Chicken antibodies specific for TR3 were generated in response to a keyhole limpet hemocyanin-conjugated peptide corresponding to residues 502–520 of human TR3.

Alkylation of TR1 with 5-IAF—The procedure for alkylation by 5-IAF of highly reactive, ROS-sensitive thiol or selenol groups in cell extracts was adapted from Wu *et al.* (26). Nearly confluent A431 cells were incubated for 16 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% fetal bovine serum (FBS), washed once with DMEM containing 0.1% FBS, and then incubated either for 0–45 min with EGF (500 ng/ml) in the same medium (Fig. 3*C*) or for 4 h in the same medium containing 100 μCi of [^{75}Se]selenite (Fig. 3*D*). The cells were washed four times with phosphate-buffered saline and frozen in liquid nitrogen. They were subsequently harvested at 4 °C by scraping, under anaerobic conditions, into an oxygen-free lysis buffer containing 50 mM Tris-HCl (pH 7.5), leupeptin (2 μg/ml), aprotinin (2 μg/ml), 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1% Nonidet P-40 detergent, and 0.04% NaN_3. Freshly prepared 5-IAF was added to a final concentration of 20 μM before scraping. The cell lysate was incubated under anaerobic conditions at 37 °C for 20 min, after which the alkylation reaction was quenched by the addition of 1 mM dithiothreitol.

After reaction with 5-IAF, the cell lysate was dialyzed against a solution containing 25 mM Tris-HCl (pH 7.5), leupeptin (2 μg/ml), and aprotinin (2 μg/ml), and TR1 was isolated from the dialysate by chromatography on ADP-Sepharose and DEAE-Sepharose columns. The purified enzyme was characterized by SDS-PAGE, PhosphorImager analysis of ^{75}Se, and immunoblot analysis with antibodies to TR1 or to fluorescein. The 5-IAF-modified enzyme (50 μg) was then further reduced with dithiothreitol in the presence of guanidine HCl, reacted with iodoacetamide, and digested with trypsin, and the resulting peptides were separated by reversed-phase HPLC on a C_{18} narrow-bore column with a gradient of acetonitrile as described previously (16). The protein mixture contained in the ADP-Sepharose flow-through fraction, which was obtained during TR1 purification, was also digested with trypsin in parallel with TR1, and the resulting peptides were separated on a C_{18} column under conditions identical to those used for fractionation of the TR1 tryptic digest. This TR1-deficient sample served as a control for the TR1 sample. The eluted peptides from the TR1 sample (Fig. 3*D*) and the TR1-deficient sample were detected by measurement of absorbance at 214 and 440 nm; absorbance at the latter wavelength was used to identify fluorescein-containing peptides. Only one major fluorescein-containing peak (fractions 56 and 57) was detected for the TR1 tryptic digest. This peak was not present in the fractions obtained from the control digest of the TR1-deficient sample, whereas several smaller peaks of absorbance at 440 nm present in the TR1 digest (Fig. 3*D*) were also apparent in the digest of the TR1-deficient sample. Fractions from the TR1 sample were analyzed for the presence of ^{75}Se with a γ-counter, and two peaks containing ^{75}Se were detected (Fig. 3*D*). The fractions containing ^{75}Se (39 and 56) were subjected to electrospray tandem mass-spectrometric analysis, which detected the COOH-terminal peptide in fraction 39 and the 5-IAF-labeled COOH-terminal peptide in fraction 56.

Regulation of TR1 Expression—A431 cells were cultured in the presence of 10% FBS to near-confluence and then incubated in DMEM containing 0.1% FBS for 16 h. The serum-deprived cells were washed once with DMEM containing 0.1% FBS, and then incubated for 4 h in DMEM containing 0.1% FBS and 20 μCi of [^{75}Se]selenite, and in the absence (control) or presence of either EGF (500 ng/ml), 0.2 mM H_2O_2, or 10–30 μM DNCB. Cells were then washed four times with phosphate-buffered saline and harvested by scraping in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), leupeptin (2 μg/ml), aprotinin (2 μg/ml), 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, sodium vanadate (2 mg/ml), 1% Nonidet P-40, and 0.04% NaN_3. The cells were sonicated and the resulting lysate was centrifuged at 15,000 $\times$ *g* for 30 min, after which the supernatant (3 μg of protein) was subjected to SDS-PAGE and either immunoblot analysis with antibodies specific for TR1 or PhosphorImager analysis.

RESULTS AND DISCUSSION

Conservation of Selenocysteine in Mammalian Thioredoxin Reductases—Only one mammalian TR isoform (TR1) has been identified to date (15). We searched the human and mouse dbEST data bases with human and rat TR1 sequences and found several homologous human and mouse sequences. We determined the sequences of the corresponding cDNAs by nucleotide sequencing of expressed sequence tag clones, and identified two new TRs (TR2 and TR3) in humans and mice (Fig. 1, *A* and *B*). The deduced sequences of the three TR isozymes share >50% identity and show conservation of all functional motifs, including two active-site redox cysteines in the NH_2-terminal region, the NADPH- and FAD-binding residues, and a dimer interface domain (Fig. 1, *A* and *B*). The 3′-untranslated region sequences of all human and mouse TR isozyme mRNAs contain selenocysteine insertion sequence elements (data not shown) that are characteristic of all mammalian selenoproteins (27). The TR isozymes are predicted to contain a selenocysteine residue encoded by UGA in a conserved COOH-terminal GCUG sequence (U, selenocysteine) (Fig. 1, *B* and *C*). For human proteins, TR3 displays 52–53% identity with either TR1 or TR2, while the latter two proteins are 70% identical. This suggests a closer evolutional link between TR1 and TR2, while TR3 is more distantly related to TR1 and TR2.

Computer search analysis of the non-redundant data base with the human cDNA sequence revealed matches to the previously determined genomic DNA sequences (accession numbers AC000078, AC000080, AC000090) and the complete human TR3 gene sequence was derived from these sequences. The human TR3 gene spans ~65 kilobases in the DiGeorge region on chromosome 22q11.2 and has an unusual gene structure. The 5′-end of this gene, including 5′-untranslated region and

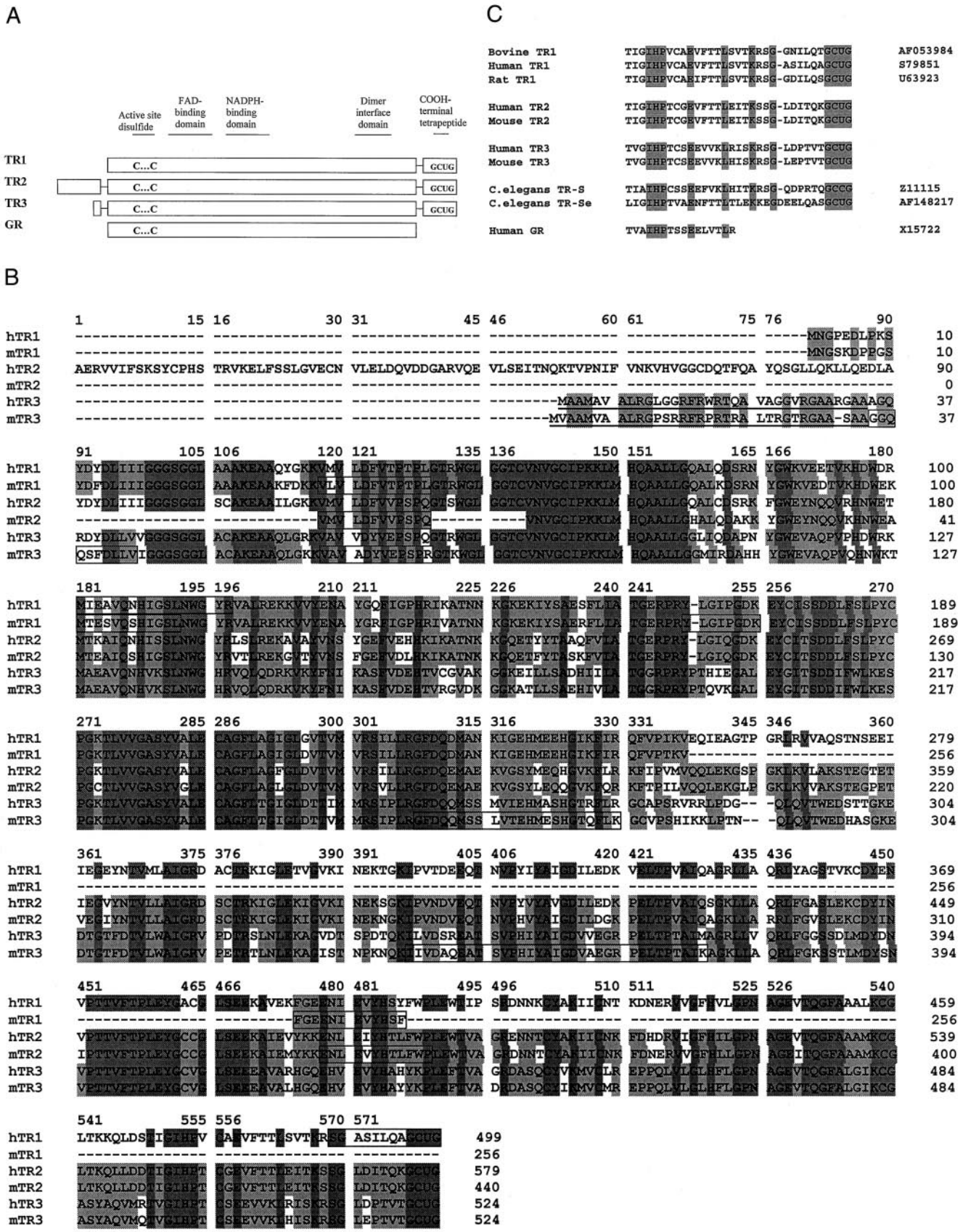

FIG. 1. **Primary structures of mammalian TR isozymes.** *A*, schematic representation of the domain organization of TR1, TR2, TR3, and glutathione reductase (*GR*). These four proteins possess active center disulfides, FAD (flavin adenine dinucleotide)- and NADPH (reduced form of nicotinamide adenine dinucleotide phosphate)-binding domains, and other features of the pyridine nucleotide-disulfide oxidoreductase family. TR1, TR2, and TR3 also possess a COOH-terminal extension containing a conserved GCUG tetrapeptide sequence. TR3 contains a short NH_2-terminal extension with a putative mitochondrial-targeting signal. TR2 contains a long NH_2-terminal extension. *B*, alignment of the deduced amino acid sequences of human TR1 (hTR1) (GenBank accession no. S79851), mouse TR1 (mTR1, partial), hTR2 (partial), mTR2 (partial), hTR3, and mTR3 sequences. Residues conserved between corresponding human and mouse isozymes are highlighted in *light gray*, and those conserved among all

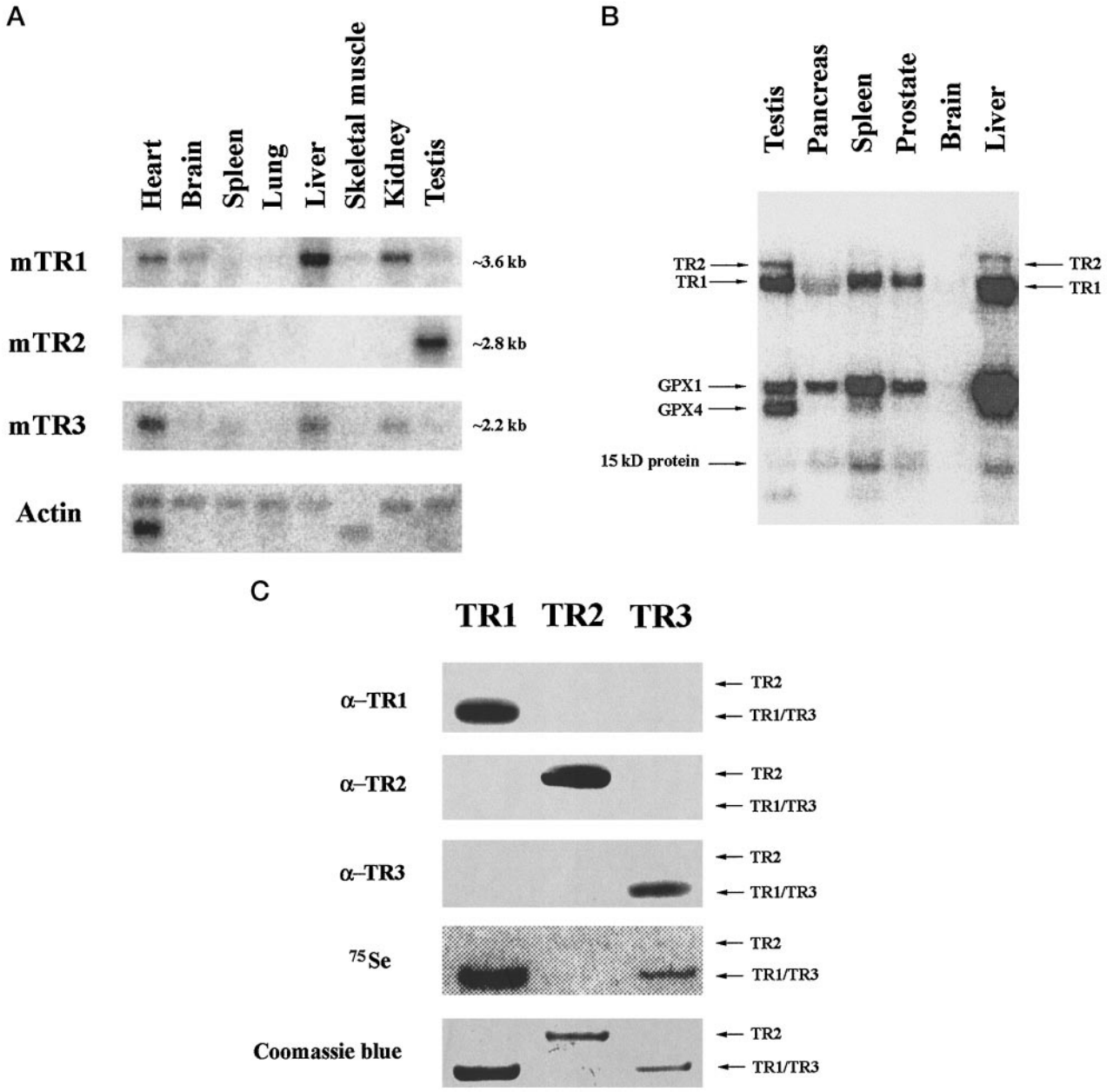

FIG. 2. **Characterization of TR1, TR2, and TR3.** *A*, abundance of TR1, TR2, and TR3 mRNAs in various mouse tissues. A mouse multiple-tissue Northern blot (CLONTECH) was developed with cDNA probes specific for mouse TR1, TR2, TR3, and actin mRNAs. The sizes of transcripts (in kilobases) are shown on the *right*. *B*, abundance of TR1 and TR2 proteins in various mouse tissues. ^{75}Se-labeled proteins in mouse tissues were separated by SDS-PAGE and detected by PhosphorImager (Molecular Dynamics) analysis. TR3 is less abundant than TR1 and is not apparent in this figure. The ^{75}Se band that appears in the liver sample slightly above the position of testis TR2 is most likely not TR2. The positions of glutathione peroxidases GPX1 and GPX4 and of the 15-kDa selenoprotein (which are the other major cellular selenoproteins), in addition to those of TR1 and TR2, are indicated. *C*, characterization of purified preparations of mouse liver TR1, testis TR2, and liver TR3. The purified proteins were subjected to SDS-PAGE and immunoblot analysis with antibodies specific for TR1 (*top panel*), for TR2 (*second panel*), or for TR3 (*third panel*); ^{75}Se detection (for ^{75}Se-labeled proteins) by PhosphorImager analysis (*fourth panel*); or Coomassie Blue staining (*bottom panel*). The ^{75}Se-labeled band for TR2 is not clearly visible because the protein was isolated from testis with a low ^{75}Se specific radioactivity. The positions of TR2 and of TR1 and TR3 are indicated on the *right*. It is not clear why TR1 mRNA is barely detectable in testis (see *A*), when the level of the corresponding protein is high in this tissue (see *B*). It should be noted that the Northern blots were carried out on a commercial filter that had been stripped and rehybridized several times and that the strains of mice used for mRNA and protein analysis were different.

the beginning of the open reading frame, overlaps in different orientations with the 5′-untranslated region of the membrane-bound catechol *O*-methyltransferase gene (data not shown). The physiological significance of this overlap and the possible relevance of TR3 to DiGeorge syndrome will require further studies.

mammalian TR isozymes are highlighted in *dark gray*. Sequences of internal tryptic peptides indicated by *boxes* were experimentally verified by tandem mass-spectrometry (hTR1 and mTR3) or Edman degradation (mTR1, mTR2, and mTR3). The putative NH_2-terminal mitochondrial signal in hTR3 and mTR3 is *underlined*. Residue numbers for each protein are shown on the *right*. *C*, alignment of the COOH-terminal sequences of mammalian and *C. elegans* TR enzymes. Human glutathione reductase, which is highly homologous to TR but lacks the COOH-terminal extension, is shown for comparison. Selenocysteine is replaced with cysteine in one form of *C. elegans* TR. Residues conserved among all TRs are *shaded*. GenBank accession numbers are indicated on the *right*. *U*, selenocysteine.

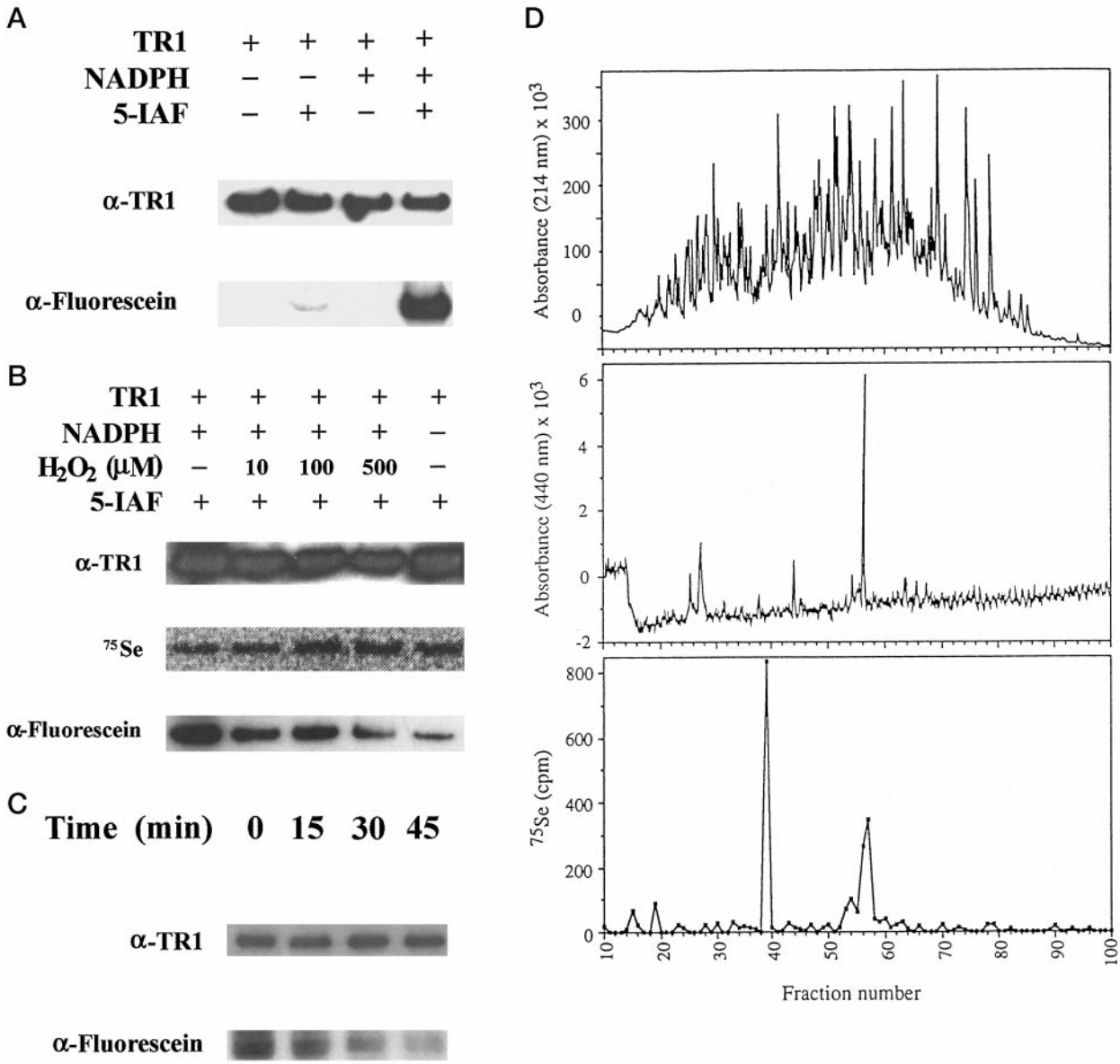

FIG. 3. **Role of TR1 in redox regulation of cell signaling.** *A*, alkylation of NADPH-reduced, but not of oxidized, TR1 by 5-IAF. NADPH-reduced or oxidized TR1 was incubated for 10 min at 25 °C in the presence or absence of 20 μM 5-IAF, and was then subjected to immunoblot analysis with antibodies to TR1 (*upper panel*) or to fluorescein (*lower panel*). *B*, oxidation by hydrogen peroxide of NADPH-reduced TR1 *in vitro*. ^{75}Se-Labeled TR1 was reduced for 10 min with 100 μM NADPH, oxidized for 10 min with the indicated concentration of H_2O_2, and then incubated for 10 min with 20 μM 5-IAF. The protein was then subjected to SDS-PAGE and either immunoblot analysis with antibodies specific for TR1 (*top panel*) or for fluorescein (*bottom panel*), or to PhosphorImager analysis of ^{75}Se (*middle panel*). *C*, effect of stimulation of A431 cells with EGF on the redox state of TR1 *in vivo*. Serum-deprived A431 cells were incubated with EGF (500 ng/ml) for the indicated times, after which cell lysates were prepared under anaerobic conditions and incubated for 20 min with 20 μM 5-IAF. TR1 was then isolated from reacted cell lysates using ADP-Sepharose and analyzed by immunoblot assay with antibodies to either TR1 or fluorescein. *D*, co-elution of ^{75}Se-labeled and 5-IAF-targeted tryptic peptides of TR1. A431 cells were incubated with [^{75}Se]selenite for 4 h, after which cell lysates were prepared under anaerobic conditions and exposed for 20 min to 20 μM 5-IAF. TR1 was then isolated from the lysates and digested with trypsin, and the resulting peptides were fractionated by reversed-phase HPLC. Absorbance at 214 nm (*upper panel*) and 440 nm (*middle panel*) was used to detect peptides and fluorescein, respectively. The *lower panel* shows the ^{75}Se radioactivity profile. *E*, specific alkylation of selenocysteine in TR1 by 5-IAF. Fraction 56 from *D*, containing both ^{75}Se and fluorescein, was subjected to mass spectrometry analysis to determine the mass of the peptide, followed by amino acid sequencing by tandem mass spectrometry. The experimentally determined mass for the 5-IAF-labeled peptide in fraction 56, $[M + 2H]^{2+} = 779.73$, matched the predicted mass for the COOH-terminal tryptic peptide of TR1 that is modified with a single 5-IAF group (shown as *circled F*) and a single iodoacetamide group (shown as *circled A*), $[M + 2H]^{2+} = 779.48$. This double-charged ion was completely fragmented and is not seen in this figure. y_n and b_n are the ions in which amino acid residues are sequentially cleaved from the NH_2-terminal and COOH-terminal, respectively. Importantly, the y_2 ion defines the selenocysteine-glycine COOH-terminal dipeptide modified with 5-IAF, and the b_{10} ion defines the SGAS-ILQAGC peptide containing cysteine that is not modified with 5-IAF. Neither the corresponding y_2 ion containing unmodified selenocysteine nor b_{10} ion containing a 5-IAF-modified cysteine were detected. This suggests that selenocysteine was modified, but cysteine was not, with 5-IAF. The predicted molecular masses for fragment ions of types b and y are shown under the sequence, and ions observed in the spectrum are *underlined*. *m/z*, mass/charge ratio. *F*, effects of stimulation of A431 cells with EGF or H_2O_2 on the expression of TR1. Cells were incubated for 4 h in the absence (*control*) or presence of EGF (500 ng/ml) or 0.2 mM H_2O_2, after which cell lysates were subjected to immunoblot analysis with antibodies to TR1 or to thioredoxin (*Trx*). *G*, effects of stimulation of A431 cells with EGF or H_2O_2 on the abundance of ^{75}Se-labeled TR1, GPX1, and GPX4. Cells were labeled with [^{75}Se]selenite in the absence (control) or presence of EGF (500 ng/ml) or 0.2 mM H_2O_2 for 4 h, after which cell lysates were subjected to SDS-PAGE and PhosphorImager analysis of ^{75}Se-labeled proteins. TR3 is less abundant than TR1 and is not apparent on this figure. *H*, effect of DNCB on the intracellular abundance of TR1. A431 cells were incubated for 4 h with [^{75}Se]selenite and the indicated concentrations of DNCB, after which cell lysates were subjected to SDS-PAGE and PhosphorImager analysis of ^{75}Se-labeled proteins.

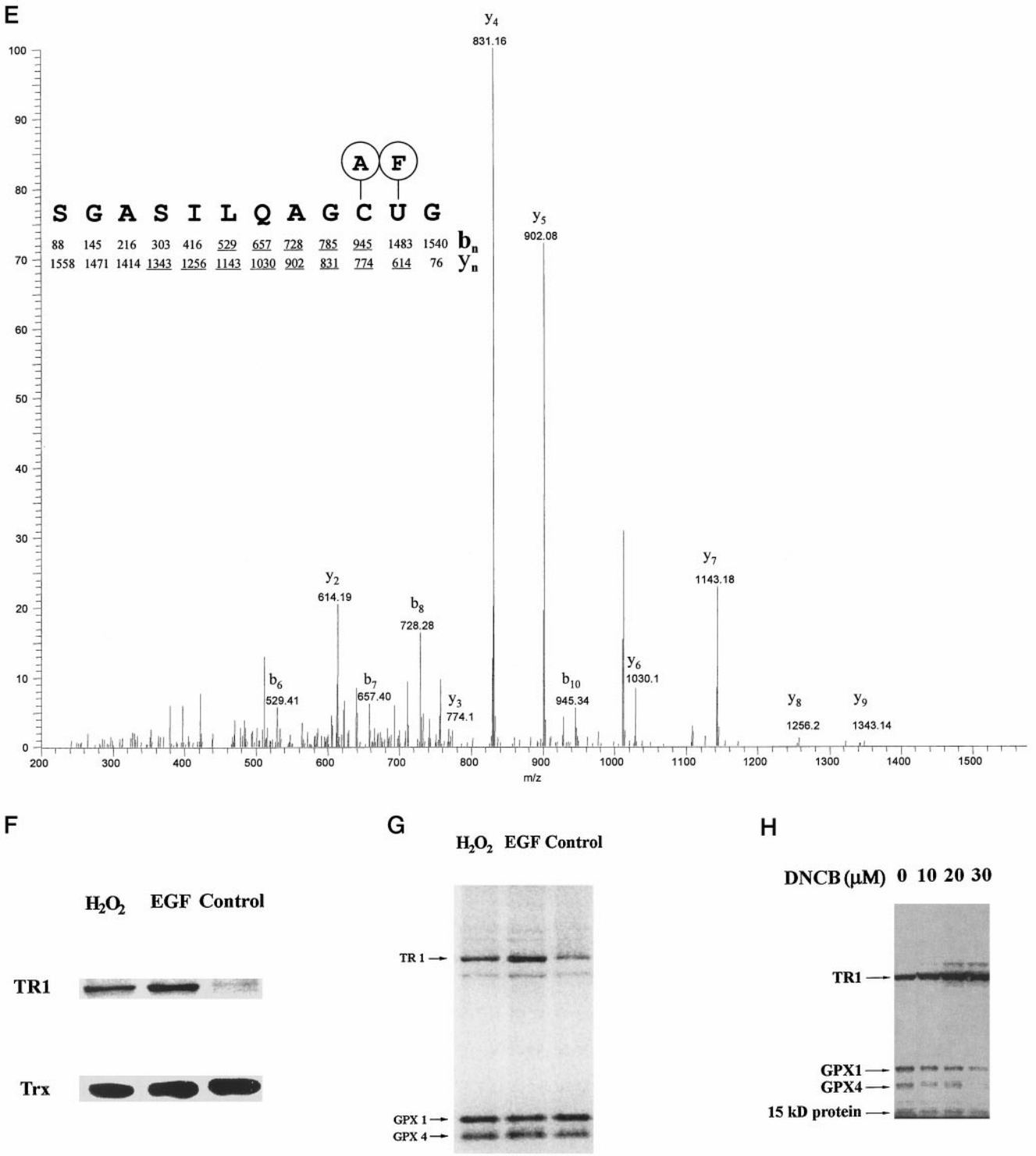

FIG. 3.—*continued*

Northern (RNA) hybridization and analysis of ^{75}Se-labeled mouse proteins suggested that TR1 and TR3 are expressed in a variety of tissues, being especially abundant in liver, whereas TR2 is preferentially expressed in testis (Fig. 2, *A* and *B*). We therefore directly purified to homogeneity, from ^{75}Se-labeled mouse tissues, TR1 from mouse liver and testis, TR2 from mouse testis, and TR3 from mouse liver (Fig. 2*C*). The identities of all TR isozymes were confirmed by immunoblot analysis with isozyme-specific antibodies to TR1, TR2, and TR3, and by comparison of the sequences of tryptic peptides with the predicted TR1, TR2, and TR3 amino acid sequences (Fig. 1*B*). In contrast to TR1 and TR3 which migrated as ~57-kDa species on SDS-PAGE gels, TR2 had a higher, ~65-kDa, molecular mass (Fig. 2*C*) that is consistent with the coding region derived from the cDNA sequence.

TR1 exists in the cytoplasm (28). TR3 has a putative mitochondrial signal (the enrichment of arginines in the NH_2-terminal structure that forms an α-helix) (Fig. 1, *A* and *B*), suggesting that it may be a mitochondrial enzyme. The experimentally determined NH_2-terminal sequence of mouse

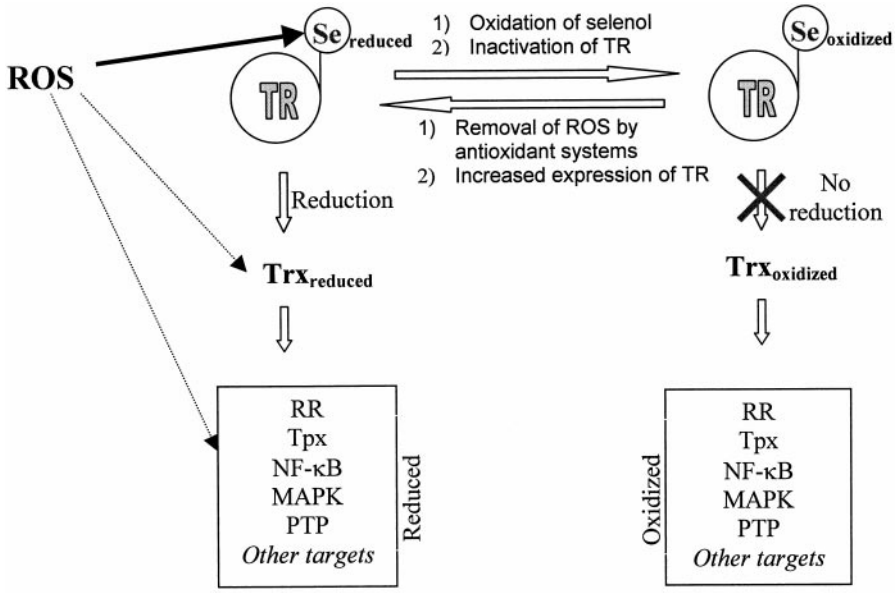

FIG. 4. **Model for the role of TR in redox regulation of cell signaling.** *Trx*, thioredoxin; *RR*, ribonucleotide reductase; *Tpx*, thioredoxin peroxidase; *MAPK*, mitogen-activated protein kinase; *PTP*, protein-tyrosine phosphatase. See text for details.

liver TR3 is GGQQSFDLLV, indicating the post-translational processing of first 34 residues. This processing is consistent with the apparently similar mobility of mouse liver TR1 and TR3 on SDS-PAGE gels. Thioredoxin reductase has recently been isolated from mitochondria, and the properties of the enzyme are different from those of TR1 (29). It is possible that three mammalian TRs have related functions in different cellular compartments.

Redox Regulation of Cell Signaling by Selenocysteine in TR1—On the basis of the redox properties of the selenocysteine in TR1 (5–7, 9, 18, 19, 22, 23) and on the basis of conservation of selenocysteine in mammalian TRs, we investigated whether oxidation of selenocysteine in TR isozymes by ROS might contribute to redox signaling. Specifically, we examined the effect of ROS on the redox state of TR1, which we monitored by alkylating thiol and selenol groups in the enzyme with 5-IAF and subsequently detecting fluorescein-modified protein by immunoblot analysis (26). We initially confirmed that purified mouse liver TR1 did not react with 5-IAF, whereas the NADPH-reduced form of the enzyme was alkylated by this compound (Fig. 3*A*). Thus, changes in the fluorescein content of 5-IAF-treated TR1 reflect the redox state of the enzyme.

With this approach, we further tested whether incubation of NADPH-reduced TR1 with hydrogen peroxide leads to oxidation of the enzyme. In the presence of 5-IAF, oxidation of the enzyme would be reflected by the decreased level of fluorescein in immunoblots with anti-fluorescein antibodies. We found that an increasing concentration of hydrogen peroxide from 0 to 500 μM indeed results in lowering the fluorescein signal (Fig. 3*B*, *bottom panel*), while not affecting the protein level (Fig. 3*B*, *upper panel*) or the amount of ^{75}Se present in the protein (Fig. 3*B*, *middle panel*). This suggests that hydrogen peroxide oxidized the thiol and/or selenol groups in the enzyme in the presence of NADPH.

To assess intracellular changes in the redox state of TR1, we used human epidermoid carcinoma A431 cells, which generate ROS (predominantly, hydrogen peroxide) in response to stimulation with EGF (10). In these cells, ROS induce tyrosine phosphorylation of proteins as a result of initial oxidation and subsequent thioredoxin-dependent reduction of PTP1B (13). The analogous reversible changes in the redox status of protein-disulfide isomerase, a thioredoxin homolog and substrate for TR1, also occur in EGF-stimulated cells (30). Exposure of A431 cells to EGF for up to 45 min resulted in a time-dependent oxidation of TR1 (Fig. 3*C*), indicating that oxidation of one or more thiol or selenol groups in TR1 occurs in response to the generation of ROS in EGF-treated cells. This is consistent with previous observation of oxidation of the 55-kDa protein, which was suggested to be thioredoxin reductase (30).

To identify the TR1 residues oxidized by ROS intracellularly, we metabolically labeled A431 cells with ^{75}Se, incubated the labeled cell lysate with 5-IAF, and then isolated TR1. The purified 5-IAF-modified TR1 was then subjected to further alkylation with iodoacetamide (to protect previously unmodified thiol or selenol groups) followed by digestion with trypsin, after which the resulting peptides were separated by reversed-phase HPLC. Analysis of fluorescein and ^{75}Se in the column fractions revealed the presence of two ^{75}Se-containing peptides but only one fluorescein-containing peptide (Fig. 3*D*), the latter of which co-eluted with the second ^{75}Se-containing peptide. Sequence analysis by tandem mass-spectrometry identified the COOH-terminal selenocysteine-containing tryptic peptide in both fractions containing ^{75}Se. Only the second eluted COOH-terminal tryptic peptide contained a fluorescein moiety, and the only site of modification was the selenocysteine residue (Fig. 3*E*). These observations indicated that the selenol group in TR1 is the only site of modification by 5-IAF, suggesting that selenocysteine is the only residue of TR1 oxidized by ROS in A431 cells.

Prolonged (4 h) incubation of A431 cells with EGF resulted in an ~5-fold increase in the intracellular abundance of TR1, as revealed by immunoblot analysis (Fig. 3*F*). The amount of TR1 was also increased as a result of incubation of A431 cells with hydrogen peroxide. The TR1 present in cells after prolonged stimulation of cells with EGF or H_2O_2 is most likely in a reduced state, given that EGF- or H_2O_2-stimulated cells exhibit a transient (<30 min) increase in ROS (10) and a corresponding transient (<1 h) oxidation of PTP1B (13) and protein-disulfide isomerase (30). The abundance of thioredoxin, the substrate of TR1, was not affected by prolonged incubation of A431 cells with either EGF or H_2O_2 (Fig. 3*F*).

The increase in the intracellular abundance of TR1 induced by prolonged incubation of cells with EGF or H_2O_2 was also apparent by labeling the cells with ^{75}Se and subsequent detection of labeled proteins by SDS-PAGE (Fig. 3*G*). The latter approach (25, 31) also detected other major antioxidant Se-containing enzymes revealing that the intracellular concentrations of cytosolic glutathione peroxidase (GPX1) and phospho-

lipid hydroperoxide glutathione peroxidase (GPX4) were not substantially affected by stimulation of cells with EGF or H_2O_2.

Incubation of A431 cells with DNCB mimicked the effect of EGF in that it increased the expression of TR1 but not that of GPX1, GPX4, and the recently discovered 15-kDa selenoprotein (32) (Fig. 3*H*). DNCB specifically inhibits TR1 through irreversible modification of the selenocysteine residue (8). Incubation of 293 cells with 20 μM DNCB for 30 min induces activation of ASK1 through oxidation of the thioredoxin system, and TR1 inactivation by DNCB has been implicated as a critical trigger for this process (12). Thus, ROS and DNCB, which possess distinct chemical properties, both target selenocysteine in TR1 and appear to induce oxidation of the thioredoxin system and a subsequent increase in the expression of TR1. These data are also consistent with a mechanism in which the intracellular ROS pathway is regulated through the selenocysteine residue in TR1, rather than through effects on thioredoxin or glutathione peroxidases.

Our results suggest a model for the role of TR in redox-regulated cell signaling (Fig. 4). The intracellular generation of ROS results in the direct oxidation of the selenol group of TR (shown as a *solid arrow* in Fig. 4) and a consequent transient decrease in enzyme activity. The resulting oxidation of thioredoxin then affects thioredoxin-dependent cellular components, including transcription factors (such as nuclear factor κB (NF-κB)) (33), protein-tyrosine phosphatases (such as PTP1B), and antioxidant enzymes (such as thioredoxin peroxidase). In addition, ROS may directly target other proteins such as thioredoxin, phosphatases, and kinases with variable efficacy (shown as *dashed arrows* in Fig. 4). The system is recycled as a result of the subsequent increased expression of TR, and possibly through the NADPH-dependent reduction of the enzyme after removal of the ROS by antioxidant enzymes.

Additional characteristics of TR1 support a critical role for this protein in redox signal transduction. Thus, TR1 is coupled with Ca^{2+}-phosphoinositide signaling pathways (34), and it exhibits oncoprotein-like properties (35). Oncogene products often regulate cell growth by simultaneously promoting cell proliferation and sensitizing cells to apoptosis (36), both of which effects have been demonstrated for TR1 and thioredoxin (37–42). Furthermore, *Caenorhabditis elegans* contains two TR genes (Fig. 1*C*), one of which encodes a selenocysteine-containing enzyme that is the major, if not the only, selenoprotein in *C. elegans* (43), whereas the other encodes a protein with cysteine in place of selenocysteine. In contrast, the bacterial-type TR enzymes (also present in plants and yeast) show limited homology to animal TRs (5, 15) and lack the COOH-terminal selenocysteine-containing extension, suggesting a different role for TR in redox regulation in bacteria. The well-characterized bacterial redox signaling protein OxyR, a transcription factor, is directly activated by H_2O_2 and is inhibited by glutaredoxin through the thiol-disulfide exchange process (44).

Concluding Remarks—Our demonstration that the selenocysteine residue of TR1 senses the presence of ROS might explain the location of this residue at the penultimate position of the COOH terminus of the protein. Consistent with its sensory role, selenocysteine is conserved in the newly discovered TR2 and TR3. Selenocysteine is generally more reactive than cysteine as a result of the ionization and redox properties of selenium (20, 21). In addition to TRs, only 11 other mammalian selenoproteins are known (20, 21, 45). Removal of either selenocysteine tRNA or thioredoxin genes from the mouse genome results in embryonic death (46, 47). Finally, whereas the TR-thioredoxin system is important in signaling by ROS, a role of the glutathione system in signaling remains to be demonstrated. Thus, the former rather than the latter is possibly the major cellular redox signaling system.

Addendum—When this manuscript was ready for submission, two papers were published that describe the cloning and characterization of rat TrxR2 (48) and human TRβ (49). Based on sequences, these proteins are apparently the same proteins as TR3. From our study, TR1 and TR2 sequences are evolutionally more related to each other than to TR3. Therefore, we suggest naming the three thioredoxin reductases as presented in this paper.

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