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Cysteine (Cys) is inserted into proteins in response to UGC and UGU codons. Herein, we show that supplementation of mammalian cells with thiophosphate led to targeted insertion of Cys at the UGA codon of thioredoxin reductase 1 (TR1). This Cys was synthesized by selenocysteine (Sec) synthase on tRNA$^{[Ser]Sec}$ and its insertion was dependent on the Sec insertion sequence element in the 3′ UTR of TR1 mRNA. The substrate for this reaction, thiophosphate, was synthesized by selenophosphate synthetase 2 from ATP and sulfide and reacted with phosphoseryl-tRNA$^{[Ser]Sec}$ to generate Cys-tRNA$^{[Ser]Sec}$. Cys was inserted in vivo at UGA codons in natural mammalian TRs, and this process was regulated by dietary selenium and availability of thiophosphate. Cys occurred at 10% of the Sec levels in liver TR1 of mice maintained on a diet with normal amounts of selenium and at 50% in liver TR1 of mice maintained on a selenium deficient diet. These data reveal a novel Sec machinery-based mechanism for biosynthesis and insertion of Cys into protein at UGA codons and suggest new biological functions for thiophosphate and sulfide in mammals.

de novo synthesis | new biosynthetic pathway | selenium deficiency

Cysteine (Cys) is one of 20 natural amino acids commonly used in protein synthesis. It is encoded by the genetic code words UGC and UGU. Catalytic redox-active Cys residues in proteins are functionally similar to selenocysteine (Sec) (1). Sec, known as the 21st amino acid in the genetic code, is encoded by a UGA codon and is inserted cotranslationally during ribosome-based protein synthesis (2–4). However, for UGA codons to dictate Sec insertion rather than termination of protein synthesis, the corresponding mRNAs must also contain a stem-loop RNA structure, called the Sec insertion sequence (SECIS) element (5). SECIS elements have different structures in the three domains of life and are located in the 3′-UTR of eukaryotic genes, in the 3′-UTR or 5′-UTR of archaeal genes, and in the coding regions of bacterial genes (6).

Another unusual feature of Sec is that it is synthesized on its tRNA, tRNA$^{[Ser]Sec}$. tRNA$^{[Ser]Sec}$ is initially aminoacylated with serine by seryl-tRNA synthetase, then the serine moiety is modified to a phosphoseryl-tRNA$^{[Ser]Sec}$ intermediate by phosphoseryl-tRNA kinase (7), and the intermediate is finally converted to Sec-tRNA$^{[Ser]Sec}$ by Sec synthase (SecS) in eukaryotes and archaea (8, 9). In eubacteria, seryl-tRNA$^{[Ser]Sec}$ is a substrate for SecS, and this pathway for Sec synthesis does not involve an intermediate (10). The selenium donor compound for the SecS-catalyzed reaction, selenophosphate ($SePO_3$), is synthesized by selenophosphate synthetase 2 (SPS2) in mammals (11), and by a homologous protein SelD in prokaryotes (12). Sec insertion into proteins is generally highly specific, but under Se-deficient conditions, Cys can occur in the Sec position, although the means of generating this Cys was not established (13). It should also be noted that the specific insertion of Sec at UGA Sec codons can be compromised under other conditions in mammalian cells, e.g., in the presence of the aminoglycoside antibiotic, G418, wherein Sec was replaced with arginine by misreading and suppressing the UGA Sec codon in glutathione peroxidase 1 (GPx1; 14).

Sec and Cys are encoded by different codons and have different biosynthetic mechanisms (i.e., Cys is not a precursor for Sec and vice versa). We recently reported that UGA codes for both Sec and Cys in *Euplotes crassus* (15). However, the insertion of these two amino acids was specific and determined by the position of UGA codons within the ORFs and the availability of the SECIS element for interaction with the ribosome.

Our previous studies have shown that SecS utilizes $SePO_3$ and *O*-phosphoseryl-tRNA$^{[Ser]Sec}$ to synthesize Sec (8, 11). We now report that Cys is also synthesized on tRNA$^{[Ser]Sec}$ in vitro when *O*-phosphoseryl-tRNA$^{[Ser]Sec}$ was incubated with mammalian SecS and thiophosphate (SPO_3), that this de novo biosynthetic pathway for Cys also occurs in mammals, that Cys is inserted in vivo in place of Sec in thioredoxin reductase 1 (TR1) and TR3, and that this process is regulated by dietary selenium and availability of SPO_3.

Results

Cys Is Synthesized de Novo by the Sec Machinery. The newly discovered pathway of Cys biosynthesis on tRNA$^{[Ser]Sec}$ using purified enzymes involved in Sec biosynthesis is shown in Fig. 1. First, we found that incubation of *O*-phosphoseryl-tRNA$^{[Ser]Sec}$ with SPO_3 and mouse SecS (mSecS) yielded Cys demonstrating that SecS can utilize SPO_3 in place of $SePO_3$ (Fig. 1*A*). In addition, Cys was synthesized on tRNA$^{[Ser]Sec}$ when *O*-phosphoseryl-tRNA$^{[Ser]Sec}$ was incubated with SecS, mouse SPS2 (mSPS2), Na_2S, and ATP (Fig. 1*B*), whereas no Cys was produced if ATP was omitted (Fig. 1*C*) or if a control protein [thioredoxin (Trx)] replaced SecS in the reaction (Fig. 1*F*). These data show that mSPS2 produces an active sulfur donor from Na_2S and ATP. A reaction containing *Caenorhabditis elegans* SPS2 (cSPS2) in place of mSPS2 also yielded Cys (Fig. 1*D*), whereas *Escherichia coli* selenophosphate synthetase (SelD) showed weak activity in the same reaction (Fig. 1*E*). These results indicate that Cys can be synthesized de novo on tRNA$^{[Ser]Sec}$ using the components of the eukaryotic Sec biosynthesis machinery in the presence of an inorganic sulfur source.

The efficiencies of Sec and Cys synthesis were evaluated in vitro under different concentrations of substrates and different

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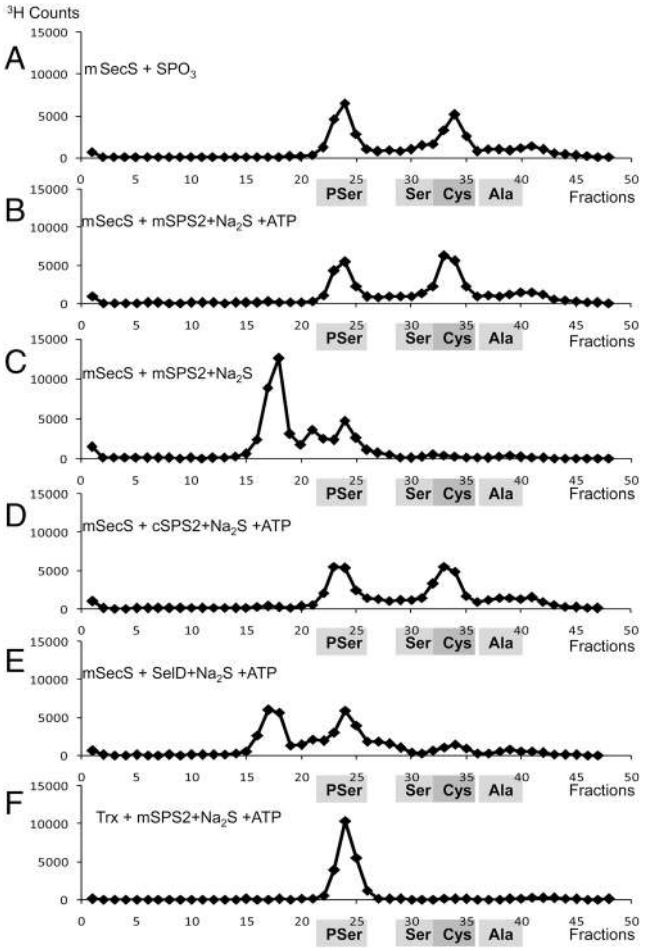

Fig. 1. In vitro Cys synthesis on tRNA$^{[Ser]Sec}$ by SecS. All reactions were carried out under anaerobic conditions in the presence of mSecS (unless otherwise indicated) and *O*-phospho-^{3}H-seryl-tRNA$^{[Ser]Sec}$. Cys synthesis was monitored by adding (*A*) SPO_3, (*B*) mSPS2, Na_2S, and ATP, (*C*) mSPS2 and Na_2S, (*D*) cSPS2, Na_2S, and ATP, (*E*) SelD, Na_2S, and ATP, or (*F*) a control protein, Trx, that included mSPS2, Na_2S, and ATP showed no activity in synthesizing Cys-tRNA$^{[Ser]Sec}$. Migration of amino acid standards is shown below each panel. Experimental details are given in *Materials and Methods*.

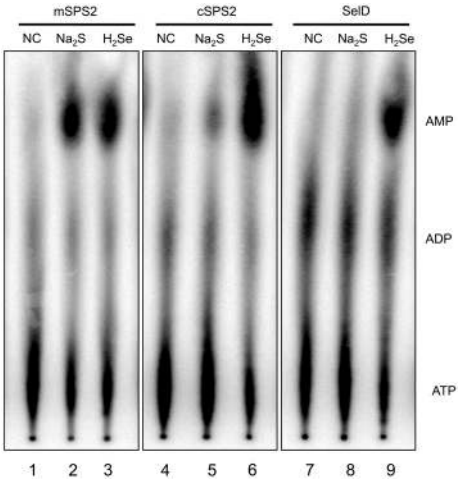

Fig. 2. Sulfide-dependent ATP hydrolysis by selenophosphate synthetases (SPSs). ATP hydrolysis reactions were carried out using [α-^{32}P]ATP in the presence or absence (designated NC, negative control) of either 5.0 mM sodium sulfide or 0.1 mM selenide. Three SPSs, mSPS2, cSPS2, and SelD, were examined. At the end of the incubation period, compounds were separated on PEI TLC plates and visualized by exposing to a PhosphorImager screen. Experimental details are given in *Materials and Methods*.

incubation times (Figs. S1 and S2). $SePO_3$ was about 5–10 times more efficient in generating Sec than SPO_3 was in generating Cys (Fig. S1, compare lanes 2 and 3 to lanes 7 and 8). When assessing Sec and Cys synthesis using *O*-phospho-[^{14}C]-seryl-tRNA$^{[Ser]Sec}$ as substrate, the generation of Sec was about twice as efficient as the generation of Cys (Fig. S2).

We further found that mSPS2 hydrolyzed ATP to AMP when Na_2S was present in the reaction mixture (Fig. 2, lane 2) or when H_2Se was present (Fig. 2, lane 3) as we have shown previously (8). cSPS2 had a weaker activity compared to mSPS2 (Fig. 2, lane 5), whereas SelD had no detectable activity (Fig. 2, lane 8). Because the product of the mSPS2-catalyzed reaction replaced SPO_3 in the biosynthesis of Cys, the data suggest that mSPS2 generated SPO_3. The efficiency of mSPS2 using selenide as substrate was approximately 50 times higher than when using sulfide as substrate (Fig. S3, compare lane 3 and 6).

Cys Is Inserted in Place of Sec in Mammalian Cells. To examine whether Cys synthesized on tRNA$^{[Ser]Sec}$ can compete with selenocysteyl-tRNA$^{[Ser]Sec}$ for insertion into proteins, we metabolically labeled NIH 3T3 cells with ^{75}Se in the presence or absence of SPO_3. Addition of SPO_3 to the medium inhibited incorporation of ^{75}Se into selenoproteins (Fig. 3*A*, *Upper*, compare lanes 1 and 2 to lanes 3 and 4). However, Western blot analyses showed that expression of some selenoproteins, such as TR1 and GPx4, was actually elevated, whereas GPx1 levels were decreased (Fig. 3*A*, *Middle*, lanes 3 and 4). The discrepancy between the levels of ^{75}Se-labeled protein and total protein suggested that an amino acid other than Sec was also inserted at the positions corresponding to UGA codons in TR1 and GPx4.

To identify the amino acid inserted at UGA in TR1, this enzyme was affinity isolated from NIH 3T3 cells grown in the presence or absence of SPO_3. Subsequent MS/MS analysis revealed that, in the presence of SPO_3, Cys was the main residue inserted and that it was 24-fold more abundant than Sec (Table 1, Exp. 1). Even in the absence of SPO_3, Cys could be detected (Sec/Cys ratio of 9). In addition, in NIH 3T3 cells transfected with a TR1 expression construct containing a His-tag, higher levels of TR1 were produced when SPO_3 was added to the medium (Fig. 3*B*, *Middle*, compare lanes 2 and 5), whereas the levels of Sec-containing TR1 were lower in SPO_3-treated cells than in nontreated controls (Fig. 3*B*, *Upper*, compare lanes 2 and 5). No full-length TR1 was produced in cells transfected with the TR1 construct lacking the SECIS element, either with or without SPO_3 treatment (Fig. 3*B*, see lanes 3 and 6, *Middle* and *Upper*). However, truncated TR1 was produced under these conditions (Fig. 3*B*, see lanes 3 and 6, *Middle*). These results indicate that the decoding of UGA by Cys requires the SECIS element and Cys-tRNA$^{[Ser]Sec}$, which can be synthesized by SecS using *O*-phosphoseryl-tRNA$^{[Ser]Sec}$ and SPO_3.

Cys Occurs in Vivo in TRs in Mammals. To determine if Cys is inserted at UGA codons in natural mammalian TRs, we affinity isolated TR1 and TR3 from livers of mice fed different selenium diets and subjected these enzymes to MS/MS analyses. Cys was detected in samples isolated from mice fed Se-deficient (0 ppm Se), Se-sufficient (0.1 ppm Se), and Se-enhanced diets (2.0 ppm Se). The amounts of Sec and Cys inserted into TR1 were about equal in the Se-deficient diet (Table 1, Exp. 2). Interestingly, in the Se-adequate diet, Cys insertion was still evident (~10% of Sec insertion). However, Cys was not detected in the case of high Se diet (2.0 ppm Se), indicating that dietary Se promotes Sec insertion while suppressing Cys insertion at UGA codons (Table 1, Exp. 2). Mitochondrial TR3 manifested somewhat similar insertion patterns of Cys and Sec in these dietary conditions (Table 1, Exp. 2). That is, Cys was found at the UGA coding site half of the

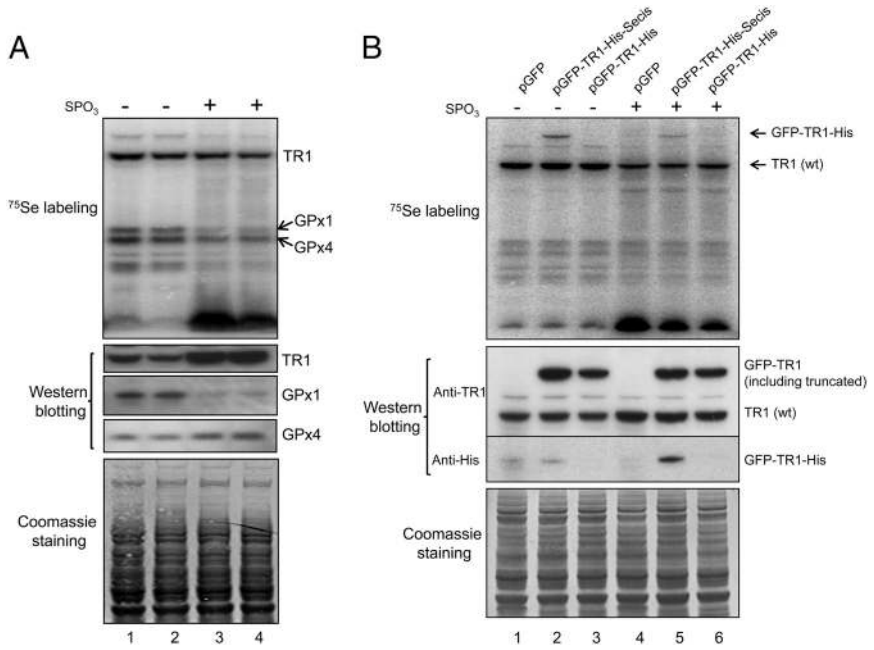

Fig. 3. Cys insertion into TR1 in NIH 3T3 cells in the presence of SPO_3. (*A*) NIH 3T3 cells were cultured without (lanes 1, 2, duplicates) or with (lanes 3, 4, duplicates) 1 mM SPO_3, labeled with ^{75}Se, and analyzed by SDS-PAGE for selenoprotein expression. (*Upper*) ^{75}Se labeling is shown, (*Center*) Western blot analyses of TR1, GPx4, and GPx1 are shown, and (*Lower*) Coomassie blue staining of proteins, used as a loading control, is shown. Experimental details are given in *Materials and Methods*. (*B*) NIH 3T3 cells were transfected with pGFP (the control vector encoding pGFP), pGFP-TR1-His-SECIS (encoding pGFP, TR1 with a His-tag at the C terminus and the SECIS element), or pGFP-TR1-His (encoding pGFP, TR1 with a His-tag at the C terminus but minus a SECIS element), grown either without (lanes 1–3) or with (lanes 4–6) 1 mM SPO_3, labeled with ^{75}Se, and analyzed by SDS-PAGE. (*Upper*) ^{75}Se labeling is shown, (*Center*) Western blot analysis with either anti-TR1 or anti-His antibody as designated is shown, and (*Lower*) Coomassie blue staining of proteins, used as a loading control, is shown. Experimental details are given in *Materials and Methods*.

time in the two TRs in Se-deficient diets, whereas both Cys and Sec were found in TR1 and TR3 in Se-adequate diets and only Sec in the Se-enriched diets.

Discussion

Our studies establish a previously undescribed pathway for Cys synthesis and insertion into proteins in mammals. The data show that in the presence of reduced inorganic sulfur, mSPS2 generates SPO_3, which can then be used by SecS to convert phosphoseryl-$tRNA^{[Ser]Sec}$ to Cys-$tRNA^{[Ser]Sec}$. This aminoacyl-tRNA form is then recognized by the Sec-specific elongation factor EFsec and inserts Cys at UGA codons in a SECIS-dependent manner. The levels of Cys in positions normally occupied by Sec are dependent on the sulfide/selenide ratio in cells. Hydrogen sulfide is a signaling molecule and a "third gas" (16). SPO_3 has recently found application for targeted thiophosphorylation of proteins (17) and was long known to be a toxic molecule. Sulfide and SPO_3 levels in mammals are low, and their increased levels

Table 1. C-terminal sequences of mouse TR1 and TR3 isolated from NIH 3T3 cells and livers of mice subjected to various Se diets*

Exp.†	Source of TRs	Protein	Peptide sequences	Sec, %‡	Cys, %‡
1	NIH 3T3, control	TR1	R.SGGDILQSGCUG	90	
		TR1	R.SGGDILQSGCCG		10
	NIH 3T3, SPO_3 treated	TR1	R.SGGDILQSGCUG	4	
		TR1	R.SGGDILQSGCCG		96
		TR1	K.RSGGDILQSGCCG		
2	Liver, 0 ppm Se	TR1	R.SGGDILQSGCUG	49	
		TR1	R.SGGDILQSGCCG		51
		TR3	K.RSGLEPTVTGCCG		
	Liver, 0.1 ppm Se	TR1	R.SGGDILQSGCUG	91	
		TR1	R.SGGDILQSGCCG		9
		TR1	K.RSGGDILQSGCUG		
		TR1	K.RSGGDILQSGCCG		
		TR3	R.SGLEPTVTGCUG		
		TR3	R.SGLEPTVTGCCG		
	Liver, 2.0 ppm Se	TR1	R.SGGDILQSGCUG	100	ND§
		TR1	K.RSGGDILQSGCUG		
		TR3	R.SGLEPTVTGCUG		
		TR3	K.RSGLEPTVTGCUG		

*TR1 and TR3 were affinity isolated from indicated sources and analyzed by MS/MS as described in *Materials and Methods*. U is Sec. Period designates the site of tryptic cleavage.

†Experiments 1 and 2 are described in the text. Typically, we consider a limit of detection at a signal-to-noise ratio of 3:1; and, thus, if the peptide ion's intensity is not greater than 3× the background noise intensity, then it is considered not detected.

‡Percent of Sec and Cys in the digested TR1 peptides were assessed by MS/MS as described in Materials and Methods.

§Not Detectable.

should interfere with Sec insertion due to the synthesis and insertion of Cys in Sec positions. Furthermore, even though Sec synthesis was much more efficient than Cys synthesis as assessed in vitro (Figs. S1–S3) in the established Sec biosynthetic pathway (8, 9), it is important to note that with adequate amounts of selenium in the diets of mice about 10% of the TR1 in liver contained Cys in place of Sec.

In mammals, Cys may arise by synthesis from methionine or transport into cells as cystine or Cys itself. However, the unique pathway for Cys biosynthesis reported herein shows that this amino acid can also be synthesized de novo from serine on a tRNA. This Cys is specifically inserted at UGA codons in place of Sec in TR1 and TR3, and its insertion is regulated by dietary selenium and availability of sulfide and thiophosphate. Cys is the main amino acid inserted when cells suffer from selenium deficiency; however, even under selenium sufficient conditions Cys insertion accounts for approximately 10% of Sec residues in TR1. Replacement of Cys for Sec in selenoproteins is known to reduce their activity manyfold, but some activity is still preserved (18–20), suggesting that Cys insertion may partially compensate for selenium deficiency. At the other end of the spectrum, selenium supplementation in the diet of mammals has often been viewed as beneficial to their health (21–23). Cys in place of Sec would indeed compromise TR activity, but whether such a reduction in activity may have additional consequences on the function of TRs, e.g., as a SecTRAP (selenium compromised thioredoxin reductase-derived apoptotic protein; 24) remains to be established. In this regard, our findings showing that enriched selenium prevents Cys insertion into selenoproteins suggests a previously undescribed role of dietary selenium in mammals: outcompeting Cys insertion in selenoproteins, thus maximizing their activity.

Materials and Methods

Materials. Materials were obtained as follows: NIH 3T3 cells were purchased from the American Type Culture Collection, [α-^{32}P]ATP (~800 Ci/mmol) and ^{3}H-serine (29.5 Ci/mmol) from PerkinElmer, Ni-NTA-agarose from Qiagen, Pfu DNA polymerase and pBluescript II from Stratagene, pET32b vector (encoding His-tagged Trx) and BL21(DE3) competent cells from Novagen, restriction enzymes from New England Biolabs, T7 RiboMAX Express Large Scale RNA Production System from Promega, 3-M filter paper from Whatman, and polyethyleneimine (PEI) TLC plates and unlabeled amino acids, sodium selenite, sodium thiophosphate (SPO_3; formula Na_3PO_3S), and sodium sulfide from Sigma-Aldrich. All other reagents were commercial products of the highest grade available.

Mice. Three-week-old, wild-type mice in a mixed background (C57BL/6/129) were placed (upon weaning) on a Torula yeast-based diet (Harlan Teklad) that was either not supplemented or supplemented with sodium selenite to obtain either 0 ppm Se, 0.1 ppm Se, or 2.0 ppm Se and maintained on the respective diets for 6 wk (25). The care of animals was in accordance with the National Institutes of Health (NIH) Institutional Guidelines under the expert direction of John Dennis [National Cancer Institute (NCI), NIH].

Protein Expression and Purification. Trx, mSecS, mouse *O*-phosphoseryl-tRNA kinase, mouse selenophosphate synthetase 2 (mSPS2) in which Cys replaced Sec in the catalytic site, cSPS2, and SelD were expressed and purified as described (8, 11). Proteins were dialyzed against Tris buffered saline for 2 h and stored at −20 °C in 50% glycerol before use.

In Vitro Cys Synthesis on tRNA$^{[Ser]Sec}$. Synthesis, purification, and aminoacylation of the tRNA$^{[Ser]Sec}$ were as previously described (7, 8). All reactions were carried out under anaerobic conditions due to the sensitivity of $SePO_3$ to oxygen in order to keep the conditions of all reactions as close to identical as possible, which were then followed by chromatographic analysis. To generate the sulfur donor for the reaction of Cys biosynthesis, a 10-μL mixture containing 20 mM ammonium bicarbonate, pH 7.0, 10 mM $MgCl_2$, 10 mM KCl, 5.0 mM Na_2S, and 2 μg of each examined SPS in the presence or absence of 2.5 mM ATP, was incubated for 1 h at 37 °C. SecS reactions were prepared in 10-μL mixtures of 20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 10 mM KCl, 1.0 μg of recombinant mouse SecS, and 5 μg (~5 μCi) of *O*-phospho-[^{3}H]-seryl-tRNA$^{[Ser]Sec}$, and either 10 μL of 0.5 mM SPO_3 or 10 μL of the SPS reactions above were added. Reaction mixtures were incubated at 37 °C for 1 h and then at 75 °C for 5 min to inactivate the enzyme, and the resulting aminoacyl-tRNAs were analyzed as described (8).

In Vitro ATP Hydrolysis Assay of SPS. The ATP hydrolysis reaction was carried out under anaerobic conditions in 20 mM ammonium bicarbonate, pH 7.0, 10 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.625 μM α-^{32}P-ATP, 2.5 μM ATP, 0.3 mg/mL of each examined enzyme, with or without 5.0 mM sodium sulfide or 0.1 mM sodium selenite. Following incubation at 37 °C for 40 min, 0.5 μL of each reaction was run on PEI TLC plates as described (8).

Construction of Recombinant TR1 Vectors. Mouse TR1 cDNA was cloned as described (26). The coding region of GFP was PCR amplified and inserted into the Nco I and EcoR V sites of the pTriEx-4 Hygro vector (designated pGFP) (Fig. S4*A*). The coding region of mouse TR1 minus the stop codon was PCR amplified and inserted between the EcoR I and Xho I sites of pGFP. This TR1 expression vector (designated pGFP-TR1-His) contained GFP at the N terminus and a 6-His-tag at the C terminus but lacked a SECIS element (Fig. S4*B*). Finally, the 3′-UTR of TR1 cDNA was PCR amplified and inserted into the Bsu36 I site after the 6-His-tag sequence. This TR1 expression vector (designated pGFP-TR1-His-SECIS) contained GFP at the N terminus, a 6-His-tag at the C terminus, and the intact SECIS element in the 3′-UTR (Fig. S4*C*). Cloning and expression of recombinant human TR1 (hTR1) and mouse TR3 (mTR3) vectors were carried out exactly as described (26).

Treatment of NIH 3T3 Cells with SPO_3. NIH 3T3 cells were cultured in DMEM supplemented with 10% FBS. For TR1 purification, cells were grown in 150 cm^2 flasks, treated with or without 1 mM of SPO_3 for 2 d, and harvested. For labeling NIH 3T3 cells with ^{75}Se, NIH 3T3 cells were cultured in 6-well plates with or without 1.0 mM SPO_3 for 24 h, 10 μCi/mL of ^{75}Se added, the cells incubated for an additional 24 h, harvested, the lysates analyzed by Western blotting, and ^{75}Se-labeled proteins visualized with a PhosphorImager as described (11).

For recombinant TR1 expression, NIH 3T3 cells were transfected with pGFP, pGFP-TR1-His-SECIS, or pGFP-TR1-His for 24 h in 6-well plates using Lipofectamine 2000 according to the manufacturer's instructions. Transfected cells were then split into two wells, 1.0 mM SPO_3 added into one well of each, and incubated for 24 h. Ten microcuries/milliliter of ^{75}Se was added to the cells and incubated for an additional 24 h. Transfected cells were harvested, cell lysates were analyzed by Western blotting, and the ^{75}Se-labeled proteins were visualized as described (11).

Purification of TR Selenoproteins and Liquid Chromatography (LC)-MS/MS. TR1 and TR3 were affinity purified on 2′,5′-ADP-Sepharose columns, the purified proteins reduced with DTT, followed by alkylation of the Cys and Sec residues with iodoacetamide as described in detail elsewhere (27, 28). Alkylated proteins were resolved by SDS-PAGE using Novex NU-PAGE system (Invitrogen) and stained with Coomassie blue. Protein bands were cut out and subjected to in-gel tryptic digestion and LC-MS/MS analysis. In-gel trypsin digestion of the destained protein bands was carried out for 16 h at 37 °C. The resulting peptide mixture was extracted from the gel slices and loaded into a fused silica microcapillary packed with Magic C18AQ beads (Michrom Bioresources). Reversed phase liquid chromatography was performed using an Agilent 1100 pump and a Famous autosampler (LC Packings). The peptides were eluted from the column with a 60-min acetonitrile gradient and detected using an LTQ-Orbitrap XL (Thermo-Fisher Scientific).

Database Analysis and Quantification. MS/MS spectra were searched against a concatenated IPI_Mouse database (version 3.60) (http://www.ebi.ac.uk/IPI/) using the Sequest algorithm (Version 28, Thermo-Fisher Scientific) and a 0.5% false discovery rate. Database search criteria were as follows: two missed cleavages, a precursor mass tolerance of 50 ppm, an MS/MS fragment ion tolerance of 0.8 Da, and the following variable modifications: oxidation (M), deamidation (NQ), and alkylation on Cys and Sec. Peptide abundances were calculated using the monoisotopic peak height from an averaged spectrum representing the entire chromatographic peak of interest. Prior to comparisons between samples, changes in total protein expression, and digestion efficiency were corrected by normalizing the abundances of the C-terminal peptides against another mTR peptide that was not observed or known to be modified and was identified with high confidence.

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1. Arner ES (2010) Selenoproteins—What unique properties can arise with selenocysteine in place of cysteine? *Exp Cell Res* 316:1296–1303.
2. Stadtman TC (1996) Selenocysteine. *Annu Rev Biochem* 65:83–100.
3. Hatfield DL, Gladyshev VN (2002) How selenium has altered our understanding of the genetic code. *Mol Cell Biol* 22:3565–3576.
4. Lu J, Holmgren A (2009) Selenoproteins. *J Biol Chem* 284:723–727.
5. Low SC, Berry MJ (1996) Knowing when not to stop: Selenocysteine incorporation in eukaryotes. *Trends Biochem Sci* 21:203–208.
6. Driscoll DM, Copeland PR (2003) Mechanism and regulation of selenoprotein synthesis. *Annu Rev Nutr* 23:17–40.
7. Carlson BA, et al. (2004) Identification and characterization of phosphoseryl-tRNA$^{[Ser]Sec}$ kinase. *Proc Natl Acad Sci USA* 101:12848–12853.
8. Xu XM, et al. (2007) Biosynthesis of selenocysteine on its tRNA in eukaryotes. *PLoS Biol* 5:e4.
9. Yuan J, et al. (2006) RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. *Proc Natl Acad Sci USA* 103:18923–18927.
10. Forchhammer K, Leinfelder W, Boesmiller K, Veprek B, Bock A (1991) Selenocysteine synthase from Escherichia coli. Nucleotide sequence of the gene (selA) and purification of the protein. *J Biol Chem* 266:6318–6323.
11. Xu XM, et al. (2007) Selenophosphate synthetase 2 is essential for selenoprotein biosynthesis. *Biochem J* 404:115–120.
12. Ehrenreich A, Forchhammer K, Tormay P, Veprek B, Bock A (1992) Selenoprotein synthesis in E. coli. Purification and characterisation of the enzyme catalysing selenium activation. *Eur J Biochem* 206:767–773.
13. Lu J, et al. (2009) Penultimate selenocysteine residue replaced by cysteine in thioredoxin reductase from selenium-deficient rat liver. *FASEB J* 23:2394–2402.
14. Handy DE, et al. (2006) Aminoglycosides decrease glutathione peroxidase-1 activity by interfering with selenocysteine incorporation. *J Biol Chem* 281:3382–3388.
15. Turanov AA, et al. (2009) Genetic code supports targeted insertion of two amino acids by one codon. *Science* 323:259–261.
16. Yang G, et al. (2008) H_2S as a physiologic vasorelaxant: Hypertension in mice with deletion of cystathionine gamma-lyase. *Science* 322:587–590.
17. Allen JJ, et al. (2007) A semisynthetic epitope for kinase substrates. *Nat Methods* 4:511–516.
18. Gromer S, et al. (2003) Active sites of thioredoxin reductases: Why selenoproteins? *Proc Natl Acad Sci USA* 100:12618–12623.
19. Kim HY, Gladyshev VN (2005) Different catalytic mechanisms in mammalian selenocysteine- and cysteine-containing methionine-R-sulfoxide reductases. *PLoS Biol* 3:e375.
20. Zhong L, Holmgren A (2000) Essential role of selenium in the catalytic activities of mammalian thioredoxin reductase revealed by characterization of recombinant enzymes with selenocysteine mutations. *J Biol Chem* 275:18121–18128.
21. Combs GF, Jr, Combs SB (1986) *The Role of Selenium in Nutrition* (Academic, Orlando, FL).
22. Rayman MP (2009) Selenoproteins and human health: Insights from epidemiological data. *Biochim Biophys Acta* 1790:1533–1540.
23. Clark LC, et al. (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *JAMA* 276:1957–1963.
24. Anestal K, Prast-Nielsen S, Cenas N, Arner ESJ (2008) Cell death by SecTRAPs: Thioredoxin reductase as a prooxidant killer of cells. *PLoS One* 3:e1846.
25. Davis CD, Zeng H, Finley JW (2002) Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice. *J Nutr* 132:307–309.
26. Yoo MH, et al. (2007) A new strategy for assessing selenoprotein function: siRNA knockdown/knock-in targeting the 3′-UTR. *RNA* 13:921–929.
27. Turanov AA, Su D, Gladyshev VN (2006) Characterization of alternative cytosolic forms and cellular targets of mouse mitochondrial thioredoxin reductase. *J Biol Chem* 281:22953–22963.
28. Sun QA, Gladyshev VN (2002) Redox regulation of cell signaling by thioredoxin reductases. *Methods Enzymol* 347:451–461.

Supporting Information

Xu et al. 10.1073/pnas.1009947107

SI Materials and Methods.

Materials. Materials were obtained as follows: NIH 3T3 cells were purchased from the American Type Culture Collection, [α-^{32}P] ATP (~800 Ci/mmol), ^{14}C-Ser (155 mCi/mmol), and ^{3}H-serine (29.5 Ci/mmol) from PerkinElmer, Ni-NTA-agarose from Qiagen, Pfu DNA polymerase and pBluescript II from Stratagene, pET32b vector [encoding His-tagged thioredoxin (Trx)] and BL21(DE3) competent cells from Novagen, restriction enzymes from New England Biolabs, T7 RiboMAX Express Large Scale RNA Production System from Promega, 3 M filter paper from Whatman, and polyethyleneimine (PEI) TLC plates and unlabeled amino acids, sodium selenite, sodium thiophosphate (SPO_3; formula Na_3PO_3S), and sodium sulfide from Sigma-Aldrich. All other reagents were commercial products of the highest grade available.

Mice. Mice were fed a selenium-deficient torula yeast basal diet supplemented with either no selenium, 0.1 ppm selenium, or 2.0 ppm selenium as described (1). The care of animals was in accordance with the National Institutes of Health (NIH) Institutional Guidelines under the expert direction of John Dennis (National Cancer Institute, NIH).

Protein Expression and Purification. Trx, mouse selenocysteine synthase (SecS), mouse *O*-phosphoseryl-tRNA kinase, mouse selenophosphate synthetase 2 (mSPS2) in which cysteine (Cys) replaced Sec in the catalytic site, *Caenorhabditis elegans* selenophosphate synthetase 2 and *Escherichia coli* selenophosphate synthetase were expressed and purified as described (2, 3). Proteins were dialyzed against Tris buffered saline for 2 h and stored at −20 °C in 50% glycerol before use.

In Vitro Cys Synthesis on tRNA$^{[Ser]Sec}$. Synthesis, purification, and aminoacylation of the tRNA$^{[Ser]Sec}$ were as previously described (2, 4). All reactions were carried out under anaerobic conditions, followed by chromatographic analysis. To generate the sulfur donor for the reaction of Cys biosynthesis, a 10-μL mixture containing 20 mM ammonium bicarbonate, pH 7.0, 10 mM $MgCl_2$, 10 mM KCl, 5.0 mM Na_2S, and 2 μg of each examined SPS in the presence or absence of 2.5 mM ATP, was incubated for 1 h at 37 °C. SecS reactions were prepared in 10-μL mixtures of 20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 10 mM KCl, 1.0 μg of recombinant mouse SecS, and 5 μg (~5 μCi) of *O*-phospho-[^{3}H]-seryl-tRNA$^{[Ser]Sec}$, and either 10 μL of 0.5 mM SPO_3 or 10 μL of the SPS reactions above were added. Reaction mixtures were incubated at 37 °C for 1 h, then at 75 °C for 5 min to inactivate the enzyme, and the resulting aminoacyl-tRNAs were analyzed as described (2). Sec and Cys synthesis was examined in the presence of different concentrations of selenophosphate or thiophosphate using 5 μg (~0.2 μCi) of *O*-phospho-[^{14}C]-seryl-tRNA$^{[Ser]Sec}$ in each reaction. For comparing Sec and Cys synthesis rates, reactions were carried out in a total volume of 20 μL of 50 mM Tris-HCl, pH 7.0, with 10 mM $MgCl_2$, 10 mM KCl and 4 μg (approximately 120 pmol) of *O*-phospho-[^{14}C]seryl-tRNA$^{[Ser]Sec}$ with either 100 μM SPO_3 or 10 μM $SePO_3$. Reactions were carried out and run on TLC as described (2) and exposed to a PhosphorImager screen. The band density of each product or substrate was quantified using ImageQuant.

In Vitro ATP Hydrolysis Assay of SPS. The ATP hydrolysis reaction was carried out under anaerobic conditions in 20 mM ammonium bicarbonate, pH 7.0, 10 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.625 μM α-^{32}P-ATP, 2.5 μM ATP, 0.3 mg/mL of each examined enzyme, with or without 5.0 mM sodium sulfide or 0.1 mM sodium selenite. For measuring the activity of mSPS2 under different concentrations of substrate, ATP hydrolysis reactions were carried out using [α-^{32}P]ATP and mSPS2 in the presence of either 0, 0.01, 0.1 mM selenide or 0.1, 1.0, 5.0 mM sodium sulfide. Following incubation at 37 °C for 40 min, 0.5 μL of each reaction was run on PEI TLC plates as described (2).

Construction of Recombinant TR1 Vectors. Mouse thioredoxin reductase 1 (TR1) cDNA was cloned as described (5). The coding region of GFP was PCR amplified and inserted into the Nco I and EcoR V sites of the pTriEx-4 Hygro vector (designated pGFP) (Fig. S4*A*). The coding region of mouse TR1 minus the stop codon was PCR amplified and inserted between the EcoR I and Xho I sites of pGFP. This TR1 expression vector (designated pGFP-TR1-His) contained GFP at the N terminus and a 6-His-tag at the C terminus but lacks a Sec insertion sequence (SECIS) element (Fig. S4*B*). Finally, the 3′-UTR of TR1 cDNA was PCR amplified and inserted into the Bsu36 I site after the 6-His-tag sequence. This TR1 expression vector (designated pGFP-TR1-His-SECIS) contained GFP at the N terminus, a 6-His-tag at the C terminus, and the intact SECIS element in the 3′-UTR (Fig. S4*C*). Cloning and expression of recombinant human TR1 and mouse TR3 (mTR3) vectors were carried out exactly as described (6).

Treatment of NIH 3T3 Cells with SPO_3. NIH 3T3 cells were cultured in DMEM supplemented with 10% FBS. For TR1 purification, cells were grown in 150 cm^2 flasks, treated with or without 1 mM of SPO_3 for two days, and harvested. The TR1 purification and alkylation were carried out as described (7). For labeling NIH 3T3 cells with ^{75}Se, NIH 3T3 cells were cultured in 6-well plates with or without 1.0 mM SPO_3 for 24 h, 10 μCi/mL of ^{75}Se added, the cells incubated for an additional 24 h, harvested, the lysates analyzed by Western blotting, and ^{75}Se-labeled proteins visualized with a PhosphorImager as described (2). For recombinant TR1 expression, NIH 3T3 cells were transfected with pGFP, pGFP-TR1-His-SECIS, or pGFP-TR1-His for 24 h in 6-well plates using Lipofectamine 2000 according to the manufacturer's instructions. Transfected cells were then split into two wells, 1.0 mM SPO_3 added into one well of each and incubated for 24 h. Ten microcuries/milliliter of ^{75}Se was added to the cells and incubated for an additional 24 h. Transfected cells were harvested, cell lysates were analyzed by Western blotting, and the ^{75}Se-labeled proteins were visualized as described (3).

Liquid Chromatography (LC)-MS/MS. Affinity isolated TR fractions were reduced with DTT, followed by alkylation of Cys and Sec residues with iodoacetamide. Alkylated proteins were resolved by SDS-PAGE using Novex Nu-PAGE system (Invitrogen) and stained with Coomassie blue. Protein bands were cut out and subjected to in-gel tryptic digestion and LC-MS/MS analysis. In-gel trypsin digestion of the destained protein bands was carried out for 16 h at 37 °C. The resulting peptide mixture was extracted from the gel slices and loaded into a fused silica microcapillary packed with Magic C18AQ beads (Michrom Bioresources). Reversed phase liquid chromatography was performed using an Agilent 1100 pump and a Famous autosampler (LC Packings). The peptides were eluted from the column with a 60-min acetonitrile gradient and detected using an LTQ-Orbitrap XL (Thermo-Fisher Scientific).

Database Analysis and Quantification. MS/MS spectra were searched against a concatenated IPI_Mouse database (version 3.60) (http://www.ebi.ac.uk/IPI/) using the Sequest algorithm (Version 28, Thermo-Fisher Scientific) and a 0.5% false discovery rate. Database search criteria were as follows: two missed cleavages, a precursor mass tolerance of 50 ppm, an MS/MS fragment ion tolerance of 0.8 Da, and the following variable modifications: oxidation (M), deamidation (NQ), and alkylation on Cys and Sec. Peptide abundances were calculated using the monoisotopic peak height from a summed spectrum representing the entire chromatographic peak of interest. Prior to comparisons between samples, changes in total protein expression and digestion efficiency were corrected by normalizing the abundances of the C-terminal peptides against another mTR peptide that was not observed or known to be modified and was identified with high confidence.

1. Davis CD, Zeng H, Finley JW (2002) Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice. *J Nutr* 132:307–309.
2. Xu XM, et al. (2007) Biosynthesis of selenocysteine on its tRNA in eukaryotes. *PLoS Biol* 5:96–105.
3. Xu XM, et al. (2007) Selenophosphate synthetase 2 is essential for selenoprotein biosynthesis. *Biochem J* 404:115–120.
4. Carlson BA, et al. (2004) Identification and characterization of phosphoseryl-tRNA$^{[Ser]Sec}$ kinase. *Proc Natl Acad Sci USA* 101:12848–12853.
5. Yoo MH, et al. (2007) A new strategy for assessing selenoprotein function: siRNA knockdown/knock-in targeting the 3′-UTR. *RNA* 13:921–929.
6. Turanov AA, Su D, Gladyshev VN (2006) Characterization of alternative cytosolic forms and cellular targets of mouse mitochondrial thioredoxin reductase. *J Biol Chem* 281:22953–22963.
7. Sun QA, Gladyshev VN (2002) Redox regulation of cell signaling by thioredoxin reductases. *Methods Enzymol* 347:451–461

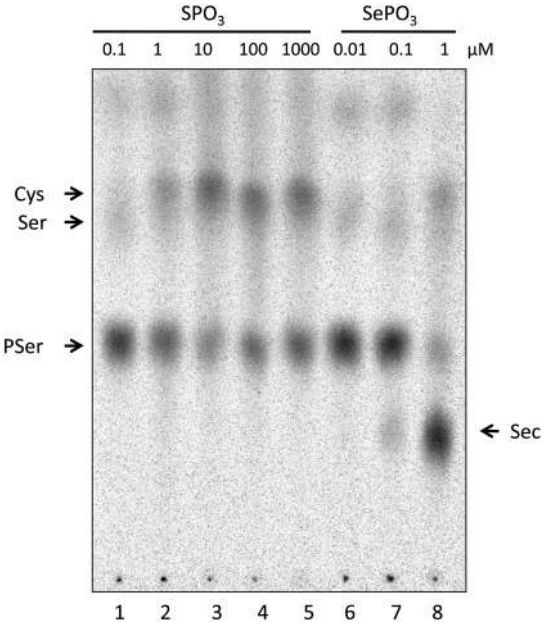

Fig. S1. Sec and Cys synthesis using varying concentrations of selenophosphate and thiophosphate. Lanes 1–5: 0.1, 1, 10, 100, and 1,000 μM of thiophosphate. Lanes 6–8: 0.01, 0.1, and 1 μM selenophosphate. Mouse SecS (mSecS) was used as enzyme, and the reaction products were separated on 3 M paper and visualized by exposing to a PhosphorImager screen. Experimental details are given in *SI Materials and Methods*.

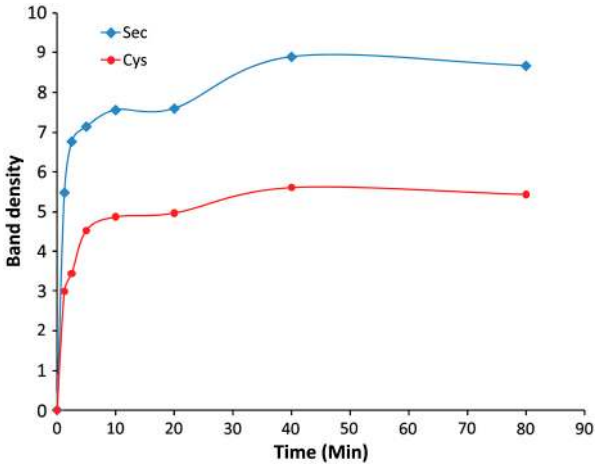

Fig. S2. Sec and Cys synthesis using *O*-phospho-[^{14}C]-seryl-tRNA$^{[Ser]Sec}$ with either 100 μM thiophosphate or 10 μM of selenophosphate. mSecS was used as enzyme and reactions were stopped at different times and the products separated on 3 M paper and visualized by exposing to a PhosphorImager screen. The band density of each product was quantified using ImageQuant. Experimental details are given in *SI Materials and Methods*.

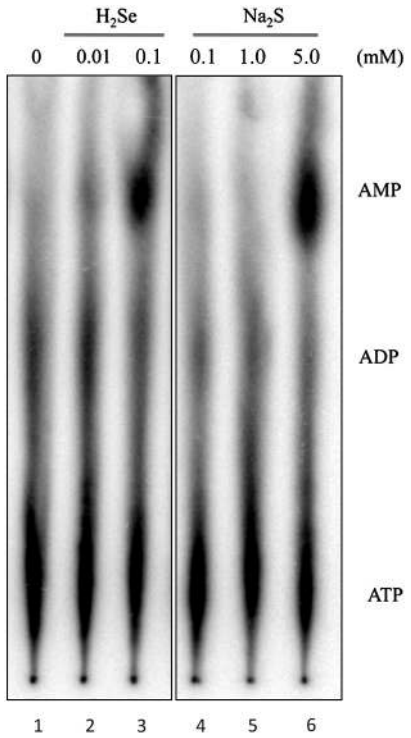

Fig. S3. Sulfide-dependent ATP hydrolysis by mouse selenophosphate synthetase 2 (mSPS2). ATP hydrolysis reactions were carried out using [α-^{32}P]ATP and mSPS2 in the presence of either 0, 0.01, or 0.1 mM selenide or 0.1, 1.0, or 5.0 mM sodium sulfide. At the end of the incubation period, compounds were separated on PEI TLC plates and visualized by exposing to a PhosphorImager screen. Experimental details are given in *SI Materials and Methods*.

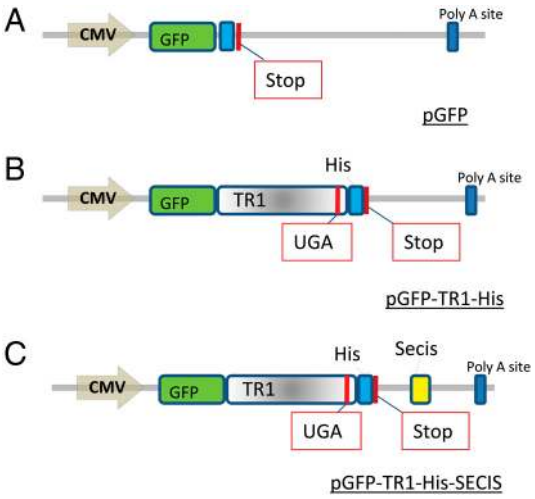

Fig. S4. Schematic illustration of recombinant TR1 vector constructs used in the study. In *A*, GFP was inserted into the Nco I and EcoR V sites of the pTriEx-4 Hygro vector (designated pGFP); in *B*, the coding region of mouse TR1 minus the stop codon was PCR amplified and inserted between the EcoR I and Xho I sites of pGFP (designated pGFP-TR1-His); and in *C*, the 3′-UTR of TR1 cDNA was PCR amplified and inserted into the Bsu36 I site after the 6-His-tag sequence (designated pGFP-TR1-His-SECIS).