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Mechanisms of the Regulation of Thioredoxin Reductase Activity in Cancer Cells by the Chemopreventive Agent Selenium[1]

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ABSTRACT

Selenium is an essential trace element, the deficiency of which is associated with an increased incidence of some human cancers. Dietary supplementation with selenium has been reported to produce a decrease in the incidence of some cancers in humans. Thioredoxin reductase (TR) is a newly discovered homodimeric selenocysteine (SeCys)-containing protein that catalyzes the NADPH-dependent reduction of the redox protein thioredoxin (Trx). Trx is overexpressed by a number of human tumors, and experimental studies have shown that Trx contributes to the growth and to the transformed phenotype of some human cancer cells. Thus, TR, by reducing Trx, could play a role in regulating the growth of normal and cancer cells. We have investigated mechanisms by which selenium, in the form of sodium selenite, added to serum-free growth medium regulates TR activity in cancer cell lines. Selenium caused a dose-dependent increase in cellular TR activity. The increase in TR activity produced by 1 μM Se compared to medium with no added selenium was: for MCF-7 breast cancer cells, 37-fold; for HT-29 colon cancer cells, 19-fold; and for A549 lung cancer cells, 8-fold. In contrast, Jurkat and HL-60 leukemia cells showed no increase in TR activity. The half-life of the time course of induction of TR in HT-29 cells after adding selenium was 10 h. The increase in TR activity was accompanied by an increase in TR protein levels up to 3-fold and an increase in the specific activity of the enzyme of 5–32-fold, depending on the cell line. Studies using ^{75}Se showed that the amount of selenium incorporated into TR increased with increasing selenium concentration up to a ratio of 1 selenium per TR monomer. There was an increase in TR mRNA levels of 2–5-fold at 1 μM selenium and an increase in the stability of TR mRNA with a half-life for degradation of 21 h compared to 10 h in the absence of selenium. Trx mRNA and protein levels and Trx mRNA stability were not affected by selenium. The results of the study show that the increase in TR activity caused by selenium is specific and due to several effects, including an increase in the stability of TR mRNA leading to increased TR mRNA levels, an increase in TR protein, but predominantly to an increase in the specific activity of TR associated with increased incorporation of selenium into the enzyme.

INTRODUCTION

Selenium is an essential biological trace element (1). Epidemiological studies have consistently shown that human populations having a low selenium intake and correspondingly low plasma or serum selenium levels have an increased incidence of a variety of cancers, including lung, stomach, bladder, ovarian, pancreas, thyroid, esophagus, head and neck, and cerebral cancers, and melanoma (2–7). Experimental evidence indicates that dietary selenium supplementation can reduce the incidence of cancer in animals (reviewed in Refs. 8 and 9). Recently, a double-blind, placebo controlled, randomized study involving a total of 1312 patients with a mean follow-up of over 6 years found that oral administration of selenium at 200 μg/day, which is three to four times the recommended daily allowance, can significantly reduce the incidence of lung, colorectal, and prostate cancer by 46, 58, and 63%, respectively (10).

The mechanism by which selenium acts to prevent cancer is unknown but has been suggested to involve either the formation of selenium metabolites that act directly to inhibit cancer cell growth (9, 11) or to the formation of critical selenoproteins (12). Selenium is specifically incorporated into proteins in the form of the unique amino acid SeCys[3] (13). Many selenoenzymes catalyze oxidation reduction reactions in which SeCys forms part of the active site (13). Eukaryotic selenoproteins include cellular and plasma glutathione peroxidases; phospholipid hydroperoxide glutathione peroxidase; types 1, 2, and 3 deiodinases; and selenoproteins P and W of unknown function (13). The chemopreventive activity of selenium has been ascribed to the ability of glutathione peroxidase to remove DNA-damaging H_2O_2 and lipid hydroperoxides (12). However, animal studies have not shown a link between alterations in glutathione peroxidase activity and the prevention of carcinogenesis (9, 14). A selenoprotein recently isolated from a human lung adenocarcinoma cell line and human T cells was found to have TR activity (15, 16). The COOH-terminal Gly-Cys-SeCys-Gly amino acid sequence of this protein (16) was identical to that predicted from the cDNA for human placental TR (17) but with TGA coding for SeCys instead of acting as a normal stop codon (15).

TR catalyzes the NADPH-dependent reduction of thioredoxin, a widely distributed redox protein (18). The thioredoxin redox system is important for cell growth and the transformed phenotype of some human cancers. Thioredoxin was first studied for its ability to reduce ribonucleotide reductase, which is the first unique step in DNA synthesis (19). More recently, thioredoxin has been shown to exert specific redox control over a number of transcription factors to modulate their binding to DNA and, thus, to regulate gene transcription. Transcription factors regulated by thioredoxin include nuclear factor-κB (20), TFIIIC (21), BZLF1 (22), and the glucocorticoid receptor (23). Thioredoxin also acts as a growth factor that stimulates the proliferation of both normal fibroblasts and human tumor cells, probably by increasing the sensitivity of the cell to endogenously produced growth factors (24). Mutant redox-inactive forms of thioredoxin lacking the active site cysteine residues are devoid of growth-stimulating activity (25). Thioredoxin has been found to be increased many fold in a number of human primary tumors compared to corresponding normal tissue (18, 26–28). Transfection of MCF-7 human breast cancer cells with a dominant-negative redox inactive thioredoxin inhibits their anchorage-independent but not monolayer growth in culture and almost completely inhibits tumor growth *in vivo* (29). Transfection of mouse WEHI7.2 thymoma-derived cells with human wild-type thioredoxin has been shown to block both spontaneous and drug-induced apoptosis and to increase tumor growth *in vivo* (30).

We have reported previously that selenium added to serum-free growth medium at concentrations similar to those found in human plasma produces an increase in TR activity in HT-29 colon cancer cells (31). Thus selenium, through its effects on TR leading to an increase in the reduction of thioredoxin, could affect the transformed

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[3] The abbreviations used are: SeCys, selenocysteine; FBS, fetal bovine serum; TR, thioredoxin reductase.

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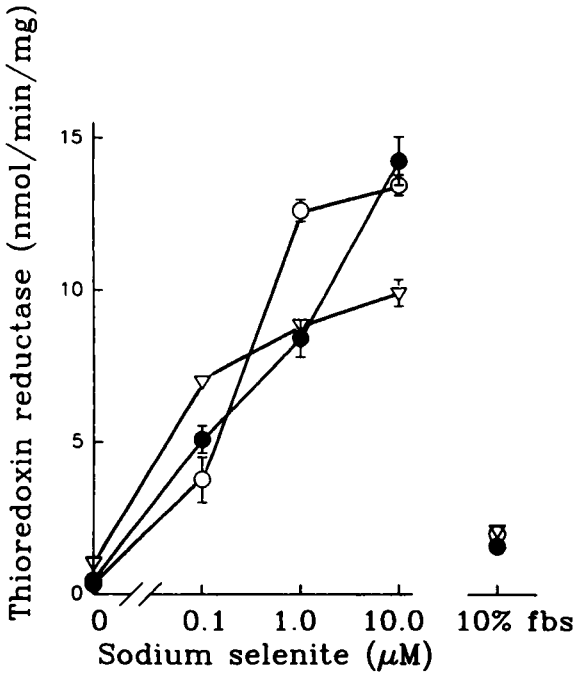

Fig. 1. TR activity of cells grown for 5 days in serum-free medium with varying concentrations of sodium selenite or with medium with 10% FBS. ▽, A549 lung cancer cells; ○, MCF-7 breast cancer cells; ●, HT-29 colon cancer cells. Each point is the mean of three determinations; *bars*, SE.

phenotype and the growth of cancer cells. The present study is an investigation of the mechanisms responsible for the increase in TR activity by selenium.

MATERIALS AND METHODS

Cell Lines. Human MCF-7 breast cancer, HT-29 colon cancer, A549 lung cancer cells, Jurkat T-cell leukemia, and HL-60 leukemia cells were obtained from the American Tissue Type Collection (Rockville, MD). MCF-7, HT-29, and A549 cells were maintained in DMEM with 10% FBS; Jurkat and HL-60 cells were maintained in RPMI 1640, both under 6% CO_2 at 37°C. Attached cells were passaged with 0.025% trypsin at 80% confluence.

Selenium Studies. For studies on the effects of selenium on TR activity, sodium selenite at concentrations of 0.1, 1.0, and 10 μM was added to 75-cm^2 culture flasks containing 10^4 cells in serum-free growth medium consisting of DMEM or RPMI containing 40 ng/ml insulin-like growth factor-1 and 40 ng/ml epidermal growth factor. At the end of the incubation, cells were washed twice with PBS (pH 7.4). Attached cells were scraped free, and leukemia cells were collected by centrifugation into 2 ml of 50 mM HEPES buffer (pH 7.6) containing 5 mM EDTA at 4°C, sonicated with a microtip probe (Heat Systems, Farmingdale, NY) for 10 s three times, and centrifuged at 110,000 × *g* for 45 min at 4°C to obtain a cytosolic fraction.

Assays. Cytosolic TR activity was measured spectrophotometrically at room temperature by the oxidation of NADPH at 339 nm in the presence of 15 μM recombinant human thioredoxin and 1 mg/ml bovine insulin, as described previously (32). Protein was measured by the dye binding method of Bradford (33). Serum selenium was measured by atomic absorption spectroscopy (Zeeman 3030; Perkin-Elmer, Norwalk, CT).

Western Blotting. Rabbit polyclonal antiserum to human TR was raised using a synthetic peptide to an internal protein sequence of human TR, as described previously (17). Rabbit polyclonal antibody to human thioredoxin was raised using recombinant protein as described previously (28). Western blots of cell extracts were visualized using the Renaissance chemiluminescence system (DuPont NEN, Wilmington, DE) and quantitated using a PhosphorImager (Molecular Dynamics, San Diego, CA). Standards of purified human placental TR (32) and recombinant human thioredoxin (18) were run on each blot.

RNA Studies. Procedures for the isolation of total cellular RNA and the preparation of Northern blots have been described previously (34). A full-length human ^{32}P-labeled thioredoxin probe was prepared as described previously (18). A TR probe was made by the random ^{32}P-labeling of purified, cloned human TR cDNA fragments, bp 1 to 3695 (17), using a DNA labeling kit (Life Technologies, Inc., Gaithersburg, MD). After hybridization with the probes for thioredoxin or TR, the blots were stripped and reprobed with histone H3.3 cDNA to normalize for unequal loading and transfer. Blots were quantified using a PhosphorImager. For studies of mRNA stability with and without 1 μM selenium, HT-29 cells were incubated with 10 μg/ml actinomycin D to inhibit new mRNA synthesis, and total cellular mRNA was prepared at various times up to 48 h.

^{75}Se Incorporation Studies. ^{75}Se (specific activity, 1.95 mCi/mg selenium) was obtained from the University of Missouri Research Reactor Facility (Columbia, MO). The incorporation of ^{75}Se into TR was measured by incubating 1.5 × 10^6 HT-29 colon cancer cells in DMEM containing 40 ng/ml insulin-like growth factor-1 and 40 ng/ml epidermal growth factor for 48 h, with ^{75}Se at 10 mCi/ml, which gave a selenium concentration of 27 nM, and with unlabeled sodium selenite at 0.1 and 1.0 μM. The cells were washed three times with PBS (pH 7.4) and harvested as described previously. The cytosol from the cells was gently mixed for 4 h at 4°C with either 0.2 ml adenosine 2′,5′-diphosphate coupled-agarose beads (Sigma Chemical Co., St. Louis, MO) or rabbit human TR antiserum coupled to protein A-Sepharose beads (Sigma). The beads were washed three times with 20 mM Tris buffer (pH 8.0), 137 mM NaCl, 10% glycerol, 0.1% Triton X-100, and then heated at 100°C for 10 min in 0.5 M Tris buffer (pH 6.8), 10% SDS, 20% glycerol, 0.1% bromphenol blue, and 3% β-mercaptoethanol prior to SDS 7.5% PAGE. Blots were transferred to a polyvinylidene difluoride membrane, and radioactivity in the immunoprecipitated TR bands was measured using a PhosphorImager; ^{75}Se was quantified by comparing to 0.2-μl aliquots of the original incubation medium. It was not possible to measure immunoprecipitated TR by Western blotting and chemiluminescent ECL visualization because of a large contaminating antibody band. Instead, TR bound to ADP-Sepharose was measured by Western blotting with standards of human placental TR, as described above.

RESULTS

TR Activity. Increasing concentrations of sodium selenite added to serum-free growth medium gave a concentration-dependent increase

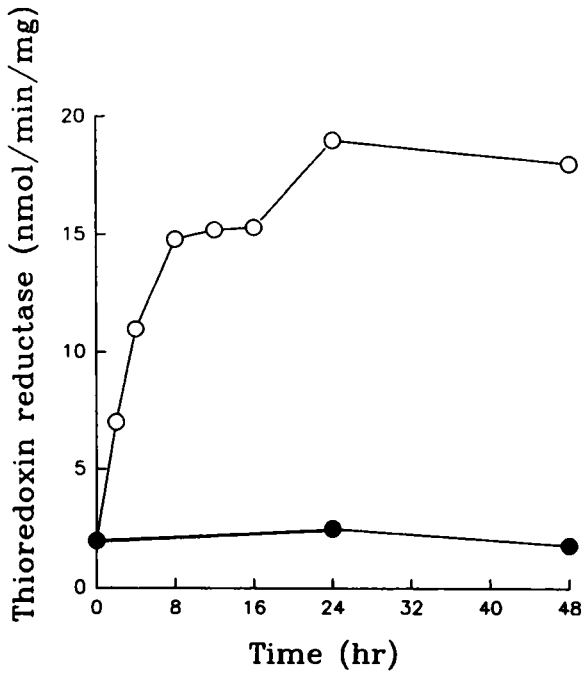

Fig. 2. Time course of the increase in TR activity in HT-29 colon cancer cells in the absence (●) and presence (○) of 1 μM selenium.

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Table 1 *Effect of selenium on TR activity without and with serum*

Cells were grown in medium with 40 ng/ml insulin-like growth-factor-1 and 40 ng/ml epidermal growth factor or medium with 10% FBS. In some cases, 1 μM sodium selenite was added. TR activity was measured after 48 h. Values are expressed as nmoles of NADPH reduced/min/mg cytosolic protein and are the means of three determinations ± SE.

	Without FBS		10% FBS	
Cell line	–	1.0 μM selenium	–	1.0 μM selenium
A549 lung	1.0 ± 0.1	8.9 ± 0.2	4.5 ± 0.5	23.2 ± 0.4
MCF-7 breast	0.4 ± 0.1	12.6 ± 0.3	2.3 ± 0.1	17.6 ± 0.3
HT-29 colon	0.4 ± 0.1	8.4 ± 0.6	2.5 ± 0.5	11.2 ± 0.4
Jurkat T cell	0.8 ± 0.0	0.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.0
HL-60 leukemia	1.6 ± 0.0	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1

in the cytosolic TR activity of A549 lung cancer, MCF-7 breast cancer, and HT-29 colon cancer cells (Fig. 1). The increase in TR activity at 1 μM selenium compared to no added selenium was: for MCF-7 cells, 37-fold; for HT-29 cells, 19- fold; and for A549 cells, 8-fold. The TR activity of cells grown in 10% FBS was almost the same as cells grown in the absence of added selenium (Fig. 1). Unlike human serum which has a total selenium concentration between 1 and 5 μM (35), the total selenium concentration in FBS was <0.1 μM, so that the selenium concentrations of DMEM with 10% FBS was <0.01 μM. Concentrations of sodium selenite over 5 μM in serum-free medium were toxic to the cells, measured by growth inhibition (36) and the occurrence of apoptosis (results not shown). Because of this toxicity, further studies were conducted at a maximum sodium selenite concentration of 1 μM. The half-life of the increase in TR activity produced by 1 μM selenium in HT-29 cells was 10 h (Fig. 2). Two human leukemia cell lines, Jurkat T-cell leukemia and HL-60 leukemia, showed no increase in TR activity at 1 μM selenium compared to medium with no added selenium (Table 1). Because it was possible that essential growth factors were missing from the serum-free medium, particularly for the leukemia cells, we also studied the effects of added selenium on TR activity of cells growing in 10% FBS (Table 1). A549 lung cancer, MCF-7 breast cancer, and HT-29 colon cancer cells showed an increase in TR activity upon addition of 1 μM selenium to medium containing 10% FBS, but there was no increase in the TR activity of the leukemia cells.

TR and Thioredoxin Protein Levels. TR protein measured by quantitative Western blotting was found to be significantly increased by selenium in A549, MCF-7, and HT-29 cell lines (Fig. 3). However, the maximum increase in TR protein at a given selenium concentration was considerably less than the increase in TR activity. The mean specific activity of the TR increased with increasing concentrations of selenium in the medium from 0.6 nmol/min/μg TR protein with no added selenium to 5.1 nmol/min/μg TR protein at 1 μM selenium (Table 2). TR protein levels in cells grown in 10% FBS were not significantly different than cells grown without selenium. The levels

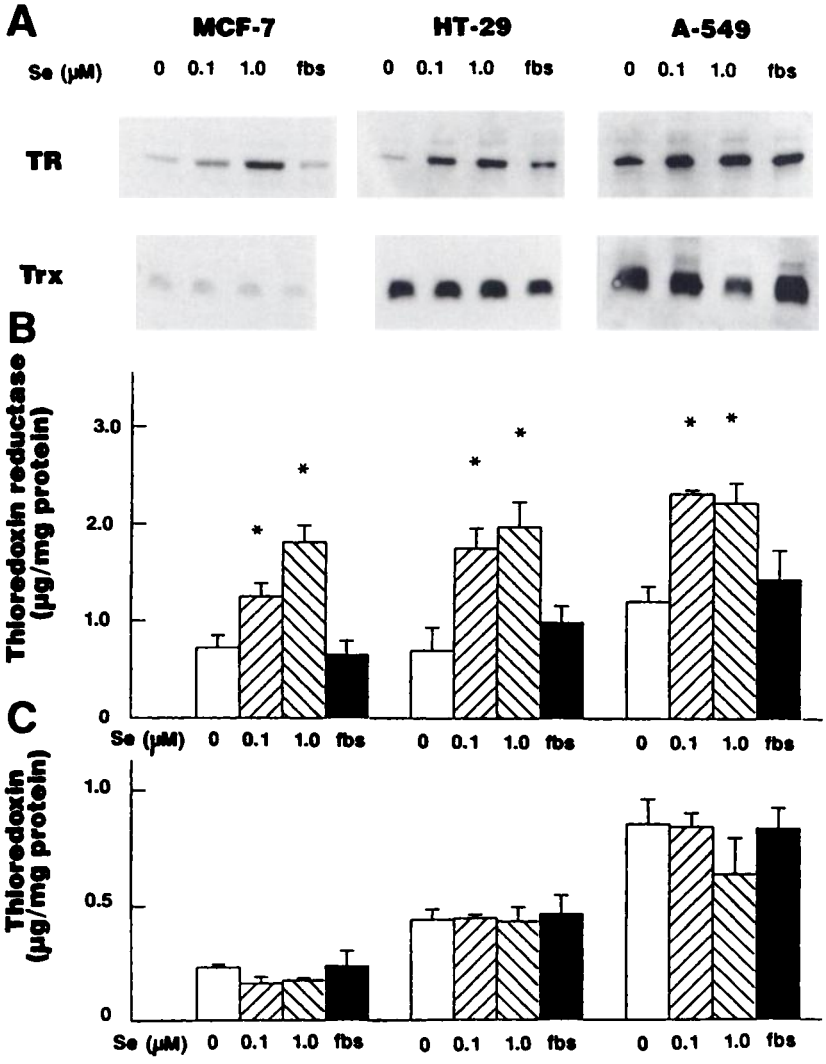

Fig. 3. TR and thioredoxin protein levels determined by Western analysis. Cells were grown for 5 days in DMEM with 0, 0.1, and 1.0 μM sodium selenite or 10% FBS, and TR and thioredoxin were determined by Western blotting as described in "Materials and Methods" using human TR and thioredoxin standards. *A*, autoradiograms of typical Western blots for TR and thioredoxin (*Trx*). *B*, TR values from three separate studies. *C*, thioredoxin values from three separate studies. *Bars*, SE. *, $P < 0.05$ compared to the control value in the absence of selenium.

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Table 2 *Specific activity of TR in cells*

The specific activity of TR in cell extracts was calculated from the data in Fig. 1 (thioreductase activity) and Fig. 3 (TR protein).

Cell line	Selenium (μM)	Specific activity nmol/min/μg protein
MCF-7 breast	0	0.22
	0.1	2.99
	1.0	6.96
HT-29 colon	0	0.63
	0.1	2.90
	1.0	4.27
A549 lung	0	0.86
	0.1	3.02
	1.0	4.00

of thioredoxin protein were not affected by selenium or 10% FBS in the medium (Fig. 3).

TR and Thioredoxin mRNA Levels. One μM selenium produced a significant increase in TR mRNA compared to medium without added selenium: in MCF-7 cells, 3.2-fold; in HT-29 cells, 4.5-fold; and in A549 cells, 1.7-fold (Fig. 4). However, there was no significant increase in TR mRNA at 0.1 μM selenium. There was also no significant increase in thioredoxin mRNA caused by selenium at 0.1 or 1 μM, except for a 2.8-fold increase seen with 1 μM selenium in HT-29 cells only. Studies on the stability of TR mRNA selenium in cells treated with actinomycin D to inhibit new RNA synthesis showed that 1 μM selenium caused an increase in the stability of TR mRNA, with a half-life for degradation of 10 h without added selenium and a half-life of 21 h with 1 μM selenium (Fig. 5). Selenium had no effect on the rate of degradation of thioredoxin mRNA, which had a half-life of 28 h both in the absence of selenium and with 1 μM selenium (results not shown).

^{75}Se Incorporation into TR. Incorporation of ^{75}Se into immunoprecipitated TR of HT-29 colon cancer cells increased with increasing concentrations of selenium in the growth medium (Table 3). The ratio of selenium to the M_r 54,000 TR monomer was 0.01 at 27 nM selenium and 0.98 at 1.027 μM selenium.

DISCUSSION

The results of the study show that sodium selenite causes a dose-dependent increase in cytosolic TR activity in MCF-7 breast cancer,

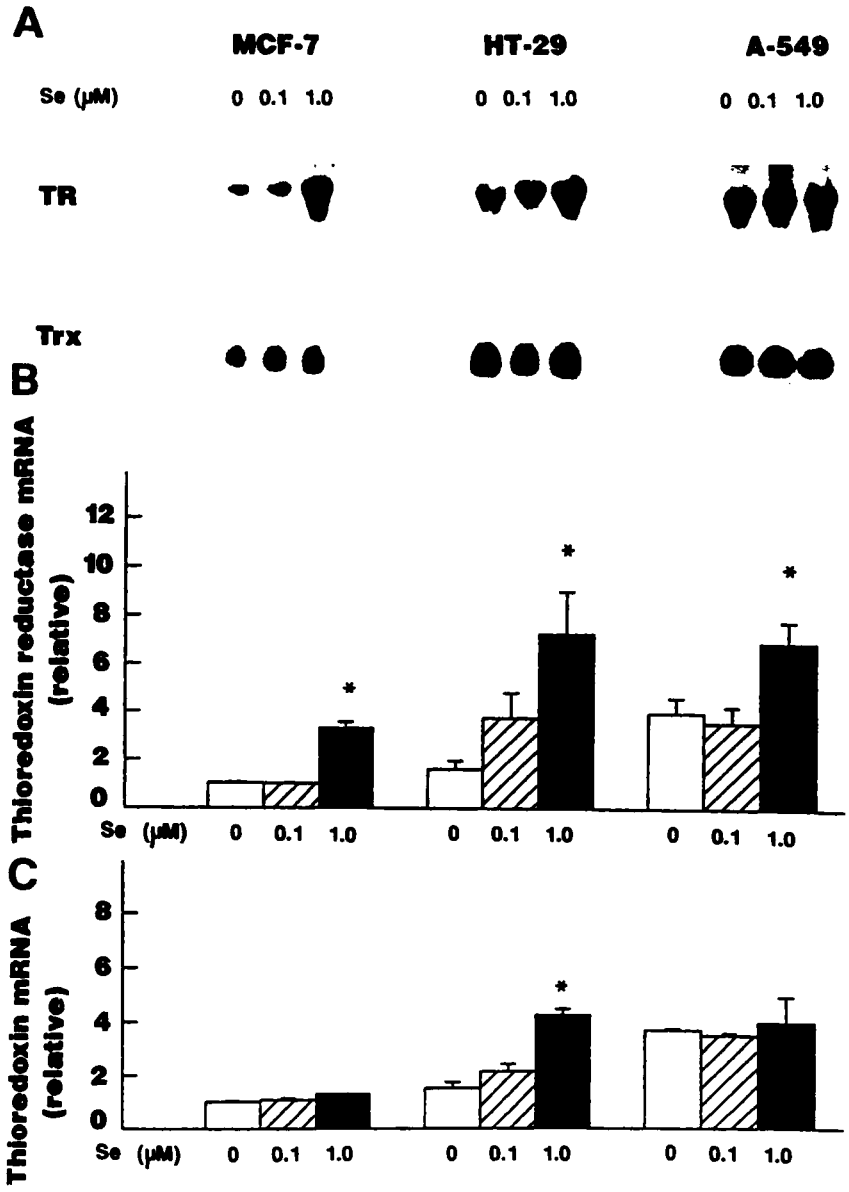

Fig. 4. TR and thioredoxin mRNA determined by Northern hybridization analysis. Cells were grown for 5 days in DMEM with 0, 0.1, and 1.0 μM sodium selenite, and TR and thioredoxin were determined by Northern blotting as described in "Materials and Methods." *A*, autoradiograms of typical Northern blots showing TR and thioredoxin (*Trx*) mRNA. *B*, TR mRNA values from three separate studies. *C*, thioredoxin mRNA values from three separate studies. *Bars*, SE. *, $P < 0.05$ compared to the control value in the absence of selenium.

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HT-29 colon cancer, and A549 lung cancer cell lines with increases of 8–37-fold at 1 μM sodium selenite. This confirms and extends our previous finding of a 28-fold increase in TR activity caused by sodium selenite in HT-29 colon cancer cells (31). The increase in TR activity was seen in both serum-free medium and with 10% FBS in the medium. However, 1 μM selenium did not increase cytosolic TR activity in two human leukemia cell lines, Jurkat T-cell leukemia and HL-60 leukemia, either in the absence or the presence of 10% FBS. Spyrou *et al.* (37) have reported a small 40% increase in TR activity with 10 μM sodium selenite in an EBV-transformed human lymphoblastoid cell line. It appears, therefore, that although cancer cells of epithelial origin can show large increases in cytosolic TR activity with added selenium, cells of lymphoid origin do not. Lymphoid-derived cell lines typically have low levels of TR; therefore, they may have a limited capacity for biosynthesis (28).

Although the increase in TR activity caused by 1 μM selenium was quite large, the increase in TR protein caused by 1 μM selenium was only between 1.8- and 2.8-fold. Thus, most of the increase in TR activity caused by selenium appears to be due to an increase in the specific activity of the enzyme. The mean specific activity of TR was 0.06 nmol/min/μg with no added selenium and 3.0 nmol/min/μg at 1 μM selenium. This is probably because SeCys, which we have shown is essential for the reduction of thioredoxin by the enzyme (36), increased as selenium in the medium increased. The amount of ^{75}Se incorporated into each M_r 54,000 TR monomer increased from 0.01 at 27 nM selenium to 0.98 at 1.03 μM selenium. This latter ratio is consistent with the value of 0.93 reported for purified human placental TR (38), indicating one SeCys residue per TR monomer as predicted by cDNA sequence data (16, 17).

Decreased synthesis of other selenoproteins has been observed under conditions of limiting selenium (39, 40) due to the mRNA UGA encoding SeCys functioning alternatively as a stop codon. A purine immediately following the UGA codon increases the frequency of protein termination relative to SeCys incorporation, which has been suggested to create a site where termination is favored over SeCys incorporation when SeCys→tRNA is limiting (41, 42). This "terminator" purine has been suggested to explain the decrease in glutathione peroxidase levels upon nutritional selenium deprivation (42). Human TR also has selenoprotein mRNAs that use codons other than UGA to specify termination (41). TR mRNA specifies termination by UAA (17). A 3′-untranslated region stem-loop sequence that could function as a putative SeCys incorporation sequence element that could regulate the incorporation of SeCys has been identified.[4] Thus, in the absence of selenium, it appears that there is premature termination or incorporation of Cys into TR in place of SeCys. The antibody we use for measuring TR recognizes an internal peptide sequence of the enzyme and cannot distinguish between wild-type, truncated, or SeCys→Cys mutant TR. Further work will be necessary to determine whether the decreased SeCys incorporation in TR at low selenium is due to truncation of the protein or Cys replacement. In addition to a decreased SeCys incorporation, we found that low selenium leads to decreased levels of TR mRNA associated with decreased relative stability of the mRNA measured in the presence of actinomycin D and decreased TR protein. Inhibition of new RNA synthesis with actinomycin D is only one method for determining mRNA half-life, and other methods were not used. It should be noted that the half-life of thioredoxin mRNA was not altered by actinomycin D. Selenium deficiency has also been shown to decrease the stability of glutathione peroxidase mRNA levels leading to lower mRNA and, thus, decreased enzyme protein levels (43).

Table 3 *Incorporation of ^{75}Se into TR*

HT-29 colon cancer cells were incubated with ^{75}Se and sodium selenite at the concentrations shown for 48 h. The incorporation of ^{75}Se into each M_r 55,000 subunit of TR is expressed as a molar ratio. Values are the means of three determinations ± SE.

Selenium concentration (nM)	Molar ratio, selenium:TR
27	0.011 ± 0.002
127	0.084 ± 0.011
1027	0.983 ± 0.097

The role SeCys plays in the biological activity of TR remains a matter of conjecture. Arscott *et al.* (38) have provided evidence that the SeCys of human placental TR is in redox communication with the catalytic site cysteine residues. Some information is available on the role of SeCys in the catalytic activity of other selenoenzymes. The reduced selenoenzyme contains an ionized selenol that reacts with the substrate H_2O_2 or organic peroxide for mammalian glutathione peroxidase (13), thyroid hormone for mammalian deiodinase (44), or glycine-protein Schiff base complex for bacterial glycine reductase selenoprotein A (45) to give oxidized enzyme and regenerated by the transfer of reducing equivalents from thiol donors. Presumably with TR, ionized selenol interacts with oxidized thioredoxin to give an enzyme-selenium-sulfur-thioredoxin complex, which then undergoes reduction to liberate reduced thioredoxin with transfer of reducing equivalents from the catalytic site cysteine residues, through FAD from NADPH.

The biological consequences of alterations in TR activity due to selenium are not known. We have found that the increase in TR activity occurs over the range of selenium found in human blood, which is between 1 and 5 μM (35), but is also seen at lower concentrations. Hill *et al.* (46) have reported that in rats fed a selenium-deficient diet, there is a decrease in TR activity in liver and kidney but not in brain. Whether changes in TR activity play a role in the effects of selenium on carcinogenesis or tumor progression is an intriguing possibility that remains to be investigated. High levels of selenite, greater than about 5 μM, are toxic to cells and cause apoptosis. Whether this increase in apoptosis is a consequence of an increase in the levels of TR above a threshold level is not known. Apoptosis has been proposed to be part of a normal surveillance mechanism for genetic damage that, when detected, eliminates the cell containing the damage by apoptosis (47, 48). Cancer cells progressively lose the ability to undergo apoptosis as they become more malignant (49). A loss of apoptosis has even been suggested to be an essential feature of

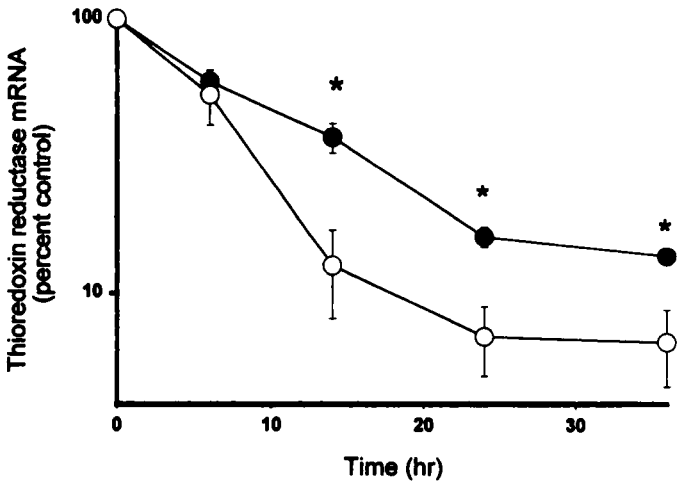

Fig. 5. Effect of 1 μM selenium on the stability of TR mRNA in HT-29 cells. Cells were incubated with 10 μg/ml actinomycin D in medium in the absence (○) or presence (●) of 1 μM sodium selenite, and total mRNA was prepared at various times. TR mRNA was determined by Northern blotting. Values are from three separate studies. *Bars*, SE. *, $P < 0.05$ compared to the control value in the absence of selenium.

[4] M. Berry, personal communication.

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a cancer cell (50). If low selenium leads to a decreased ability of cells to undergo apoptosis, this also might lead to an increased transmission of genetic damage and increased risk of developing cancer, thereby explaining the association of low selenium with an increased incidence of human cancer (51–53). Whether TR plays a role in this process, however, remains to be elucidated.

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