Overexpression of Enzymatically Active Human Cytosolic and Mitochondrial Thioredoxin Reductase in HEK-293 Cells

EFFECT ON CELL GROWTH AND DIFFERENTIATION*

Ivan Nalvarte‡, Anastasios E. Damdimopoulos‡, Christina Nystömi‡, Tomas Nordman§, Antonio Miranda-Vizuete‡, Jerker M. Olsson§, Lennart Eriksson§, Mikael Björnstedt‡, Elias S. J. Arnér‡, and Giannis Spyrou‡,‡ systems

From the ›Department of Biosciences at Novum, Center for Biotechnology, Karolinska Institutet, SE-141 57 Huddinge, Sweden, the $Department of Laboratory Medicine, Division of Pathology, F46, Karolinska Institutet, Karolinska University Hospital Huddinge, SE-141 86 Huddinge, Sweden, the ‡Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden, and ‡Institute of Biomedical Research, Academy of Athens, Soranou Efessiou 4, 11527 Athens, Greece

The mammalian thioredoxin reductases (TrxR) are selenoproteins containing a catalytically active selenocysteine residue (Sec) and are important enzymes in cellular redox control. The cotranslational incorporation of Sec, necessary for activity, is governed by a stem-loop structure in the 3′-untranslated region of the mRNA and demands adequate selenium availability. The complicated translation machinery required for Sec incorporation is a major obstacle in isolating mammalian cell lines stably overexpressing selenoproteins. In this work we report on the development and characterization of stably transfected human embryonic kidney 293 cells that overexpress enzymatically active selenocysteine-containing cystolic TrxR1 or mitochondrial TrxR2. We demonstrate that the overexpression of selenium-containing TrxR1 results in lower expression and activity of the endogenous selenoprotein glutathione peroxidase and that the activity of overexpressed TrxRs, rather than the protein amount, can be increased by selenium supplementation in the cell growth media. We also found that the TrxR-overexpressing cells grew slower over a wide range of selenium concentrations, which was an effect apparently not related to increased apoptosis nor to fatally altered intracellular levels of reactive oxygen species. Most surprisingly, the TrxR1- or TrxR2-overexpressing cells also induced novel expression of the epithelial markers CK18, CK-Cam5.2, and BerEP4, suggestive of a stimulation of cellular differentiation.

Thioredoxin reductases (TrxRs) are members of the nucleotide-disulfide oxidoreductase family and are ubiquitously found in mammalian tissues. Two main isoforms of thioredoxin reductases exist in mammals: the classical cytosolic form (TrxR1, the TXNRD1 gene product) (1, 2), and the mitochondrial form (TrxR2) (3, 4). A third form, thioredoxin and glutathione reductase, is mainly expressed in testis (5). All mammalian TrxRs are homodimeric selenocysteine-containing enzymes (6) that share high sequence homology between each other and contain an NADPH binding domain and a FAD binding domain per subunit (7). TrxRs reduce and thereby activate thioredoxins (Trxs), which are small ubiquitous protein-2 (SBP2) (23–25), binds the SECIS element and is involved in a variety of reactions, such as redox regulation of transcription factors (8, 9), reduction of hydroperoxides (4, 10), and modulation of cell growth (11) and apoptosis (12, 13). The selenocysteine residue (Sec) that is found at the C terminus in TrxRs (-Gly-Cys-Sec-Gly-COOH) is encoded by a UGA codon that is normally identified as a stop codon, but which can be transcribed as Sec in the presence of a selenocysteine insertion sequence (SECIS) element in the 3′-UTR of the corresponding mRNA (14). The C-terminal Cys-Sec motif is located on a presumably flexible arm of the enzyme that is kept reduced by the N-terminal redox active motif (-Cys-Val-Asn-Val-Gly-Cys-) of the other subunit of the dimere (15). The combination of a low pKₐ of Sec (typically 5.25) (7) and an easily accessible C-terminal localization of the Sec-containing active site should explain why reduced TrxRs are highly reactive at physiological pH and display a broad substrate specificity (16–18).

The intricate mammalian selenoprotein synthesis machinery imposes a major obstacle in constructing stable selenoprotein-overexpressing mammalian cell lines. First, the SECIS element motif in the 3′-UTR of a mammalian selenoprotein-encoding gene is conserved to some few critical nucleotides in a stem-loop structure that needs to be positioned within a functional distance hundreds of nucleotides downstream of the in-frame UGA codon (19–22). Second, at least two distinct proteins are needed for successful translation of the UGA codon to a Sec residue. The first protein factor, termed SECIS binding protein-2 (SBP2) (23–25), binds the SECIS element and is believed to recruit a second protein, the Sec-tRNA-Sec-specific elongation factor (EFsec), which in turn delivers a third translated region; WT1, Wilms’ tumor 1 protein; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species.
critical factor, the Sec-tRNA\textsubscript{Sec} to the ribosome (26–28). The selenocysteine-specific tRNA is originally aminoaacetylated with a seryl moiety and subsequently needs to be converted to selenocysteinylated tRNA to acquire functionality. The success of this machinery is ultimately limited by the availability of selenium in the surrounding medium and the conversion of selenium metabolites to selenide, the active species of selenium in selenoprotein synthesis (29). The unique role of TrxR in relation to selenoprotein synthesis is underscored by the fact that TrxR may reduce major selenium metabolites, e.g. selenodiglutathione (30), selenite (31), and methyselenolinate (32), hence liberating selenide and thereby itself providing this necessary substrate for selenoprotein synthesis. The importance of selenium incorporation into TrxR is further underlined by the recent discovery that selenium-compromised TrxR1 can cause induction of cell death by a yet unknown mechanism (33). In addition, different human TrxR1 transcripts encode a number of splice variants that translate into different N-terminal domains of yet unknown function (2). Furthermore, TrxR1 (but not TrxR2) mRNA contains six AU-rich instability elements (34, 35), which prompts rapid turnover of TrxR1 mRNA and are typical for transiently expressed genes such as proto-oncogenes, growth factors, and cytokines. Removal of either three or all of these AU-rich elements gives a significant increase in TrxR1 production (35).

Recently we reported the first success in isolating stable mammalian cell lines overexpressing TrxR1 (18). Here we describe the construction and characterization of these HEK-293 cells, stably overexpressing either active TrxR1 or TrxR2 in a selenium dose-dependent manner. We show that the overexpression of these selenoproteins is down-regulating the activity and production of the endogenous selenoprotein glutathione peroxidase (GPx), illustrating the high hierarchy of TrxR expression within the selenoprotein family. Furthermore, overexpression of TrxR1 surprisingly leads to a slower growth rate, a phenotypic characteristic that seemed to be independent of the enzymatic activity of TrxR. In addition, TrxR1 and TrxR2 overexpression in these cells affected the cellular differentiation as illustrated by novel expression of gene products regarded as epithelial markers.

**EXPERIMENTAL PROCEDURES**

Chemicals— Dulbecco’s modified Eagle’s medium, F-12 nutrient mixture, and fetal bovine serum for cell culturing were purchased from Invitrogen. G418 was obtained from Calbiochem. EDTA, NADPH, phenylmethylsulfonyl fluoride (PMSF), polyethyleneimine (PEI), sodium selenite, 5,5′-dithiobis-2-nitrobenzoate, glycerol, staurosporine, phenylmethylsulfonyl fluoride (PMSF), polyethyleneimine (PEI), 10% fetal bovine serum, and production of the endogenous selenoprotein glutathione peroxidase (GPx), illustrating the high hierarchy of TrxR expression within the selenoprotein family. Furthermore, overexpression of TrxR1 surprisingly leads to a slower growth rate, a phenotypic characteristic that seemed to be independent of the enzymatic activity of TrxR. In addition, TrxR1 and TrxR2 overexpression in these cells affected the cellular differentiation as illustrated by novel expression of gene products regarded as epithelial markers.

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Characterization of Stable Cell Lines Overexpressing TrxR

**FIG. 1.** Effect of 72-h incubations with increasing amounts of selenite in HEK-293 growth medium on TrxR1 and TrxR2 activity and overexpression. A, the TrxR1 activity in 50-µg cytosolic extracts from HEK-RES (white bars) and HEK-TrxR11 (black bars) read spectrophotometrically at 412 nm using the insulin reduction assay. B, the TrxR2 activity in 12-µg mitochondrial extracts from HEK-RES (white bars) and HEK-TrxR2.14 (black bars). Each activity measurement represents the mean of triplicates with blank samples (no Trx) subtracted (error bars correspond to S.D.). Overexpression of TrxR1 in HEK-TrxR11 (C) and TrxR2 in HEK-TrxR2.14 (D) compared with HEK-RES control cells as detected by Western blot analysis and 75Se labeling of TrxR1 or TrxR2 in the cytosolic or mitochondrial extracts, respectively. Western blots and autoradiographies were performed on the same membranes for the respective cell lines.

Plate (BD Biosciences) containing 1 ml HEPES-NaOH, pH 7.5, 4 mM EDTA, 200 µM NADPH, and 1 mg/ml insulin yielding a final volume of 100 µl. Reactions were started by the addition of 10 µM E. coli Trx (Promega) to TrxR1-containing wells or 4 µg of Trx2 (13) to TrxR2-containing wells followed by incubation at 37 °C for 20 min. Blank sample was treated in the same way except for the addition of Trx or Trx2. The reaction was terminated by the addition of 200 µl 6 M guanidine HCL in 0.2M Tris-HCl containing 0.4 mg/ml 5,5'-dithiobis-2-nitrobenzoate producing 2-nitro-5-thiobenzoate. The absorbance was read spectrophotometrically at 412 nm (Bio-Tek PowerWaveX, Winooski, VT), and the blank value was subtracted from each sample.

**Glutathione Peroxidase Activity**—The GPx activities were determined by a coupled assay as described by Lawrence and Burk (44). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.2 mM NADPH, 1 mM NaHCO3, 1 mM glutathione reductase, 1 mM GSH, and 50 µl of cytosol. The oxidation of the NADPH was followed spectrophotometrically (Shimadzu PC2501, Japan) at 340 nm, after addition of 0.25 mM H2O2 to the reaction mixture. The activities were calculated using an extension coefficient for NADPH of 6.22 mA·cm⁻¹·µM⁻¹.

**Western Blot Analysis**—Indicated amounts of cytosolic or mitochondrial extracts from HEK-293 cells were analyzed by Western blotting using 1:5000 dilution of rabbit anti-TrxR1 antibody or 1:2000 dilution of rabbit anti-TrxR2 antibody that was purified as described earlier (45). An anti-rabbit antibody coupled to horseradish peroxidase was used as a secondary antibody at 1:5000 dilution. To assess relative protein amounts in each sample, the membranes were also incubated with an anti-β-actin monoclonal antibody, at 1:5000 dilution, for which an anti-mouse horseradish peroxidase-coupled antibody was used at 1:5000 dilution. The GPx amount in each sample was detected by using a 1:200 dilution of anti-GPx antibody raised in sheep. A donkey anti-sheep horseradish peroxidase conjugated antibody was used at 1:5000 dilution as secondary antibody. The cytochrome c amounts in cytosolic or mitochondrial fractions were detected by using mouse anti-cytochrome c antibodies at 1:5000 dilution, and an anti-mouse horseradish peroxidase-coupled secondary antibody was used at 1:5000 dilution. The membranes were then exposed to ECL hyperfilm (Amersham Biosciences) for visualization.

**Cell Growth and Viability**—The cell number was adjusted to 25,000 cells/well. Sodium selenite was added to the cells at the indicated concentrations, and the cells were subsequently split into 24-well plates (BD Bioscience) to 1 ml of cells in culture medium per well. The cells were then grown for 7 days at 37 °C in 5% CO2, and were counted each day and assessed for viability using trypan blue exclusion after trypsinization. At days 4–6, the medium was changed to fresh medium containing the same sodium selenite concentrations. For detection of apoptosis, cells were grown in 175-cm² flasks with medium as above supplemented with 0, 0.1, or 10 µM sodium selenite and incubated for 72 h at 37 °C in 5% CO2. One additional flask of each cell line was incubated for 48 h and then with 1 µM staurosporine for 12 h at 37 °C in 5% CO2. All cells were then harvested, and cytosolic and mitochondrial fractions were extracted as above.

**Assessment of Intracellular Reactive Oxygen Species Levels**—Cells were cultured in 11-cm² culture dishes (BD Biosciences) in plain medium, as described above, or medium supplemented with 0.1 or 10 µM sodium selenite and incubated for 72 h at 37 °C in 5% CO2. The cells were then harvested, and cytosolic and mitochondrial fractions were extracted as above.

**Immunohistochemistry**—Cells were cultured until confluence in medium as described above, without any sodium selenite supplementation. The cells were harvested by scraping and were sedimented. The cell pellet was washed in culture medium once, sedimented, and then fixed in 4% formaldehyde for 24 h. The cell pellet was embedded in paraffin and sectioned 2.5-µm thick and routinely stained immunochemically according to accredited standard laboratory procedures at the Immunolaboratory at Karolinska University Hospital (Huddinge, Sweden).

**RESULTS**

Characterizing the Selenium Dose Dependence for TrxR1 and TrxR2 Protein Overexpression and Activity in Stably Transfected HEK-293 Cells—Characterization of the stable cell lines concerning overexpression, activity, and selenium dependence was performed to confirm successful selenoprotein synthesis. This selenium dependence was examined by incubating the HEK-293 cells stably overexpressing TrxR1 (HEK-TrxR11),...
TrxR2 (HEK-TrxR2.14), or cells transfected with the empty vector (HEK-IRES control cells) with different amounts of sodium selenite for 72 h. The activities of TrxR1 and TrxR2 were measured in cytosolic (Fig. 1A) and mitochondrial (Fig. 1B) extracts, respectively. A clear difference in TrxR1 activity was seen between HEK-IRES control cells and HEK-TrxR11. This difference was further increased upon supplementation of selenium and displayed a clear dose-dependent response. A supplementation with 0.05 μM sodium selenite gave more than 15-fold higher TrxR1 activity in HEK-TrxR11 cells compared with HEK-IRES control cells, whereas a 5-fold higher activity was seen in the absence of additional selenium supplementation above the concentration in the standard culture medium (≈20 nm selenium; see “Experimental Procedures”). The TrxR1 activity seemed to be saturated at supplementation with more than 0.1 μM sodium selenite. The HEK-TrxR2.14 cell line, overexpressing mitochondrial TrxR2, showed a basal TrxR2 activity in mitochondrial extracts that was about 7-fold higher than in the control HEK-IRES cells. Although the TrxR2 activity was elevated slightly by additional selenium supplementation, this effect was not as pronounced as with TrxR1 (compare Fig. 1, A and B). The highest TrxR2 activity was seen at 2 μM sodium selenite, indicating a less saturable uptake of selenium for TrxR2 as compared with TrxR1.

The overexpression pattern analyzed by Western blot was to a large extent coherent with the activity measurements, both for HEK-TrxR11 (Fig. 1C) and HEK-TrxR2.14 (Fig. 1D). The ability of the overexpressing cell lines to incorporate selenium in TrxR was further demonstrated by incubating the cell lines with radioactive 75Se. Here the 75Se-labeled protein was detectable at 0.05 μM selenite[75Se], and the labeled amount did not change much in the range between 0.1 and 2 μM selenite[75Se]. This pattern correlated well with the activity measurements from these cell lines. Taken together, we found a clear overexpression of both TrxR1 and TrxR2 in the stably transfected clones, indicating seemingly sufficient selenoprotein synthesis machinery for overexpression of these selenoproteins in HEK-293 cells. Furthermore, the activity rather than the protein amount was elevated with additional selenium supplementation, illustrating that the selenium supply was a limiting factor in standard culture medium.

Upon selenium deficiency the “less important” selenoproteins lose activity in favor of more important ones (46, 47). This hierarchy might be regulated by means of mRNA stability governed by the 3′-UTR region and/or mRNA-binding proteins (48). An increase in TrxR1 production in HEK-TrxR11 cells would possibly decrease the availability of selenium for endogenous selenoproteins, such as glutathione peroxidase (GPx), if total selenium availability becomes a limiting factor. Alternatively, because TrxR1 reduces selenium metabolites and provides selenide for selenoprotein synthesis, it could also be hypothesized that TrxR1 overexpression would augment synthesis of other selenoproteins if intracellular selenide supply was the limiting factor. To assess this question, we measured the GPx activity in these cell lines under increasing selenium concentrations in media. We thus found a clear difference in GPx activity between HEK-TrxR11 and the HEK-IRES control cells, with lower GPx activity in TrxR1-overexpressing cells (Fig. 2A). The largest difference was seen at low selenium supplementation (0.01 μM) where the GPx activity was almost 3.5-fold higher in HEK-IRES cells compared with HEK-TrxR11. At suppletions of 0.05–2 μM sodium selenite, the GPx activity in HEK-IRES was found to be constant at a level of ~1.3 times that in HEK-TrxR11. The endogenous glutathione reductase activity provides reduction of GSSG necessary for all glutathione-dependent enzymes. This was also measured and found to be around 5 nmol/min/mg protein, with no apparent difference between selenium concentrations or the different transfected HEK-293-derivative cell lines studied here (data not shown). Determining the GPx protein amounts in the cytosolic extracts by Western blot (Fig. 2B) and its 75Se incorporation (Fig. 2C) confirmed the considerable down-regulation of GPx upon TrxR1 overexpression.

Characterization of Stable HEK-293 Cells Overexpressing TrxR1 or TrxR2—Different reports have been published suggesting an inhibitory effect of TrxR1 on cell growth (35, 36, 49–51). We therefore examined the effect of TrxR1 and TrxR2 overexpression at different levels of selenium supplementation on cell viability in the stably transfected HEK-293 cells. In the range of 0–2 μM sodium selenite supplementation, the HEK-IRES cells reached confluence at day 5, whereas the TrxR1- and TrxR2-overexpressing cells grew considerably slower. HEK-TrxR11 reached confluence at day 6, and HEK-TrxR2.14 reached confluence at around day 7 (Fig. 3, A–C). Most interestingly, the effect on growth was similar from 0 to 2 μM selenite supplementation (Fig. 3) which implied that the effect on cell growth was not strictly related to

![Image](http://www.jbc.org/)

**Fig. 2.** The effect of TrxR1 overexpression in HEK-293 cells on GPx activity and amount. A, GPx activity in the cytosol (50 μg) of HEK-IRES (empty bars) and HEK-TrxR11 cells (black bars) incubated in indicated amounts of sodium selenite for 72 h. The activity was read spectrophotometrically at 340 nm detecting NADPH oxidation upon glutathione and glutathione reductase addition. Each value represents the mean of duplicates repeated three times (error bars correspond to S.D.). *, p < 0.1; ***, p < 0.001 compared with HEK-TR11 using Student’s t test. B, Western blot of GPx content in 50-μg cytosolic extracts from HEK-IRES and HEK-TrxR11 cells grown in 0, 0.1, or 2 μM sodium selenite. C, autoradiography of 75Se labeling of GPx and TrxR1.
TrxR activity, because the TrxR activity was markedly increased at increased selenite concentrations (Fig. 1). At a concentration of 10 μM, sodium selenite proved to be toxic (Fig. 3D). However, under these toxic conditions, the HEK-TrxR11 and HEK-TrxR2.14 cells were more resistant to selenite than the HEK-IRES cells. Furthermore, although the TrxR1- or TrxR2-overexpressing cells grew slower than the control cells, they also reached a higher number of cells per well than the control cells did during growth under the toxic 10 μM sodium selenite treatment (Fig. 3D). This could suggest a cytoprotective role of TrxRs at high selenium concentrations.

We reasoned that the slower growth of the TrxR-overexpressing cells could indicate that the Sec incorporation machinery is not completely sufficient for the overexpression, resulting in production of TrxR proteins truncated at the position of the Sec-encoding UGA codon. This truncated, selenium-compromised form of at least TrxR1 (but not the fully active selenium-containing enzyme) was recently shown to provoke directly apoptosis in A549 cells (33). However, we could not detect any significant cytochrome c translocation to the cytoplasm from the mitochondria, a well-used marker of apoptotic cell death, in any of our cell lines (HEK-TrxR11, HEK-TrxR2.14, or HEK-IRES control cells) upon incubation with increasing selenite concentrations, not even when using 1 μM of the strong apoptosis inducer staurosporine, illustrating resistance to apoptosis as an inherent property of HEK-293 cells (Fig. 4A). Another reason for the slower growth could be that the intracellular redox status in the TrxR-overexpressing cells becomes shifted, consequently affecting the function of different proliferation factors and ROS-dependent signaling pathways. To assess the overall ROS levels in the cells overexpressing TrxRs as compared with the control cells, we used the cell-permeable ROS indicator carboxy-H2DCFDA. This compound is nonfluorescent until intracellular esterases cleave off its lipophilic blocking groups and subsequent oxidation occurs, resulting in a charged fluorescent form trapped within the cell. However, by using this method we could not detect any significant difference in ROS amount in cells overexpressing TrxR1 or TrxR2 compared with the control cells (Fig. 4B). Preincubating the cells with 0.1 μM selenite also led to increased TrxR activity, because the TrxR activity was markedly increased at increased selenite concentrations (Fig. 1). At a concentration of 10 μM, sodium selenite proved to be toxic (Fig. 3D). However, under these toxic conditions, the HEK-TrxR11 and HEK-TrxR2.14 cells were more resistant to selenite than the HEK-IRES cells. Furthermore, although the TrxR1- or TrxR2-overexpressing cells grew slower than the control cells, they also reached a higher number of cells per well than the control cells did during growth under the toxic 10 μM sodium selenite treatment (Fig. 3D). This could suggest a cytoprotective role of TrxRs at high selenium concentrations.
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Table 1

| Cell line             | CD10 | BerEP4 | CK7 | CK-Cam5.2 | CK18 | CK19 | EMA | CD56 | WT1 | Vimentin |
|-----------------------|------|--------|-----|-----------|------|------|-----|------|-----|----------|
| HEK-IRES              | -a   | -      | -   | -         | -    | -    | -   | +    | () | +        |
| HEK-TrxR11            | -a   | -      | +   | +         | +    | (+)  | -   | +    | (+) | +        |
| HEK-TrxR2.14          | -    | -      | -   | +         | +    | -    | -   | +    | (+) | +        |

*a* = negative; +, positive; () weak positive.

**DISCUSSION**

Successful overexpression of selenoproteins is to a great extent hampered by the intricate and low efficiency translation machinery for incorporation of Sec into a growing polypeptide chain. Nevertheless, some prior reports have described stable overexpression of mammalian selenoproteins. The first such report described overexpression of mitochondrially targeted GPx (57), and recently we reported the isolation of HEK-293 cells stably overexpressing TrxR1 (18) and TrxR2 (45). Müller et al. (48) managed to isolate human hepatoma cells (HepG2) and baby hamster kidney cells (BHK-21) stably overexpressing various variants of GPxs, whereas Patenaude and co-workers (58) isolated several stable mammalian cell lines overexpressing TrxR2. Here we have described the successful construction of mammalian cells stably overexpressing active TrxR2 or TrxR1. The success in producing stable TrxR-overexpressing cell lines could be due in part to the use of the pIRESneo vector, but the inherent characteristics of the host cell line are probably also important determinants for selenoprotein overexpression, possibly more pronounced in the case of TrxR overexpression. Muller et al. (48) used a pcDNA3 vector (Invitrogen) in their experiments to successfully stably overexpress GPx variants in HepG2 and baby hamster kidney cells, illustrating that the features of the pIRESneo vector are not essential for stable selenoprotein expression per se, which also Nakagawa and co-workers (57) and Patenaude and co-workers (58) showed by using yet other vectors. Most importantly, we attempted to create stable cells lines overexpressing TrxR1 in Jurkat, SH-HeLa, and U1285 cells, but in those cases we could not isolate stably overexpressing clones. It is possible that TrxR1 and particularly selenium-compromised TrxR2 (33) carries intrinsic toxic effects provoking apoptosis in those cell lines. The fact that apoptosis is not easily induced in HEK-293 cells could thereby help explain the successful establishment of TrxR1-overexpressing HEK-293 cells. These difficulties were not seen with the TrxR2 gene.

In the present study we found that the specific activity of overexpressed TrxR1 was drastically elevated upon supplementation of sodium selenite in the medium, which was less obvious for TrxR2. The lower responsiveness of TrxR2 activity toward selenium supplementation indicates a more complicated post-transcriptional uptake of selenium for this mitochondrial selenoprotein compared with its cytosolic counterpart. One explanation for this may be that as TrxR2 probably is an important redox protein in the mitochondria (4), any increase or decrease in TrxR2 activity because of fluctuations in extracellular selenium concentration would disrupt the crucial redox balance important for a functioning respiratory chain. In fact, the 3′-UTRs of TrxR2 differ not only in distance to the SECIS element compared with TrxR1 but also lack AU-rich elements, indicating a different post-transcriptional regulation for this enzyme. The details of the transcriptional and translational machinery of TrxR2 are still to be elucidated.

In our experiments, elevated expression of TrxR1 clearly lowered the endogenous GPx expression and activity, espe-
cally under limited selenium availability, which was somewhat surprising to us. This may be a reflection of the higher hierarchy of TrxR1 synthesis over GPx synthesis, presumably governed by differences in yet unidentified regulatory sequences in the 3′-UTR mRNA of these selenoproteins (38, 48). In fact, others reported similar findings (59), where GPx was found to be repressed in tumor cells where TrxR1 was overexpressed. Other studies (60, 61) showed that limited selenium availability leads to a priority for the selenoenzyme 5′-deiodinase production over GPx.

Earlier reports (51, 58, 62) have suggested an important function of the thioredoxin system in redox regulation of cell growth. In our study, we found a significantly slower growth of the TrxR1- and TrxR2-overexpressing cells as compared with their controls. We initially believed that this slower growth could be ascribed to the cytotoxicity of selenium-compromised TrxRs (33), but the fact that the effect on cell growth was similar at all selenium concentrations, whereas the activity of the enzyme clearly increased at higher selenium concentrations in the medium, argued against this mechanism. In addition, we could not detect any apoptotic effect of TrxR1 or TrxR2 overexpression in our HEK-293 cells, as judged from mitochondrial cytochrome c release. However, because this was not provoked by staurosporine either, the HEK-293 cells seem to be remarkably resistant to induction of apoptotic cell death. An alternative explanation for the slower growth may be linked to more efficient antioxidant systems dependent upon TrxR, which would yield lower intracellular H₂O₂ concentrations. Elimination of H₂O₂ could subsequently disrupt growth factor-mediated pathways augmenting cell proliferation where H₂O₂ acts as second messenger (63), which could account for the slower growth of TrxR-overexpressing cells. Furthermore, Kim et al. (64) observed that HeLa cells transiently overexpressing dominant negative TrxR2, transcribed from DNA lacking the SECS1 element in the 3′-UTR, showed an increase in cell proliferation. This could then be suggested to be due to a higher intracellular H₂O₂ concentration, resulting from an altered redox balance as the inactive TrxR2 may possibly inhibit the endogenous full-length TrxR2. On the other hand, we could not detect any difference in intracellular ROS amounts in cells overexpressing TrxR1 or TrxR2 compared with control cells, using an intracellular fluorescent ROS indicator.

Most surprising was the novel expression of several markers for epithelial differentiation in the TrxR1- and TrxR2-overexpressing cells. The finding is, however, coherent with the elevated TrxR activity in mesothelioma cells with an epithelial differentiation compared with mesothelioma cells with a sarcomatoid phenotype (54). Although the mechanism behind a possible TrxR-mediated differentiation signaling is not known, it has been shown previously that the levels and activities of both TrxR1 and TrxR2 are elevated in several epithelial tumors (52, 54). It should be noted also that overexpression of TrxR2, resident in the mitochondria, yielded expression of cell surface proteins regarded as markers for epithelial differentiation, although the pattern was not identical to that seen with TrxR1. In contrast to HEK-TrxR1 cells, the HEK-TrxR2.14 cells did not express BerEP4, an epithelial marker often seen in tissues with neuroendocrine differentiation pattern. However, none of the cell lines expressed the general epithelial protein EMA nor the epithelial antigens CD10 and CK7, which indicates an intermediate differentiation status of HEK-TrxR1 and HEK-TrxR2.14. The complex mechanism of cell differentiation in response to environmental stimuli involves modulation of transcription factors and thereby differential expression of specific genes. The redox status in the cell is a major regulator of differentiation and, in this context; the ability of the Trx system to scavenge ROS and reduce functional cysteines in different transcription factors can affect the differentiation status in the cell. This could be true even if the overall redox status of the cell remains unaltered. Both TrxR1 and TrxR1 may enter the nucleus by unknown mechanisms (52) and there modulate the DNA binding and transcriptional activity of several important redox sensitive transcription factors, including nuclear factor-κB (NFκB) (65, 66), activator protein-1 (AP-1) (67, 68), SP1 (69), and tumor suppressor protein p53 (70). Such effects could possibly explain the higher degree of differentiation in the TrxR1-overexpressing cells. In addition, it has been demonstrated that TrxR1 can positively regulate the transcriptional activity of estrogen receptor β (71), proposing a role for TrxR1 as an indirect regulator of genes under expression control of this nuclear receptor. Furthermore, estrogen receptors are also present in the mitochondria (72, 73), a fact that may be related to the effects on differentiation in the TrxR2-overexpressing cells, i.e. TrxR2 may also be involved in redox regulation of estrogen receptor resident in the mitochondria.

One aspect further adding complexity to the thioredoxin system is the existence of several different splicing variants of both TrxR1 (2, 71) and TrxR2 (74, 75), targeted to both the mitochondria and cytosol, respectively. Little is still known about these splice forms, and future studies are needed to assess whether other effects on differentiation patterns would be obtained by overexpression of other splice variants of TrxR1 or TrxR2 than those utilized in this study.

The construction of stable cell lines overexpressing thioredoxin reductases expands the possibilities of biological analysis of selenoproteins in general, and TrxRs in particular, using the convenient and amenable in vitro system. Although the intricate translation machinery of selenoproteins has yet to be explored further, availability and the characterization of these cell lines may assist in the continued studies of mammalian selenium metabolism, selenoprotein synthesis, and selenium pathophysiology. The unexpected effects on differentiation by TrxR1 or TrxR2 overexpression also deserve further scrutiny.

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