An Alternative Splicing Variant of the Selenoprotein Thioredoxin Reductase Is a Modulator of Estrogen Signaling*

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The selenoprotein thioredoxin reductase (TrxR1) is an integral part of the thioredoxin system. It serves to transfer electrons from NADPH to thioredoxin leading to its reduction. Interestingly, recent work has indicated that thioredoxin reductase can regulate the activity of transcription factors such as p53, hypoxia-inducible factor, and AP-1. Here, we describe an alternative splicing variant of thioredoxin reductase (TrxR1b) containing an LXXLL peptide motif, implicated in direct binding to nuclear receptors. In vitro interaction studies revealed direct interaction of the TrxR1b with the estrogen receptors α and β. Confocal microscopy analysis showed nuclear colocalization of the TrxR1b with both estrogen receptors α and β in estradiol-17β-treated cells. Transcriptional studies demonstrated that TrxR1b can affect estrogen-dependent gene activation differentially at classical estrogen response elements as compared with AP-1 response elements. Based on these results, we propose a model where thioredoxin reductase directly influences the estrogen receptor-coactivator complex assembly on nonclassical estrogen response elements such as AP-1. In summary, our results suggest that TrxR1b is an important modulator of estrogen signaling.

Thioredoxin reductase (TrxR)1 is a selenocysteine containing enzyme (1) that catalyzes the reduction of thioredoxin (Trx) in the presence of NADPH constituting the so called thioredoxin system (2). In mammalian cells three TrxRs have been characterized, a cytoplasmatic one (TrxR1), a mitochondrial specific TrxR (TrxR2) (3), and more recently, a testis-specific one that can function both as a thioredoxin reductase and as glutathione reductase (4). TrxR1 purified from rat liver exists as a dimer of two identical 58-kDa subunits (5). In addition to reducing Trx, TrxR1 can reduce proteins such as protein-disulfide isomerase and plasma glutathione peroxidase (6). Furthermore, it has a wide range of low molecular weight targets such as dehydroascorbic acid, lipoic acid, selenite, and selenocysteine, it can cleave S-nitrosogluthathione (GSNO) to glutathione and nitric oxide (6) and can scavenge peroxynitrite (7). Recently, we also reported the reduction of ubiquinone by TrxR1 (8).

It has also been reported that TrxR1 is involved in the regulation of transcription factors such as p53, hypoxia-inducible factor and AP-1. Prostaglandins have been shown to covalently modify and inhibit the function of TrxR1 leading to the repression of p53 and hypoxia-inducible factor (9). Furthermore, TrxR1 is involved in promoting the cell death effect of combined interferon and retinoic acid (10, 11), caused by the TrxR1-dependent activation of p53-mediated gene expression (12, 13). In addition, p53-dependent gene expression was inhibited in yeast lacking TrxR because of the oxidation and inactivation of Trx, suggesting that the effect of TrxR1 on p53 activity is mediated by the reduction of Trx (14, 15). Recently, TrxR1 has also been shown to regulate the activation of AP-1 although this is also thought to occur through its function of reducing Trx1 (16). These examples indicate that TrxR1, alone and/or through Trx, may impact various transcriptional pathways.

Estrogen receptors (ERs) belong to the steroid/thyroid hormone receptor superfamily of nuclear receptors that upon binding of ligand are activated and bind to specific DNA response elements to initiate transcription of target genes (17). Apart from promoting DNA binding, the ligand-induced conformational change of the ligand binding domain of the receptors enables them to recruit co-activators such as GRIP1 and SRC-1 and the histone acetyltransferases p300/CBP and pCAF to the response elements, leading to activation of transcription (18, 19). In the case of ERs, it has been reported that, apart from the classical ER activation pathway that depends on the binding of ERs to the EREs (estrogen response elements), ER can regulate transcription even via AP-1 and SP-1 sites without ER binding to DNA (20–24).

We describe here a TrxR1 variant, referred to in this paper as TrxR1b, with an extension at the N terminus. Within the extra amino acid sequence we found the presence of a nuclear receptor motif (NR box) with the consensus sequence LXXLL, which mediates the binding of co-activators to their nuclear receptors (25). Indeed, the presence of an LXXLL motif in the TrxR1b leads to the direct interaction of the protein with ERα and ERβ. As a result of the TrxR1b binding to the ERs, the reductase is translocated into the nucleus and colocalizes with the ERs. Furthermore, whereas TrxR1b enhances the transcriptional activity of the ERs at the classical EREs it effectively represses ER activity at AP-1 sites, suggesting that TrxR1 could be an important regulator of ER activity and ER-dependent cellular growth and differentiation.

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1 The abbreviations used are: TrxR, thioredoxin reductase; AP-1, activator protein 1; E2, estradiol; ER, estrogen receptor; ERE, estrogen response element; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GR, glucocorticoid receptor; NR box, nuclear receptor box; Trx, thioredoxin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; TR, thyroid receptor.
**Materials and Methods**

**Plasmids**—For the construction of the glutathione S-transferase (GST)-TrxR1b42–56 fusion, which includes the LXXL motif found in the long form TrxR1, the oligos KDRF5 (5′-GCAGTGGATCCCATGTCCCCTATACTAGGTTATTGGAA-3′) and KDRF5′ (5′-GATCTATCCAGGAGGAGCAGTCACTAAAAAGCTATACAAA-TGAAAGGCCCCTTAAG-3′) were annealed and subsequently ligated into an EcoRI- and BamHI-digested pGEX-3x vector. For the construction of the full-length protein–GST fusions, TrxR1a was amplified employing standard PCR techniques and the primers hTrxR1′ NdeI (5′-GCCGTGCTATATGCGGCCGCTGAAGGTCTATTCCCC-3′) and hTrxR1′ BamHI (5′-CCATGCAGTGCAGCAGGAGCGGTCGTCGAGGGAG-3′), whereas TrxR1b was amplified with the primers KDRF5′ NdeI (5′-GCAGTGCATATGCTGAGGAGCGGTCGTCGAGGGAG-3′) and hTrxR1′ BamHI. The PCR products were purified and digested with NdeI and BamHI and then ligated into the pET11c vector. The open reading frame for GST was amplified from the pGEX-3x vector with the GST′ BamHI (5′-CAAGCTGATCCATGCGCTGATGAGG-3′) and GST′ BamHI (5′-CTGAGGATCCATGCGCTGATGAGG-3′) primers. After purification the PCR products were digested with BamHI and ligated in-frame into the pET11c vectors containing either TrxR1a or TrxR1b, resulting in a fusion where the GST protein is located at the C terminus of the products so that it should not interfere with the LXXL motif located at the N terminus of the TrxR1. For the construction of GFP fusions the open reading frames of TrxR1a and TrxR1b were amplified with the hTr1 GFP-F1 (5′-GCGGGATTACCATGGCAGCCCGTCGAGGATGAGGAGCAGGACCGCATGCGCCATGCGCCCTACATAGTGGATTGGA-AATTAAG-3′) and GST′ BamHI (5′-CTGAGGATCCATGCGCTGATGAGG-3′) primers from the pREd1-N1 vector and replaced the green fluorescent protein in the pEGFP-N3 by digestion with BamHI and NotI.

Other plasmids used are described elsewhere: pIRESneo-TrxR1 (8), pIRESneo-TrxR1a (8, 52), pIRESneo-TrxR1b (52), pET11c vectors containing either TrxR1a or TrxR1b, resulting in a fusion where the GST protein is located at the C terminus of the reported transfections we used 500 ng of the reporter plasmid, 250 ng of pSg5-ERE or -β, and 5 μg of the pIREsneo-TrxR1a or -b or pIREsTrx1 and used polyethylene glycol as the transfection agent. The amount of the DNA was kept constant by the addition of empty pIREsneo vector. For the GFP analysis, cells were transfected with 1 μg of the indicated DNA. After transfection, the cells were kept for 2 days in the presence of ligand, 25 μM E2 or 100 μM ICI 182, 780. Luciferase was measured using the luciferase assay kit from Promega. The GFP analysis was performed with a Leica laser scanning confocal microscope. For the expression of the GFP we used the 488-nm laser line and light was collected between 500 and 540 nm, whereas the excitation of HcRed was achieved with the 568-66 nm laser line and the emitted light was collected between 580 and 640 nm. Both live and fixed cells were used for the localization studies. For fixed cells studies, cells were seeded onto coverslips and transfected after 2 days. Cells were fixed in 3.7% paraformaldehyde for 30 min in room temperature and mounted using Fluorescent Reagent (Calbiochem). For the fluorescence recovery after photobleaching (FRAP) studies cells were seeded onto tissue culture dishes for 2 days prior to transfection. Live cells were point bleached by exposing a limited area of the cell to a high intensity laser beam for 30 s using the 488-nm laser line and then images were acquired every 15–20 s.

**Results**

**Expression of TrxR1a and -b**—Several miRNA splicing variants are known for human TrxR1 (28). However, their translation results in only two different proteins. One of those, referred to as TrxR1b in this paper, contains an extra 52-amino acid sequence at the N terminus (Fig. 1, A and B). To study the specific expression of TrxR1b we raised an antibody (anti-TrxR1b) against amino acids 28–42 (KQRKGGHGTPTLKY) within the extra amino acid sequence. The specificity of the anti-TrxR1b was demonstrated with Western blot analysis using extracts from HEK-293 cells transfected with expression plasmids for TrxR1a and -b and with fusions of the TrxRs with GFP. Two Western blots were performed in parallel, one developed with the specific anti-TrxR1b antibody and the other with an anti-TrxR1a antibody (Fig. 1C). An enhanced signal can be seen in the cells transfected with TrxR1b and TrxR1b-GFP, whereas cells transfected with TrxR1a and TrxR1a-GFP had the same signal as the untransfected cells when developed with the TrxR1b antibody. However, in the Western blot developed with the anti-TrxR1a we saw an increased signal in all the transfected cells further confirming the specificity of the anti-TrxR1b antibody as no TrxR1a is detected in the anti-TrxR1b blot (Fig. 1C, lanes TrxR1a and TrxR1a-GFP). The SDS analysis of in vitro translated TrxR1a and TrxR1b is shown in Fig. 1D.

To verify the expression of TrxR1b in human tissues we used extracts from different sources. As shown in Fig. 2A, TrxR1b is expressed in all tissues analyzed with particularly high expression in testis, testis, ovary, and prostate. We also analyzed the expression of the protein in various cell lines (Fig. 2B). HeLa cells and HT cells express TrxR1b at high levels, whereas the level of expression in the other cell lines was lower. Furthermore, in tissues like testis and kidney as well as in cell lines such as HEK-293, HeLa, and HT, two bands are detected. The lower band seems to be a degradation product from TrxR1b. Even the bands seen in the cells transfected with TrxR1b and TrxR1b-GFP, whereas cells transfected with TrxR1a and TrxR1a-GFP had the same signal as the untransfected cells when developed with the TrxR1b antibody.
provides an explanation as to why we sometimes detect two bands. Because our antibody reacts to amino acids 28–42, the protein cleaved at amino acid 24 still contains the crucial amino acids for its detection; when cleaved at amino acid 49, however, the recognition sequence is discarded making the detection of the third expected band impossible.

To compare the expression levels of TrxR1a and -b we used a polyclonal antibody raised against TrxR1a. Fig. 2A shows that the antibody can recognize both forms of the protein when cells were transfected with respective expression plasmids. However, in the untransfected and TrxR1a-transfected cells only one band is visible, corresponding to TrxR1a, whereas just a faint shadow is visible at the place one would expect TrxR1b, suggesting that TrxR1a is much more abundant in HEK-293 cells, however, at significantly lower amounts. In conclusion, the TrxR1b appears to be widely expressed in both human tissues and cell lines, and furthermore, to be specifically cleaved at the N terminus in several tissues and cell lines.

TrxR1b Binding to the Estrogen Receptors—The most distinct feature of the extra amino acid sequence in TrxR1b is the presence of an NR box at amino acids 47–51 with the consensus sequence LXXLL (Fig. 1A). The LXXLL motif is present in the majority of nuclear receptor coregulators and is responsible for their binding to their respective nuclear receptor (25). The presence of an NR box in TrxR1b raises the question of whether the protein is able to bind to nuclear receptors. A fusion of GST with a peptide comprising amino acids 42–56 of TrxR1b (GST-TrxR1b42–56), including the NR box, was constructed to be used as bait to isolate interacting nuclear receptors. Subsequent GST pull-down experiments showed that both ERs could interact with GST-TrxR1b42–56. Furthermore, this interaction was estrogen dependent (Fig. 3A).

Fusions of the entire TrxR1a/b proteins with GST were used to confirm these results. As shown in Fig. 3B, TrxR1b was able to bind both ERs, and the binding was ligand dependent as...
containing the peptide, amino acids 42 to 56, of TrxR1b was expressed although not with the same strength as observed with ER
XX
LL motif at the N terminus. To further confirm the specificity of the binding to the LXXLL motif of TrxR1b, A, the NR box

nuclear Trafficking—ERα/β and Their Sub-nuclear Trafficking—ERα/β and TrxR1a/b reside in different compartments of the cell. ERα/β are localized mainly in the nucleus, whereas TrxR1a/b are mainly cytoplasmic (Fig. 5A) when these proteins are expressed in cells as fusions with GFP and HcRed, respectively. However, we show that, although TrxR1 is mainly a cytoplasmic protein, there are always low amounts of the protein present in the nucleus and ∼5% of the cells have quite substantial amounts, with ∼10% of the total amount of TrxR1a/b protein present in the nucleus (Fig. 5A). When cotransfecting GFP-ERα/β with TrxR1b-HcRed, in the presence of E2, the localization pattern of TrxR1b in the nuclear changes, from mainly a homogeneous distribution to distinct spots colocalizing with ERα/β (Fig. 5B). Furthermore, we observed that in the presence of ER and E2, the nuclear localization of TrxR1b would become more pronounced so ∼35% of the cells would have more than 10% of the protein in the nucleus and furthermore, in ∼10% of the cells, it was exclusively in the nucleus; this was not observed without cotransfection. TrxR1a, however, shows the same pattern as when transfected alone (Fig. 5C), i.e. it is mainly cytoplasmic with the nuclear portion of the protein homogeneously distributed in the nucleus with no apparent colocalization with ERα/β.

The pull-down experiments revealed that the interaction between TrxR1b and ERs was estrogen dependent, therefore we investigated whether estrogen is important for colocalization of the proteins. For that reason we used the antiestrogen ICI and performed similar transfection experiments as described above. Fig. 5B shows that ICI effectively abolished nuclear colocalization of the ERs and TrxR1b. TrxR1b was no longer restricted to areas of the nucleus where ER was localized but was distributed throughout the entire nucleus. This suggests that the interaction of the ERs and TrxR1b is essential for their colocalization observed in the presence of E2.

To obtain quantitative data from the localization studies we analyzed the images and produced graphs of the fluorescent intensity over a certain area of the image, a fluorescence profile. The analysis was performed on the cells shown in Fig. 5B.
in the presence of either E₂ or ICI. As shown in the graphs (Fig. 6) obtained from the cells in the presence of E₂, the profile of TrxR1b and ERα/β overlap, whereas in the presence of ICI the TrxR1b fluorescence assumes a more flat profile, no longer resembling the ERα/β distribution, further demonstrating the importance of E₂ for the colocalization pattern of the ERs and TrxR1b in the nucleus. However, this interaction does not seem to be necessary for the presence of TrxR1b in the nucleus, because part of the TrxR1b pool is in the nucleus even during ICI treatment (Fig. 5B) as well as in the absence of ERs (Fig. 5A). On the other hand, we were unable to find any cells where ERs and nuclear TrxR1b were not colocalized during E₂ treatment. Furthermore, the fact that TrxR1b can be found almost exclusively in the nucleus of cotransfected cells suggests that the ER/TrxR1b interaction, although not essential for nuclear import of TrxR1b, might result in an accumulation of TrxR1b in the nucleus. Hence, both ERα/β and E₂ are required for TrxR1b to assume its structured/dotted nuclear pattern seen when

**FIG. 5.** ERα/β both colocalize with TrxR1b. A, cells were transfected with either GFP-ERα/β or TrxR1a/b-HcRed1 expression plasmids in the presence of E₂. After 2 days cells were fixed and analyzed with a

**FIG. 6.** Fluorescence profile of cotransfected ERα/β and TrxR1b in the presence of either E₂ or ICI. The area used for creating the profile is shown as a white line in the merged cell pictures.

Leica confocal laser scanning microscope. B, cells were cotransfected with GFP-ERα/β and TrxR1b-HcRed1 and kept for 2 days in media containing 25 nM E₂ or 100 nM ICI. After the incubation period cells were fixed and analyzed. C, cells were cotransfected with GFP-ERα with TrxR1a-HcRed1 and kept for 2 days in media containing 25 nM E₂.
colocalized with the ERs as the presence of only ER or E₂ alone does not significantly change the cellular pattern of TrxR1b. Furthermore, the presence of the 52 extra amino acid sequence is required as the cellular localization of TrxR1a lacking those extra amino acids was not influenced by the presence of ERs and E₂.

Using the FRAP technique showed that ER/H9251 appears to be very mobile within the nucleus. Furthermore, this mobility is ligand-dependent but also dependent on proteasomes as the use of proteasome inhibitors causes the immobilization of ER (31). We performed FRAP experiments to further investigate interactions between TrxR1b and the ERs. Live cells cotransfected with ERα and TrxR1b were point bleached in the nucleus and images were captured every 15 s. In the presence of E₂, ERα was colocalized with TrxR1b; after bleaching ERα recovers very fast, in a matter of seconds, and the same was true for TrxR1b (Fig. 7). Moreover, when point bleached for longer periods of time, the entire nucleus would become bleached, further confirming the pronounced mobility of both TrxR1 and ERα. Similar results were obtained with cotransfection of ERβ. This demonstrates that both ER and TrxR1b are mobile proteins and that the presence of TrxR1b does not seem to interfere with the dynamic intranuclear behavior of the ER.

Effect of TrxR on ER Transcriptional Activity—Because ER appears to relocate TrxR1b in the nucleus to areas where it resides itself we wondered whether TrxR1b could affect the transcriptional activity of ER. Cotransfections of ERα/β with TrxR1a/b were performed and ER transcriptional activity was measured using a luciferase reporter vector under the regulation of an ERE-TATA. In the case of ERα, TrxR1b was capable of enhancing the transcriptional activity of the receptor 2-fold, whereas TrxR1a did not significantly affect ERα activity (Fig. 8A). In the case of ERβ, a 3-fold induction of its transcriptional activity was obtained in the presence of TrxR1b. However, in contrast to ERα, the ERβ activity was also enhanced by TrxR1a in a similar manner to TrxR1b (Fig. 8A). We then examined whether the ERβ activity could be enhanced by the cytosolic Trx as has been demonstrated for ERα (32). Indeed, as shown in Fig. 8B, Trx1 is a potent inducer of ERβ as it is for ERα, with a 4-fold induction of the ER transcriptional activity, suggesting that ERβ just like ERα can be redox regulated.

In addition to the classical ERE-dependent ER pathway, it has been shown that ER can affect transcription at AP-1 sites (20–22). Moreover, TrxR1a has been reported to regulate AP-1 activity (16). Could TrxR1a/b have any influence on the alternative pathway of ER transcriptional activity through AP-1? When the AP-1 reporter was transfected with either TrxR1a or -b, no differences could be observed between the two proteins. However, when either of the two ERs was added, a significant difference was found between TrxR1a and -b with respect to the AP-1 response (Fig. 9). Whereas in the presence of TrxR1a, both ERs were capable of inducing the AP-1 activity 2-fold, but both ERs failed to induce the AP-1 in the presence of TrxR1b and instead a decreased AP-1 activity was observed. Thus, both TrxR1a and -b can act as an activator of ERα and -b in the classical pathway by enhancing its transcriptional activity, most probably because of an increased reduced nuclear environment and through the reduction of Trx as is the case with several other transcription factors, whereas TrxR1b acts...
as a suppressor for both ERs in their alternative transcriptional pathways involving AP-1 sites. This effect can be explained by the physical protein-protein interaction shown in our proposed model (Fig 10), where TrxR1b could interfere with the assembly of AP-1 ER complex.

DISCUSSION

Studies on the genomic organization and mRNA expression of TRXR1 revealed that there are many splicing variants resulting from the 5’ end of the TRXR1 gene (28). There are several exons present at the 5’-untranslated region of TRXR1, with the ATG for the original TrxR present in exon 4. When exons 1 and 2 together recombine with exon 4, a new ATG is formed that is in-frame with the start codon for the original TrxR1 and gives rise to a protein with a 52-amino acid extension. Could this additional N-terminal amino acid extension provide a novel function to TrxR1? When analyzing the protein sequence we found the presence of an NR box; this box has the consensus sequence LXXLL and is responsible for the binding of coregulatory proteins to nuclear receptors (25). This suggested that TrxR1b might bind to nuclear receptors. Furthermore, it has been shown that peptides selected on the basis of a strong affinity to ERα often had an arginine before the first leucine in the LXXLL motif (34), just like the NR box in TrxR1b. In addition, peptides that can bind activated ERα have been divided into three subgroups according to their amino acids flanking the LXXLL motif (35). The TrxR1b motif did not fall into any of those classes, although it has a charged amino acid (Arg) upstream of the LXXLL motif, which is consistent with the class I peptides, it lacks the conserved serine found in the second position upstream of the NR box. TrxR1b bound to both ERs and apart from a weak binding to ERβ in the absence of ligand it did not display any preference for any of the ERs as demonstrated for TRAP220 (30) and the class II peptides that contain a proline as the second amino acids upstream the LXXLL motif (35).

To understand the biological significance of the in vitro interaction between ERα/β and TrxR1b, the intracellular localization of these two proteins was investigated. In several studies it has been shown that the estrogen receptor is a nuclear protein (31, 36–38) and that ligand is important for the intranuclear distribution of the estrogen receptor (36, 38). In our experiments both ERαs behaved in a similar manner giving rise to a structured nuclear pattern in HEK-293 cells in the presence of E2. On the other hand, TrxR1 has been considered to be a cytoplasmic protein, similar to our results, however, low levels of the proteins could be detected in the nucleus. Interestingly, histological studies on lung carcinoma biopsies have demonstrated strong immunoreactivity against TrxR1 in the nuclei of non-small lung cells (39). It is unclear how TrxR1 enters into the nucleus because neither TrxR1a nor TrxR1b have any classical nuclear localization signals.

Cotransfection of fluorescent ERαs and TrxR1a gave similar
results as when these constructs were transfected individually. However, cotransfection of TrxR1b and ER changed the nuclear pattern of TrxR1b. TrxR1b relocates from a cytosolic or homogeneous nuclear distribution, and forms a dotted pattern colocalizing with both ERs. A similar nuclear redistribution has previously been reported for peroxisome proliferator-activated receptor γ under the influence of RXRα (40). However, unlike TrxR1b, which is a cytosolic protein, peroxisome proliferator-activated receptor γ is predominantly localized in the nucleus. A protein more relevant to TrxR1b would be the short form of metastatic tumor antigen 1 that similarly to TrxR1b is localized in the cytoplasm. It has been reported that metastatic tumor antigen 1 can bind ER but unlike TrxR1b the interaction of metastatic tumor antigen 1 and ER leads to the retention of the receptor in the cytoplasm (41). However, in our cotransfections, we could not detect any cytoplasmatic ER suggesting that TrxR1b is not able to retain ER into the cytoplasm and that ER is the dominant partner. When the experiments were performed in the presence of ICI, the localization pattern of TrxR1b once again changed. The presence of ER seems to be essential for the intranuclear distribution of TrxR1b but not for its nuclear localization, because cells that had only TrxR1b or TrxR1b and ER in the presence of ICI, still showed the presence of TrxR1b in the nucleus. Interestingly, when cotransfected with ER and TrxR1b, several cells showed TrxR1b exclusively in the nucleus, an observation that was never made when TrxR1b was transfected alone. Apparently, ER can bind to TrxR1b and change its subnuclear pattern but probably also inhibits its export from the nucleus leading to an accumulation of the protein in the nucleus.

ERα has been shown to be highly mobile within the nucleus in the absence of ligand (31). In the presence of E2, the mobility is reduced, which is thought to be caused by the binding of ER to the nuclear matrix, however, even though the mobility is reduced the protein is still dynamic. Similarly, it has been shown that GR is also highly mobile and undergoes rapid exchange with DNA-binding sites (42). The question was then if TrxR1b had the same dynamic intranuclear behavior as ER or if it could influence that of ER. To clarify this point we used the FRAP technique to study the intranuclear trafficking of the proteins. Both ERs and TrxR1b were found to be mobile. After bleaching, the recovery rate was similar for both proteins, indicating that the nuclear colocalization of ER and TrxR1b represents a dynamic process where both proteins retain their mobility. Furthermore, results obtained from transfections studying the transactivation function of the ERs in the presence of TrxR1b cannot be attributed to changes in the dynamics of the ERs because their mobility is intact.

Although ERs seem to have a drastic impact on the intranuclear localization of TrxR1b, one might ask whether TrxR1b has any effect on ER function. The activity of ERs has been found to be redox regulated with Trx as a mediator (32). Can TrxR1b directly affect ER or is Trx necessary to reduce ER and enhance transcription? In transient cotransfection experiments using an ERE TATA plasmid with luciferase as a reporter for ER activity, TrxR1b enhanced the transcriptional activity of ER. This might be explained by TrxR1b creating a reduced environment in the immediate vicinity of ER in the nucleus. In the case of ERα, only TrxR1b was active, whereas for ERβ both forms of TrxR1 act as coactivators. In our system ERβ has an almost 7-fold lower activity than ERα on the ERE TATA reporter (data not shown); therefore even the low amounts of TrxR1a and Trx1 that could be present in the nucleus might influence ERβ activity through their reducing activity. In addition, TrxR1 has been shown to promote the nuclear localization of Trx1 (16), which is an activator of ERα (32). However, this has not yet been shown for ERβ, so we performed a cotransfection experiment to determine whether Trx1 is capable of enhancing ERβ activity. Indeed, Trx1 activated both ERs 4-fold as compared with their basal activity. Because in our experiments ERβ has so much lower basal activity than ERα, even the presence of endogenous Trx1 could be sufficient to enhance the activity of ERβ, whereas for ERα, which is a very potent activator of the ERE TATA reporter, the endogenous Trx1 would affect the ERα activity only marginally.

We also examined the effect of TrxR1b on the alternative transcription pathway of ERs at AP-1 sites. In contrast to the classical pathway, TrxR1b appeared to diminish the activation of AP-1 by the ERs, whereas TrxR1a enhanced ER activity. It has been proposed that ER activates AP-1 by binding to coactivators that have already bound to the AP-1 and then triggers them to higher activity (43). We propose that TrxR1b could inhibit this activation by binding to ER and preventing it from binding to the AP-1 coactivator complex. In the presence of TrxR1b, the amount of ERs could thus be decreased at AP-1 sites and shifted toward ERE containing genes, thereby selectively enhancing the classical estrogen response pathway instead of the alternative AP-1 pathway.

The presence of an LXXLL motif in TrxR1b might also counteract its export from the nucleus and thus increase its concentration in the nucleus. It has been reported previously that TrxR1b is translocated into the nucleus under conditions that induce oxidative stress such as during UV irradiation (44). We provide here a mechanism by which TrxR1b could accumulate in the nucleus to provide reducing equivalents to both Trx and nuclear redoxin, a nucleus-specific Trx-like protein that has been found to regulate transcription factors such as AP-1 and NFκB (45, 46). Thereby TrxR1b could indirectly regulate also redox-sensitive transcription factors other then ER, such as GR (47), NFκB (48), AP-1 (49), SP1 (50), and p53 (51). However, the physical binding of TrxR1b to nuclear receptors suggests that TrxR1b might have a much more direct role in transcriptional regulation by interfering with the assembly of transcription complexes. TrxR1b could also be involved in chromatin regulation, as many proteins associated with ERs are proteins involved in chromatin remodeling and modification (52–55). Future studies are needed to clarify whether TrxR1b might be involved in the chromatin remodeling that occurs during ER activation of transcription.

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