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Thioredoxin, a regulator of gene expression

Maria Kontou[1], Rainer D Will[2], Caroline Adelfalk[1,3], Rainer Wittig[2], Annemarie Poustka[2], Monica Hirsch-Kauffmann[1,3] and Manfred Schweiger*[,1]

[1]*Institut für Biochemie, Freie Universität Berlin, Thielallee 63, Berlin D-14195, Germany;* [2]*Department of Molecular Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Im Neuenheimer Feld 580, Heidelberg D-69120, Germany*

Cancer cells have high levels of thioredoxin (Trx) and of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Cells from patients with the cancer-prone disease Fanconi anemia (FA) exhibit reduced Trx levels. We found the activity of GAPDH to correlate directly with the endogenous Trx content and mRNA transcripts for GAPDH and TRx reduced in FA cells. The treatment of cells with reduced human Trx stimulated the synthesis of GAPDH mRNA. Similarly, the transfection of cells with an expression plasmid for Trx increased GAPDH mRNA synthesis. Trx treatment of cells and subsequent analysis of the differential gene expression by human cDNA arrays containing about 50 000 different PCR products resulted in more than 300 up- or downregulated genes. Two representative genes, GAPDH and IκBα/MAD-3, were further investigated to confirm their stimulation by Trx. Trx besides being the major carrier of redox potential of cells is also a regulator of gene expression on the transcriptional level. By regulation via Trx, cells are able to adapt to the prevailing redox conditions. These findings also enlighten the pathophysiology of FA in the respect that the characteristic diminution of Trx that results in the dysregulation of gene expression is a basis for the major symptoms of this disease.
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Introduction

Thioredoxin (Trx) is the major carrier of redox potential in cells. Recently, we showed in cells from patients with the fatal hereditary disease Fanconi anemia (FA) that the pathological elevation of oxygen radicals (ROS) is accompanied by a significant decrease in Trx (Kontou *et al*., 2002). In addition to its antioxidative function, Trx acts as a cofactor for essential enzymes as, for example, nucleoside diphosphate reductase, the enzyme responsible for the production of DNA precursors (Holmgren, 1989), for T7 DNA polymerase (Huber *et al*., 1987) and for 3′phosphoadenosylsulfate reductase (Lillig *et al*., 1999). Further, Trx is involved both in protein repair via methionine sulfoxide reductase (Brot and Weissbach, 1991) and in the reduction of protein disulfides (Stewart *et al*., 1998).

Mammalian Trx participates in the action of the transcription factor NF-κB (Schreck *et al*., 1992; Hayashi *et al*., 1993; Schenk *et al*., 1994; Sen and Baltimore, 1996; Dalton *et al*., 1999), and also interacts with the DNA-binding proteins AP-2, AP-1, p53, PEBP2/CBF as well as glucocorticoid and estrogen receptors (Powis and Montfort, 2001a). These actions of Trx result in its involvement in apoptosis, tumorigenesis and oxidative stress. It is, therefore, of interest to elucidate the precise role Trx plays in gene expression. In this regard, it was reported that the synthesis of manganese superoxide dismutase (MnSOD) is stimulated in cells with the addition of Trx (Das *et al*., 1997). In order to obtain a better insight into the role of Trx in gene expression, we focused on the regulation of specific RNAs and enzymes under the regimentation of Trx. DNA arrays were used to assess the potential of Trx to up- or downregulate gene expression. In this context, the regulation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analysed more extensively.

GAPDH is an important 'housekeeping' enzyme in glucose metabolism. Other important functions of this enzyme in DNA repair, apoptosis and oxidative stress serve to emphasize the diversity of roles this enzyme plays. GAPDH acts as a cellular kinase (Kawamoto and Caswell, 1986) and is regulated by PKC-, EGF kinase- and Ca^{2+}-dependent protein kinase II (Ashmarina *et al*., 1988, Reiss *et al*., 1996). GAPDH apparently binds to 5′- or 3′-UTR mRNA sequences, thus playing a role in the translational regulation of gene expression (Nagy and Rigby, 1995, Schultz *et al*., 1996). GAPDH is a tubulin-binding protein, which catalyses tubulin polymerization into microtubules (Durrieu *et al*., 1987, Muronetz *et al*., 1994), thereby promoting membrane fusion. This function may be of special interest in the case of FA since, as we have observed previously, membranes are altered in FA fibroblasts (Willingale-Theune *et al*., 1989). The capability to bind Ap4A

*Correspondence: M Schweiger, Current address: Max-Planck-Institut für Molekulare Genetik, Ihnestr. 73, Berlin D-14195, Germany; E-mail: schweigm@molgen.mpg.de
[3]Current address: Max-Planck-Institut für Molekulare Genetik, Ihnestr. 73, Berlin D-14195, Germany
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implies a role of GAPDH in DNA replication and repair (Baxi and Vishwanatha, 1995). Actually, it also works as a uracil DNA glycosylase (Meyer-Siegler *et al*., 1991). It is worth noting that GAPDH plays a role in apoptosis similar to that shown for cerebellar granular cells (Sunaga *et al*., 1995; Ishitani *et al*., 1996; Ishitani *et al*., 1998). In these cells, apoptotic death is age induced and accompanied by the formation of the GAPDH protein. GAPDH is also involved in organizing cellular responses to oxidative stress (Dastoor and Dreyer, 2000) by binding NADH–dichlorophenol–indophenol oxidoreductase in neuronal plasma membranes. The complex has a high impact on major cellular processes. Furthermore, GAPDH is a target for nitric oxide (Messmer and Brune, 1996).

In this paper, we demonstrate that Trx strictly regulates the expression of GAPDH. The implication of this finding with respect to FA, a human hereditary disease exhibiting endogenously low levels of Trx, is discussed.

Results

In the course of studies on the Trx content in cells, we observed that cells with low amounts of Trx exhibit low enzyme activity of the housekeeping enzyme GAPDH. Previously, we have found that fibroblasts from patients suffering from the hereditary disease FA have lower Trx contents than those from unaffected individuals (Kontou *et al*., 2002). FA cells indeed also have significantly lower GAPDH activities (Figure 1). The reduced level of GAPDH activities in FA cells corresponds fairly well to the reduced concentrations of Trx (Kontou *et al*., 2003): 50% lower Trx compared to 50% lower GAPDH activity.

Immunoblots show that the GAPDH protein content in FA cells is reduced. This was best demonstrated in one of the cell lines, GM6935, with a very low Trx content (Kontou *et al*., 2003). Western blot analysis revealed that the GAPDH antigen of these cells was indeed extremely weak, whereas the internal control protein β-actin was comparable to all other cells namely, the FA cell line GM6914 and the controls analysed on the same blot (Figure 2).

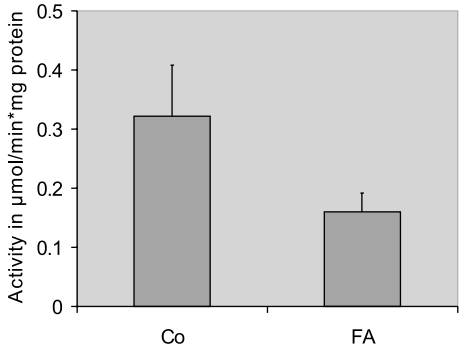

Figure 1 GAPDH activity measured in cell extracts of two FA fibroblast lines (GM6914, GM 6935) and two controls (Wi38, GM637). The activity is expressed in μmol NADH/min/(mg protein) and is given as means of five independent experiments with standard deviations

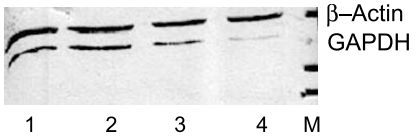

Figure 2 GAPDH in SV40-transformed fibroblasts of two FA patients and two healthy controls. Whole-cell extracts (20 μg) were loaded on a 12.5% denaturing gel and immunoblotted with antibodies against GAPDH. β-actin served as internal control. Control fibroblasts lane 1: GM637; lane 2: Wi38; FA-fibroblasts lane 3: GM6914; lane 4: GM6935; M = marker

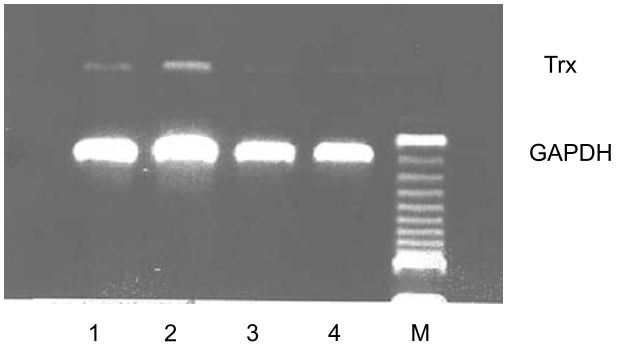

Figure 3 RT–PCR of GAPDH (30 cycles) and Trx (30 cycles) using 1 μg RNA of primary fibroblasts. Lanes 1 and 2: healthy control (Ko-1); lanes 3 and 4: FA-G (1424); lane 5: 100 bp marker

RT–PCRs indicate that the difference in the amounts of GAPDH protein in FA and control fibroblasts is due to differences in mRNA (Figure 3). RNA of control cells exert a distinct band at 350 bp, which corresponds to the mRNA of Trx. This 350 bp band is too weak to be seen under identical conditions in the FA strain 1424 (FA-G). Also a band at 700 bp, corresponding to GAPDH mRNA, is significantly stronger in RNA from controls than in RNA from FA cells. This result supports the suspicion that Trx might regulate GAPDH mRNA synthesis.

To further analyse the possible regulation of GAPDH gene expression by Trx, control fibroblasts were incubated for 24 h with either a solution of purified reduced human Trx (rhTrx) or with buffer (Figure 4). The GAPDH mRNA of the cells was analysed by semiquantitative RT–PCR. As can be seen clearly in lanes 1 and 5, no GAPDH mRNA can be found in buffer-treated cells (lane 1), whereas in rhTrx-treated cells a GAPDH PCR product clearly becomes visible (lane 5). Similarly, in lanes 2 and 6 or 3 and 7, it could be shown that under rhTrx treatment cells synthesized more GAPDH mRNA than without.

Since RT–PCR might be subject to artifacts, the amount of GAPDH mRNA was directly determined by Northern blot analysis (Figure 5b). Comparison of lanes 1 and 3 confirmed the stimulation of GAPDH mRNA expression by Trx. Lane 3 exerted much less mRNA

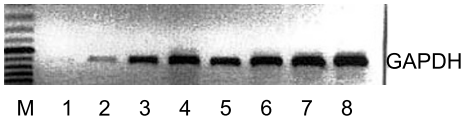

Figure 4 PCR kinetik. The expression of GAPDH after 24 h treatment of control fibroblasts (ko-2) with 1 μM reduced human rh Trx. Fibroblasts were treated either with buffer (lanes 1–4) or with buffer containing 1 μM rhTrx (lanes 5–8). PCR kinetik was allowed for the following cycles: 24 (lanes 1 and 5), 26 (lanes 2 and 6), 28 (lanes 3 and 7) and 30 (lanes 4 and 8). The treatment of cells with 1 μM rhTrx leads to an increased expression of GAPDH (lanes 5–8) compared to the buffer-treated cells (lanes 1–4)

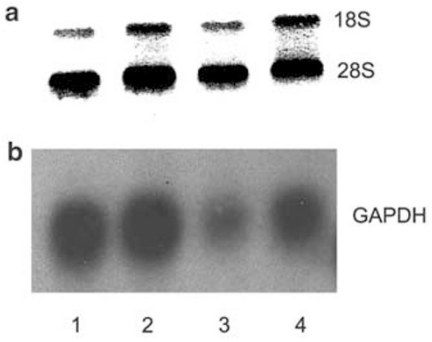

Figure 5 Northern blot of Trx and buffer-treated fibroblasts (Ko-1). 2 μg (lanes 1 and 3) and 4 μg (lanes 2 and 4) of total RNA (lanes 1 and 2: cells rhTrx treated; lanes 3 and 4: buffer treated) were loaded on a 1.2% formaldehyde gel. (**a**) Ribosomal RNA as internal standard. (**b**) Autoradiogram of the Northern blot after the hybridization of the RNA with ^{32}P-labeled GAPDH. Lanes 1 and 3 and 2 and 4 (treated and untreated probes) have to be compared to note the increase in GAPDH RNA in cells with higher Trx content

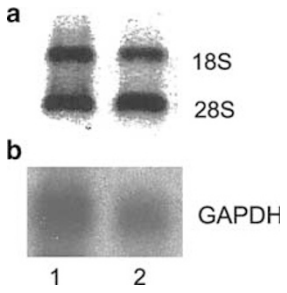

Figure 6 Northern blot of Trx and mock-transfected fibroblasts. SV40-transformed fibroblasts (Wi38) were transiently transfected either with the nuc/Trx plasmid (lane 1) or the nuc plasmid, lacking Trx (lane 2). (**a**) Total RNA (2 μg) were loaded on a 1.2% formaldehyde gel. (**b**) Autoradiogram of the Northern blot after the hybridization of the RNA with ^{32}P-labeled GAPDH

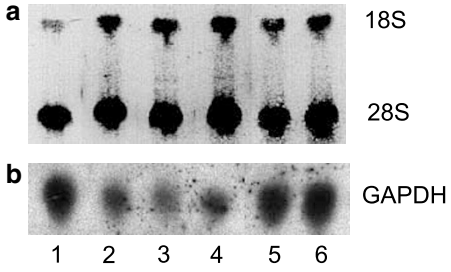

Figure 7 Northern blot of primary and SV40-transformed fibroblasts. Total RNA (2 μg) from primary fibroblasts and from untransfected and transfected SV40-transformed fibroblasts were loaded on a 1.2% formaldehyde gel. Lane 1: Ko-1(control); lane 2: GM1309(FA); lane 3: 1424(FA); lane 4: FLP(FA); lane 5: Wi38 mock transfected; lane 6: Wi38 transiently transfected with nuc/Trx, (**a**) ribosomal RNAs as internal control. No differences in the amount of ribosomal RNAs became apparent between those cells. (**b**) Autoradiogram of the blot after hybridization with ^{32}P-labeled GAPDH

transcript (buffer treatment) than lane 1 (Trx treatment). Ribosomal RNAs (18S and 28S) served as internal controls. Since the amounts of these RNAs are comparable in untreated and rhTrx-treated cells (Figure 5a), the effect of Trx was specifically restricted to mRNA synthesis.

Feeding cells with Trx assumes that this small protein can enter cells from the outside and subsequently regulate gene expression. To verify this type of experiment, Trx was overexpressed in cells by transient transfection and the synthesized GAPDH mRNA subsequently analysed by Northern blot (Figure 6). As expected, overexpressed Trx (lane 1) induced more GAPDH mRNA synthesis than could be found in mock-transfected cells (lane 2). The 18S and 28S ribosomal RNAs served as internal standards proving that comparable amounts of RNA were analysed.

In order to examine the regulation of GAPDH by Trx *in vivo*, the dependence of GAPDH transcription on the endogenous amount of Trx in different fibroblast lines was analysed (Figure 7). Northern blot analysis verified that primary fibroblasts with low concentrations of Trx such as FA cells of the complementation groups A and G produced significantly less GAPDH mRNA (lanes 2–4) than did healthy control cells (lane 1). Since in the described experiments SV40-transformed fibroblasts as well as untransformed fibroblasts were used, it was necessary to show that SV40-transformed fibroblasts (Wi38, line 5) have as much GAPDH mRNA as untransformed fibroblasts (lane 1). The transfection of Wi38 cells with Trx resulted in an elevation of GAPDH mRNA above normal (lane 6).

To study the generality of this regulative potential of Trx, RNA from either Trx- or buffer-treated cells were analysed. Differential gene expression was examined on DNA arrays with about 50 000 different PCR products. A number of 304 candidate genes resulted of which 101 were up- and 203 were downregulated at least by a factor of two. That means that on the transcriptional level Trx stimulated many genes beside GAPDH, whereas many others were suppressed. Lists of upregulated as well as downregulated candidate genes are given in Table 1a and b.

One of the stimulated genes was IκBα/MAD-3. This RNA and that of GAPDH were selected to become further investigated by Northern blot analysis (Figures 7 and 8) both because of their close relation to oxidative stress – IκBα is known to regulate the availability of NF-κB, a transcription factor that coordinates a whole set of genes depending upon the oxidative state of the cell – as

Table 1 Thloredoxin regulated candidate genes: (a) Upregulated candidate genes after treatment with 1 μM thioredoxin threshold value 2.0 and (b) Down-regulated candidate genes after treatment with 1 μM thioredoxin threshold value 0.5

Gen-Alias	*Description*	*NCBI REFSEQ*
(*a*)		
ARD1	ARD1 homolog, *N*-acetyltransferase (*S. cerevisiae*)	NM_003491
ARHC	Ras homolog gene family, member C	NM_175744
ATP5G2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2	NM_005176
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	NM_021813
DRAP1	DR1-associated protein 1	NM_006442
ENO1	Enolase 1, (alpha)	NM_001428
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase	NM_002046
KLK10	Kallikrein 10	NM_002776
LHX1	Lim homeobox 1	NM_005568
MT1L	Metallothionein 1x	NM_005952
NF?BIA	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	NM_020529
RPL18A	Ribosomal protein L18a	NM_000980
RPS19	Ribosomal protein S19	NM_001022
SELT	Selenoprotein T	NM_016275
SOD2	Manganese superoxide dismutase 2, mitochondrial	NM_000636
(*b*)		
AIP-1	Abl-interactor 2	NM_005759
AP1G1	Hypothetical protein FLJ20151	NM_017689
HCCS	Holocytochrome *c* synthase (cytochrome *c* hemelyase)	NM_005333
LTC4S	Leukotriene C4 synthase	NM_000897
MRPL16	Mitochondrial ribosomal protein L16	NM_017840
MYO1C	Myosin IC	NM_033375
NXPH4	Neurexophilin 4	AK094231
ORP150	Hypoxia upregulated 1	NM_006389
PBK1	PDZ-binding kinase	NM_018492
RPS4Y	Ribosomal protein S4, Y linked	NM_001008
SCAP2	Src family-associated phosphoprotein 2	NM_003930
SH3GLB1	SH3-domain GRB2-like endophilin B1	NM_016009
SLC4A1	Solute carrier family 4, anion exchanger, member 1 (erythrocyte membrane protein band 3, Diego blood group)	NM_000342
SRP19	Signal recognition particle 19 kDa	NM_003135
SRP46	Splicing factor, arginine/serine -rich, 46 kDa	NM_032102
SUDD	RIO kinase 3 (yeast)	NM_003831
TYMS	rTS beta protein	NM_017512

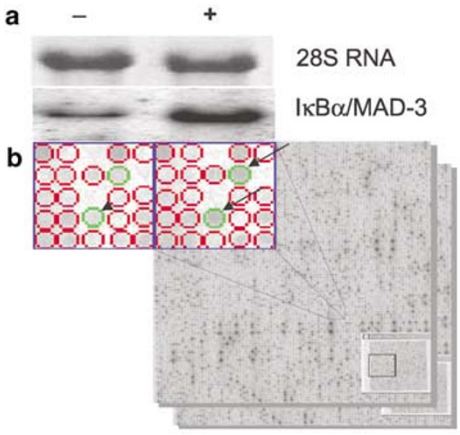

Figure 8 Complex hybridization of total RNA from ±rhTrx-treated Wi38 fibroblast cells on RZPD HumanUnigene2 Filters. (**a**) Validation of the IκBα hybridization results by Northern blot analysis. The 28S rRNA bands of the gel before blotting served as a quality control for equal loading. (**b**) Magnification of a 5×5 spotting pattern containing the cDNA corresponding to IκBα/Mad-3 (arrows). The hybridization signal for IκBα of Trx-treated cells (right part) shows a stronger intensity in comparison to the reference spots of the buffer-treated cells (left part)

well as to reconfirm the validity of the method. Once again the results elucidate the enormous impact of Trx as a general regulator of gene expression on the transcriptional level.

Discussion

Trx is a small ubiquitous protein with diverse biochemical functions (Arnér and Holmgren, 2000). Besides, as the cofactor of essential enzymes and as the major carrier of redox potential, Trx plays a central role in the maintenance of cellular oxidative equilibrium and of cell functions. The diversity of functions under Trx control can be coupled to the subcellular localization of this protein. Trx in the intercellular space acts as a signaling molecule (Silberstein *et al*., 1993; Nakamura *et al*., 1997; Bertini *et al*., 1999). Translocated to the nucleus, Trx increases the binding capacity of transcription factors, for example, NF-κB to DNA. This intervention with transcription factors has an impact on human diseases as we recently showed for FA (Kontou *et al*., 2003). As we demonstrated, this disease is accompanied by a

deficiency of Trx that might entail a dysfunction of the transcription factor NF-κB. Consequently, FA cells cannot successfully overcome their oxidative burden. Thus, Trx has an important role on the pathophysiology of FA patients.

The analysis of the mRNA expression pattern of cells with and without Trx treatment by the use of DNA arrays enabled us to evaluate Trx function not only as a major carrier of redox potential in the cell but, simultaneously, as a major regulator of gene expression, thereby adjusting the cell to the requirements needed under the prevailing redox conditions. Out of the 50 000 genes tested, a remarkable amount of genes responded to Trx either by up- or downregulation. This observation confirms and exceeds results published by Das *et al*. (1997) concerning the antioxidative enzyme MnSOD.

Of the many genes responding to Trx, we analysed two: GAPDH and IκBα/MAD-3 in more detail. Both proteins function to combat oxidative stress. IκBα is the inhibitor of NF-κB, one of the major transcription factors supporting cells in their defence against oxidative burden. This protein only acts after being transported to the nucleus where its binding to DNA is facilitated by Trx. In the cytoplasm, however, NF-κB is bound to its inhibitor protein, IκBα that hinders NF-κB from entering the nucleus. During oxidative stress conditions IκBα becomes ubiquitinylated and subsequently degraded by the 26S proteasome complex. It was shown that Trx inhibits the degradation of IκBα (Hirota *et al*., 1999). We demonstrate here that Trx also induces the expression of IκBα/MAD-3. Therefore, Trx plays not only a role in stabilizing the IκBα protein, but also in regulating its gene expression. Trx itself regulates the efficiency of NF-κB-dependent genes: in the nucleus it promotes the DNA binding of the transcription factor. By promoting the transcription of its inhibitor it concurrently reduces the concentration of transportable NF-κB in the cytoplasm. This again confirms that Trx functions as a main mediator of cellular regulation.

GAPDH in its tetrameric form acts as a glycolytic enzyme in the cytoplasm and is involved in energy production. We show (Figures 1–3) that GAPDH activity, protein content as well as mRNA transcripts all are reduced in FA cells. The diminution of GAPDH content in these cells is apparently dependent on the reduced Trx. The transfection of cells with cDNA for Trx or addition of Trx to the medium of cultured cells (Figures 4–8) proved this to be the case. From these experiments, we can conclude that Trx indeed regulates the gene expression of GAPDH.

We report on the stimulation of the housekeeping enzyme GAPDH by Trx. Since GAPDH is a central enzyme in glycolysis, its increased synthesis leads to a more overall rate of glycolysis. Elevated synthesis of Trx has been reported for cancer cells (Powis *et al*., 2000, Powis and Montfort, 2001b) and activated transcription of genes encoding glycolytic enzymes has been observed (Semenza, 1998). The observation of anaerobic glycolysis in tumor cells (Warburg and Christian, 1943) can easily be explained by the impact of Trx on gene expression presented here.

Furthermore, the importance of GAPDH becomes evident with respect to its function in the nucleus where it acts as a uracil DNA glycosylase, a repair enzyme of the base excision system (Das *et al*., 1997). In this capacity, it removes uracil bases generated by oxidative deamination of cytosine bases from the DNA. Thus, GAPDH on the one hand produces redox potential in glycolysis and on the other participates in the repair of oxidative damages.

In our experiments, the Trx level of fibroblasts was manipulated either by the transfection and overexpression of Trx cDNA or by the addition of the reduced protein directly to the culture medium, implying that Trx can enter the cell from the outside. Several publications have reported that Trx can indeed leave the cell (Nakamura *et al*., 1996, Pekkari *et al*., 2000, Sumida *et al*., 2000). The fact that Trx can enter and leave cells, thereby, transmitting information about the oxidative state of the cells, and can adjust gene expression, makes Trx an indispensable second messenger. Moreover, Trx when excreted from cells can be transported from the intercellular space to the serum.

Since Trx reflects the oxidative state of cells, alterations of the serum concentrations of Trx should be an indication for 'oxidative stress' diseases. The elevation of Trx concentrations in the serum of HIV-infected patients was shown (Nakamura *et al*., 1996) and serves as an indicator for the induction of Trx in viral-infected cells. Thus, Trx as a regulator of gene expression may become a valuable measure both for prophylaxis and diagnosis of oxidative stress-related diseases.

Materials and methods

Chemicals and antibodies

Chemicals were purchased from Merck, Germany, Roth, Germany or Invitrogen, Germany unless otherwise indicated. Polyclonal antibodies against recombinant human Trx and GAPDH were raised in rabbit and purified against the antigen over a cyanobromide-activated sepharose column. Goat anti-actin antibodies were purchased from Santa Cruz.

Cell lines and culture conditions

Cell lines used: Primary fibroblasts: KO-1, KO-2 (normal, established by MH-K); 1424 (FA-G, established by K Sperling); FLP (FA-G, established by MH-K); GM1309 (FA-A, Human Genetic Mutant Cell Repository, Cambden, NJ, USA, USA). SV40-transformed fibroblasts: Wi38 (normal, American type culture collection); GM637 (normal), GM6914 (FA-A), GM6935 (FA), Human Genetic Mutant Cell Repository, Cambden, NJ, USA). Cells were grown in minimal essential medium with 10% fetal calf serum (MEM-Earle, Biochrom, Berlin, Germany) and cultivated at 37°C with 5% CO_2 in air. All cells were free of mycoplasms.

Plasmids

PCR fragments were cloned in the TOPO TA cloning vector pCR2.1 (Invitrogen, Karlsruhe, Germany). The Trx fragment was subcloned into the mammalian expression vector pCR/CMV/myc/nuc (Invitrogen).

Cloning and DNA sequencing

Trx was cloned and sequenced as described (Kontou *et al*., 2002).

Transient transfection

A plasmid purification kit (Qiagen, Hilden, Germany) was used to purify the DNA before transfection. Confluent cells (80%) were transfected by the calcium phosphate precipitation method as described (Ramirez *et al*., 1999). At 48 h after transfection, cells were harvested for RNA isolation.

Northern blot analysis of GAPDH

Total RNA was isolated from cultured cells using RNA Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (2–4 μg) were loaded and electrophorized on 1.2% formaldehyde gels and transferred to a nylon membrane. After hybridization with ^{32}P-labeled cDNA of GAPDH (Invitrogen), the probes were detected by autoradiography. The position and amount of 28S and 18S ribosomal RNA as a marker for molecular weight and equality of loaded samples were checked by ethidium bromide staining.

Northern blot analysis of IκBα

Northern blot analysis was carried out as described (Wittig *et al*., 2002). In brief, 15 μg total RNA were separated by electrophoresis and transferred to Hybond N + Nylon membranes (Amersham Biosciences) by capillary transfer overnight and immobilized by UV crosslinking. Hybridization was performed with ^{32}P-labeled cDNA probes at 65°C overnight. After washing membranes were exposed to X-ray films and imaging plates. In the latter case, image acquisition was carried out using a Fuji FLA3000 phosphoimager (Raytest GmbH).

RT–PCR

For amplification, cytoplasmic RNA was extracted by the use of the Qiagen RNA extraction kit following the manufacturer's manual. RNA (1 μg) was reverse transcribed with MMLV-reverse transcriptase (Promega) after priming with an OligodT primer. The reverse transcribed products were diluted directly into PCR buffer and amplified with the 5′ primer (CGGGATCCTGAAGCAGATCGAGA) and the 3′ primer (TCAGTAGACTTTGACTAATTCATTAATGGTG) for Trx.

GAPDH enzyme activity assay

For preparation of cell extracts cells were treated with lysis buffer (200 mM Tris-HCl, pH 8.0, 300 mM NaCl, 100 mM KCl, 10 mM EDTA, 0.1 mM NP40, 0.5 mM PMSF), incubated on ice for 30 min and centrifuged at 12 000 *g* for 5 min at 4°C. The protein concentration was measured with the Bio-Rad protein assay (Bio-Rad, München, Germany). The assay was performed in a final volume of 1 ml containing 20 μg protein of cell extracts, 1 M Tris-HCl, pH 8.0, 500 mM potassium phosphate, 100 mM fructose 1,6-biphosphate, 2 g/ml trioseisomerase and 10 mg/ml aldolase, whereby the reaction was started by the addition of 500 mM potassium phosphate. GAPDH activity was monitored by the reduction of NAD^+ to $NADH/H^+$ at 340 nm for 5 min and is expressed in μmol $NADH/H^+$/min/(mg protein) using 6.22/mM/cm as the absorption coefficient for NADH.

Western blot

Cells were harvested by trypsinization, washed with PBS and whole-cell extracts were prepared as described above. Protein (20 μg) from cell extracts was loaded on a 12.5% SDS gel, electrophorized and blotted onto a nitrocellulose membrane (Schleicher and Schuell, Kassel, Germany). GAPDH and β-actin antibodies were used to visualize the respective proteins. Detection was achieved by the use of alkaline phosphatase-conjugated antibodies.

cDNA arrays

The cDNA arrays used for the hybridizations were Human Unigene Set RZPD 2, PCR_HumUnigene 2_5 × 5 (filter 1 + 2). All filter parts were derived from the same batch of production and were handled according to the manufacturer's instructions (RZPD GmbH, Berlin, Germany).

Complex cDNA hybridization

Total RNA was extracted after Trx treatment (Kerblat *et al*., 1999) using Trizol (Invitrogen) following the manufacturer's protocol. Quality and concentration of the total RNA were confirmed by gel-electrophoresis using the RNA 6000 LabChip® Kit (Agilent Technologies, Böblingen, Germany). For the generation of complex cDNA probes, 20 μg total RNA were used consisting of equal amounts of RNA pooled from three different preparations. Reverse transcription and labeling of the cDNA probes was carried out as described previously (Boer *et al*., 2001). After separation of unincorporated label, the specific activity and volumes of the probe were adjusted to ascertain equivalent conditions for the array hybridizations. The arrays were hybridized under the conditions described (Boer *et al*., 2001).

Image acquisition and analysis of array hybridizations

For the analysis of arrays ArrayVision 6.0. Software (Imaging Research) was used. After spot finding using an automated algorithm, the signal intensities were calculated as mean pixel values minus a regional background calculated for each 5 × 5 spot group, respectively. For array comparisons, signal intensities were normalized to the mean of all human cDNA-containing spots on one filter, and the expression ratios for single spots were calculated. Ratios of 2.0 and 0.5 were set as thresholds to identify differentially expressed genes according to the detected expression level of MnSOD that had been shown to be differentially expressed in Trx-treated cells (Das *et al*., 1997). As a further criteria, only those genes were selected that showed differential expression at least in three of four spots and did not exceed a factor of one in comparison of the ratios from their expression levels.

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