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Interaction of mitochondrial thioredoxin with glucocorticoid receptor and NF-κB modulates glucocorticoid receptor and NF-κB signalling in HEK-293 cells

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Trx2 (mitochondrial thioredoxin) is an antioxidant and anti-apoptotic factor essential for cell viability. Trx1 (cytoplasmic thioredoxin) is a co-factor and regulator of redox-sensitive transcription factors such as the GR (glucocorticoid receptor) and NF-κB (nuclear factor κB). Both transcription factors have been detected in mitochondria and a role in mitochondrial transcription regulation and apoptosis has been proposed. In the present study, we show using SPR (surface plasmon resonance) and immunoprecepitation that GR and the p65 subunit of NF-κB are Trx2-interacting proteins. The interaction of Trx2 with GR is independent of the presence of GR ligand and of redox conditions. The p65 subunit of NF-κB can interact with Trx2 in the oxidized, but not the reduced, form. Using HEK (human embryonic kidney)-293 cell lines with increased or decreased expression of Trx2, we show that Trx2 modulates transcription of GR and NF-κB reporter genes. Moreover, Trx2 overexpression modulates the mRNA levels of the *COX1* (cytochrome oxidase subunit I) and *Cytb* (cytochrome *b*), which are known to be regulated by GR and NF-κB. Increased expression of Trx2 differentially affects the expression of Cytb. The glucocorticoid dexamethasone potentiates the expression of Cytb, whereas TNFα (tumour necrosis factor α) down-regulates it. These results suggest a regulatory role for Trx2 in GR and NF-κB signalling pathways.

Key words: glucocorticoid receptor (GR), mitochondria, nuclear factor κB (NF-κB), reactive oxygen species (ROS), thioredoxin, transcription.

INTRODUCTION

Thioredoxins are a class of ubiquitously expressed redox proteins containing a conserved consensus amino acid sequence (Cys-Gly-Pro-Cys) in their catalytic centre. They exist either in a reduced dithiol form or in an oxidized form. The oxidized form of thioredoxin is reduced, and thereby activated, by TrxR (mitochondrial thioredoxin reductase) and NADPH, the so called thioredoxin system [1]. Thioredoxins are involved in many cellular processes, such as cell growth and proliferation, gene regulation and apoptosis. They act as antioxidants facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Therefore they serve as electron donors for enzymes such as ribonucleotide reductases, thioredoxin peroxidases (peroxiredoxins) and methionine sulfoxide reductase. Thioredoxins are also regulators of redox-sensitive transcription factors, such as NF-κB (nuclear factor κB) and GR (glucocorticoid receptor) [1]. Regarding NF-κB activation, Trx1 (cytosolic thioredoxin) plays dual and opposing roles in its activation. In the cytoplasm, Trx1 blocks the degradation of IκB (inhibitor of NF-κB) that could lead to nuclear NF-κB translocation and activation. In the nucleus, however, Trx1 enhances NF-κB transcriptional activity by reducing a disulfide bond involving Cys^{62} in the DNA-binding loop of the p50 subunit [2]. Restoration of the GR activation in cells under oxidizing conditions is also attributed to Trx1. Trx1 directly interacts with both the ligand and the DNA-binding domains of GR, keeping the receptor in a reduced transcriptionally active form [3,4]. In mammalian cells, other, non-cytosolic, members of the thioredoxin family have been described, including a mitochondrial thioredoxin system [Trx2 (mitochondrial thioredoxin) and TrxR2 (mitochondrial thioredoxin reductase)] [5].

Mitochondria are the major source of ROS (reactive oxygen species) generated during both physiological respiration and under pathological conditions, such as inflammation, in response to cytokines [6,7]. Therefore the existence of the mitochondrial thioredoxin system, together with other mitochondrial antioxidant defence systems, such as mitochondrial glutathione-peroxidase, MnSOD (manganese superoxide dismutase) and Prx (mitochondrial peroxiredoxin) is of utmost importance for cell viability [8]. Trx2 plays a crucial role in protection against oxidative stress and in regulating the mitochondrial apoptosis signalling pathway [8–11]. Moreover, absence of Trx2 causes massive apoptosis, exencephaly and early embryonic lethality in homozygous mice [12]. Trx2 is also involved in the regulation of hypoxia by attenuating hypoxia-evoked HIF (hypoxia-inducible factor)-1α accumulation and NO levels [13]. The exact mechanism by which Trx2 exerts its function in mitochondria and the identity of the Trx2-interacting molecules involved in these cellular processes are not yet known.

Abbreviations used: AMS, 4-acetoamido-4′-maleimidylstilbene-2-2′-disulfonic acid; ANT, adenine nucleotide translocase; COX I, cytochrome oxidase subunit I; COX III, cytochrome oxidase subunit III; Cytb, cytochrome *b*; DEX, dexmethasone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GR, glucocorticoid receptor; GST, glutathione transferase; HEK, human embryonic kidney; IPTG, isopropyl β-D-thiogalactoside; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; RLU, relative light units; ROS, reactive oxygen species; SDH, succinate-ubiquinone oxidoreductase; siRNA, small-interfering RNA; SPR, surface plasmon resonance; TNFα, tumor necrosis factor; Trx1, cytosolic thioredoxin; Trx2, mitochondrial thioredoxin; TrxR, mitochondrial thioredoxin reductase.

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The mitochondrial localization of orphan, thyroid and steroid receptors, including GR and, more recently, other transcription factors, such as NF-κB, has been demonstrated in several cell types [14–20]. Novel functions of these factors in mitochondria have been proposed, including involvement in mitochondrial transcriptional regulation (by binding to mitochondrial DNA hormone-response elements to regulate the expression of oxidative phosphorylation gene expression) and in mitochondrial-dependent apoptosis (through interaction with pro-apoptotic or anti-apoptotic factors) [19,21]. In the present study, we explored the role of Trx2 in the regulation of GR and NF-κB functions. We demonstrate a direct interaction of Trx2 with GR and the p65 subunit of NF-κB and we characterize these interactions. In addition, we provide evidence for a direct or indirect involvement of Trx2 in the modulation of GR and NF-κB transcriptional activation.

EXPERIMENTAL

Chemicals

DMEM (Dulbecco's modified Eagle's medium) and FBS (fetal bovine serum) were obtained from Gibco-BRL. Molecular-mass protein markers and complete protease inhibitors cocktail were purchased from Fermentas and Roche respectively. Glutathione–Sepharose 4B was from Amersham Biosciences, AMS (4-acetoamido-4′-maleimidylstilbene-2-2′-disulfonic acid) and Lipofectamine™ 2000 were from Invitrogen. Human GR and the p65 and p50 subunits of NF-κB recombinant proteins were obtained from Panvera and Active Motif respectively. All other chemicals, including Protein A–Sepharose and RU-486, were purchased from Sigma–Aldrich.

Antibodies

The following affinity-purified polyclonal antibodies commercially provided by Santa Cruz Biotechnology were used: GR-H300, which recognizes an epitope corresponding to amino acids 121–420 of human GR, and sc-109, an antibody raised against the p65 subunit of NF-κB. For the Trx2 immunoprecipitation and detection, a monoclonal antibody, provided by Abcam, and an affinity-purified polyclonal antibody were used [9]. The anti-β-actin antibody and the anti-SDH (succinate-ubiquinone oxidoreductase) antibodies were provided by Sigma–Aldrich and Invitrogen respectively.

siRNA (small-interfering RNA)-mediated knockdown of Trx2 in HEK (human embryonic kidney)-293 cells

HEK-293 cells were maintained in DMEM, supplemented with 10 % (v/v) FBS. Cells were grown at 37 °C in a humidified atmosphere with 5 % CO_2. On the day before transfection, HEK-293 cells were plated in 60-mm-diameter culture dishes so that they reached 50–70 % confluency at the time of transfection. To obtain stable expression of the siRNA sequence, and hence reduced expression of Trx2, an oligonucleotide (5′-CAGTGA-GACACCAGTGGTT-3′) directed against nucleotides 228–246 of the human Trx2 cDNA (GenBank® accession number AF480262) was designed. The duplex, produced by annealing the forward and reverse oligonucleotides 5′-gatccccCAGTGA-GACACCAGTGGTTttcaagagaAACCACTGGTGTCTCACTG - ttttttggaaa-3′ and 5′-agcttttccaaaaaCAGTGAGACACCAGTGGT-TtctcttgaaAACCACTGGTGTCTCACTGggg-3′, was inserted between BglII and HindIII sites in the pSuper vector and was transfected into the HEK-293 cells together with the pcDNA3neo plasmid vector (Clontech) carrying the Tn5 neo aminoglycoside phosphotransferase gene (pSuper/pcDNA3neo at a ratio of 3:1) using Lipofectamine™ 2000, according to the manufacturer's instructions. Control cells were prepared by co-transfection of the empty pSuper with the pcDNA3neo vectors in a 3:1 ratio. After 24 h incubation at 37 °C, cells were trypsinized and passaged at a 1:5 dilution in selective growth medium [DMEM, supplemented with 10 % (v/v) FBS, containing 1.5 mg/ml of Geneticin (G418; Calbiochem)]. On the next day, the medium in all plates was replaced with fresh selective growth medium and cells were cultured for 2 weeks. G418-resistant colonies were established in 24-well plates, expanded, cloned independently and analysed by Western blotting to confirm the knockdown of Trx2.

Expression of recombinant proteins in bacteria

GST (glutathione transferase) fusion proteins

For the construction of the expression plasmid for GST–Trx2, the DNA fragment encoding human Trx2, without the mitochondrial signal (nucleotides 181–501), was amplified by PCR and ligated in frame into the pGEX4T-3 plasmid (Amersham Biosciences). GST or GST–Trx2 fusion proteins were expressed in *Escherichia coli* BL21(DE3), by induction with 0.3 mM IPTG (isopropyl β-D-thiogalactoside) for 3 h at 37 °C. Cells were centrifuged for 20 min at 3000 ***g***. Cell pellets were suspended in PBS containing 1 % (v/v) Triton X-100 and 5 mM DTT (dithiothreitol) and were subsequently sonicated using a probe tip sonicator ten times for 10 s each, with 1 min rests between sonications, to lyse the cells. Lysates were centrifuged at 12000 ***g*** for 30 min at 4 °C and supernatants were incubated with glutathione–Sepharose 4B beads for 3 h at 4 °C. Beads were washed three times with PBS and bound proteins were eluted with 50 mM Tris/HCl, pH 8.0, containing 10 mM glutathione.

Hexahistidine (His_6) tagged fusion proteins

Trx1 and Trx2 recombinant proteins were expressed in *E. coli* as the N-terminal fused form with a His_6- tag, using the pET-15b expression plasmids (Clontech). Trx2 was expressed without the mitochondrial localization signal. We have previously shown that both endogenous [5] and overexpressed [13] Trx2 are targeted to mitochondria where they lose the targeting signal. *E. coli* strain BL21 was transformed with each pET expression vector. *E. coli* were cultured at 37 °C with vigorous shaking and were treated for 4 h with 0.5 mM IPTG. Pelleted cells were lysed in phosphate buffer (25 mM sodium phosphate, pH 7.0, containing 150 mM NaCl) containing 0.5 mg/ml lysozyme, sonicated, using a probe tip sonicator ten times for 10 s each, with 1 min rests between sonications, and clarified by centrifugation at 12000 ***g*** for 30 min. Supernatant containing His_6 tagged Trx1 or Trx2 was loaded on to an Ni^{2+}-NTA (nitrilotriacetate)–agarose column. The column was washed in PBS and the fusion protein was eluted by PBS containing 150 mM imidazol. The eluted protein was dialysed overnight at 4 °C against PBS.

SPR (surface plasmon resonance) interaction studies

SPR was carried out using a BIAcore2000 instrument. Using amino coupling, approx. 2500 RU (resonance units) of purified His_6–Trx2, His_6–Trx1 and GST proteins were immobilized on to a CM5 censor chip (BIAcore). Subsequently, various molar quantities of recombinant human GR and 77 nM of p65 were injected over the surface. All proteins were diluted in 10 mM Hepes, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA and

0.005 % (v/v) Tween-20. Trx2–GR interactions were examined in the presence or absence of GR ligand [10^{-6} M DEX (dexamethansone)] or GR antagonist (10^{-5} M RU-486). The GST surface was used for background correction.

GST pull-down experiments with *in vitro*-translated GR

Human GR was amplified by PCR using suitable primers, carrying T7 polymerase-binding sites and kozak sequences, and was used as a template for *in vitro* translation. The protein was synthesized *in vitro* using the T7 RNA polymerase rabbit reticulate lysate-coupled transcription/translation kit (TNT; Promega) and was labelled with [^{35}S]methionine (Amersham Biosciences). Recombinant proteins were subjected to pull-down experiments as follows. GST or GST–Trx2 protein was incubated with glutathione–Sepharose 4B beads in PBS at 4 °C for 3 h. After being washed in PBS three times, the GST- or GST-Trx2-conjugated beads were incubated with rotation for 4 h at 4 °C with *in vitro*-translated GR protein in the presence or absence of ligand (10^{-6} M DEX), in incubation pull-down buffer (50 mM potassium inorganic phosphate, pH 7.4, containing 100 mM NaCl, 1 mM $MgCl_2$, 10 % (v/v) glycerol and 0.1 % Tween 20) containing 1.5 % (w/v) BSA. Subsequently, the beads were washed with pull-down incubation buffer three times for 15 min, resuspended in an equal volume of 2 × SDS sample buffer, boiled for 5 min and pelleted in a microcentrifuge (1500 ***g*** for 2 min). A portion (10 μl) of each of the supernatants was subjected to SDS/PAGE (10 % gels). The gel was dried under vacuum and subjected to autoradiography.

Immunoprecepitation

HEK-293 cells overexpressing Trx2 (HEK-Trx2) [9] grown in DMEM on 15-cm-diameter dishes were rinsed twice in PBS, harvested in PBS and centrifuged at 500 ***g*** for 5 min. Pellets were lysed in RIPA buffer [(10 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 1mM EDTA and 0.1 % Nonidet P40) supplemented with protease inhibitors] and sonicated three times for 20 s. Insoluble material was removed by centrifugation at 13 000 ***g*** for 15 min and the supernatants were pre-incubated for 1 h with 20 μl of Protein A–Sepharose at 4 °C. Samples were centrifuged for 2 min at 1500 ***g***, and the supernatants were further incubated with the rabbit anti-Trx2 antibody [9] or the mouse anti-Trx2 antibody (Abcam). Protein A–Sepharose (20 μl) was added to each sample and the mixture was incubated for 3 h, at 4 °C. Protein A–Sepharose was pelleted by centrifugation at 1500 ***g*** for 2 min and washed three times in PBS. The samples were boiled in SDS/PAGE loading buffer, in the presence of 5 % (v/v) 2-mercaptoethanol, for 4 min and centrifuged at 1500 ***g*** for 2 min. The resulting supernatants were subjected to SDS/PAGE (10 % gel), and Western blotting was performed with specific antibodies against Trx2 (rabbit polyclonal) and GR. Membranes were reprobed with antibodies against the p65 subunit of NF-κB.

Subcellular fractionation

Cells were homogenized and subjected to subfractionation as previously described in [15]. Briefly, cells grown on 15-cm-diameter dishes were washed twice with ice-cold PBS, harvested in 5 vol. of homogenization buffer [20 mM Hepes/KOH, pH 7.5, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.1 mM PMSF and 250 mM sucrose, with protease inhibitors (cocktail from Roche supplemented with 10 μg/ml of chymostatin, 2.5 nM pepstatin)] and homogenized at 4 °C, with 20 strokes of a glass Potter–Elvejhem homogenizer using a Teflon pestle. The homogenate (called the total extract) was centrifuged for 5 min at 1000 ***g*** and the supernatant was further centrifuged at 10 000 ***g***, for 20 min, to give the crude mitochondrial pellet. The resulting supernatant was subject to ultracentrifugation in a Sorvall ultracentrifuge using a T-8100 rotor at 40 000 rev./min for 1 h to give the cytosolic fraction. The crude mitochondrial pellet was submitted to protease treatment 4 °C, using 1 μg of proteinase K per 10 μg of protein in buffer B (20 mM Tris/HCl, pH 7.5, containing 0.07 M sucrose, 0.21 M mannitol, 1.5 mM $MgCl_2$, 2.5 mM EDTA and 2.5 mM EGTA). The reaction was stopped with the addition of 1 mM PMSF after 10 min. Mitochondria were diluted with 10 vol. of Buffer B and were subsequently centrifuged at 12 000 ***g*** for 20 min. The resulting pellet was washed with buffer B twice and centrifuged at 12 000 ***g*** for 20 min. Pellets of mitochondria were suspended in RIPA buffer with addition of protease inhibitors, placed on ice for 30 min and sonicated with an ultrasonic homogenizer twice for 20 s at level 40 to give the mitochondrial (called the Mito) fraction. The protein concentration of the subcellular fractions (TE, Cyt and Mito) was determined according to Bradford [21a]. For Western blotting, 30 μg of protein was separated by SDS/PAGE (15 % gels), transferred on to nitrocellulose filters and analysed using specific antibodies against Trx2, SDH and β-actin.

NF-κB and GR activity

HEK-293 cells growing on 24-well plates were co-transfected with NF-κB-Luciferase (NF-κB-Luc), an MMTV (murine-mammary-tumour virus) promoter-driven luciferase construct (GR-Luc reporter gene construct) and a β-galactosidase reporter construct. Cells were treated either with TNFα (tumour necrosis factor α; 10 ng/ml–60 ng/ml) for various time periods (2, 4, 6 or 12 h) or with 10^{-6} M DEX for 4 h, lysed in report lysis buffer (Promega) following the manufacturer's protocol, and assayed for the expression of luciferase and β-galactosidase activity. The light emission was measured in RLU (relative light units) using a chemiluminometer (LB 9507; Berthold) and adjusted to the β-galactosidase activity of the sample. Transfection efficiency was expressed as RLU.

Trx2 redox state analysis

The redox state of Trx2 was determined by Western blot analysis of cell extracts pre-incubated with AMS as described previously in [22]. Briefly, cells were precipitated with 10 % ice-cold trichloroacetic acid for 30 min at 4 °C. Samples were centrifuged at 12 000 ***g*** for 30 min, resuspended in 100 % acetone and incubated at 4 °C for 30 min. Following centrifugation at 12 000 ***g*** for 10 min, the acetone was removed and protein pellets were dissolved in 20 mM Tris/HCl, pH 8.0, containing 15 mM AMS and incubated at room temperature (25 °C) for 3 h. Trx2 redox forms were separated by SDS/PAGE (15 % gel) in the presence of non-reducing loading buffer.

Real-time PCR

The expressed levels of mRNA were quantified using real-time RCR as described previously in [20]. Briefly, total RNA was extracted using TRizol® followed by DNase treatment (Promega) and reverse transcription into cDNA, using random primers and superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed after mixing the cDNA with SYBR GreenER qPCR super mix universal (Invitrogen) and appropriate primers for COX I (cytochrome oxidase subunit I), COX III (cytochrome

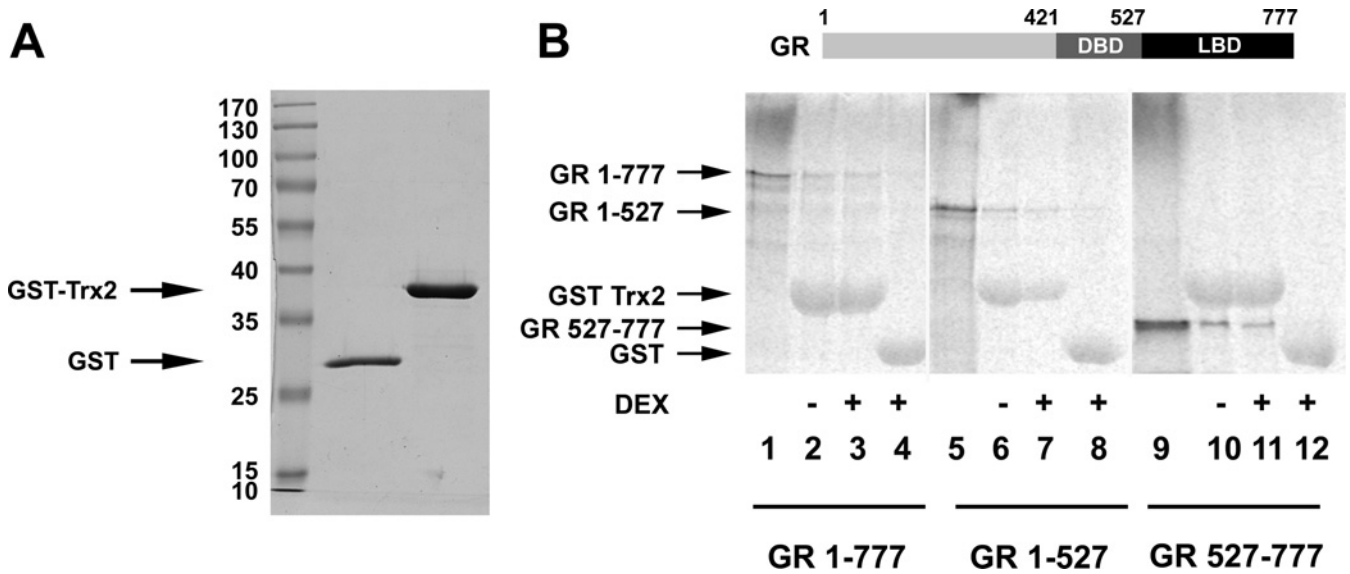

Figure 1 Binding of GR to Trx2

(**A**) Expression and purification of GST and GST–Trx2 proteins. The Trx2 gene was inserted into the pGEX vector. The GST and GST–Trx2 proteins were expressed in BL21 cells. The cells were lysed and the purified proteins were visualized by SDS/PAGE and silver staining. (**B**) Pull-down experiments with GST and GST–Trx2 recombinant proteins. *In vitro*-translated full-length GR (GR 1–777), as well as GR truncated forms carrying either the GR DNA-binding (DBD; GR 1–527) or ligand-binding (LBD; GR 527–777) domains, revealed interaction of Trx2 with all GR forms, in the presence or absence of 10^{-6} M DEX. Lanes 1, 5 and 9 show 30 % of the input; lanes 4, 8, and 12, show control GST-interacting proteins; lanes 2, 6 and 10, show GST–Trx2-interacting proteins in the absence of DEX; lanes 3, 7 and 11, show GST–Trx2-interacting proteins in the presence of DEX.

oxidase subunit III), Cytb, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and β-actin [20]. Products were quantified with a Chromo4 Real-Time System (Bio-Rad Laboratories). PCR was performed with an initial step at 52 °C for 2 min and 95 °C for 2 min followed by 35 cycles of 95 °C for 15 s and 60 °C for 40 s, followed by a final step of 60 °C for 10 min.

Statistical analysis

All results are expressed as means $\pm$ SD ($n = 3$–5). Data were analysed both by independent t test and Mann–Whitney test. Statistical analysis was performed with SPSS software, version 11.5 and differences were considered significant at a two-tailed P-value < 0.05.

RESULTS

GR is a Trx2-interacting protein

GR interaction with cytosolic Trx1 has been reported previously and further studies have shown that both the ligand and the DNA-binding domains of GR could be targets for Trx1 [3,4]. In order to examine whether Trx2 could also interact with GR, pull-down experiments were performed with *in vitro*-translated full-length GR (GR 1–777), and with truncated forms carrying either the ligand- (GR 527–777) or the DNA- (amino acids 421–526; GR 1–527) binding domain [23] against human GST–Trx2 or GST immobilized on to glutathione–Sepharose beads. The GST and GST–Trx2 proteins were expressed and purified as described in the Experimental section. The purity of the isolated proteins was analysed by SDS/PAGE and silver staining (Figure 1A). The binding affinity of the different forms of GR with GST and GST–Trx2 was studied in the presence or absence of 10^{-6} M DEX, the GR agonist. None of the GR variants showed binding to GST (Figure 1B, lanes 4, 8 and 12). However, the full-length GR (Figure 1B, lane 2) as well as its truncated forms carrying either the DNA-binding (Figure 1B, lane 6) or the ligand-binding domain (Figure 1B, lane 10) were able to bind to GST–Trx2. The presence of DEX did not affect the interaction of either GR variant form with Trx2 (Figure 1B, lanes 3, 7 and 11) demonstrating that the Trx2–GR interaction is ligand-independent.

Characterization of Trx2 and Trx1 interaction with GR

The binding of GR to Trx2 in pull-down experiments prompted us to further characterize the GR–Trx2 interaction and to perform comparative studies with Trx1. For that purpose, His_6–Trx1 and His_6–Trx2 recombinant proteins were immobilized on a Biacore sensor chip for SPR. Various concentrations of GR (0.7–5.6 nM) were analysed for their binding affinity to Trx1 and Trx2. Equilibrium dissociation constants (K_d) of the GR–Trx1 or GR–Trx2 associations were calculated using a 1:1 binding kinetic model. Analysis of the SPR data showed that Trx2 interacts with GR in the presence of 1 mM DTT ($K_d = 0.5 \pm 0.03$ nM) (Figure 2A). In agreement with the previously reported observations [4], Trx1 interacts with GR. However, this interaction is weaker under reducing conditions ($K_d = 26 \pm 0.4$ nM) (Figure 2B). In order to further elucidate whether the redox conditions affect the binding affinity of GR to Trx1 and Trx2, the experiments were repeated under oxidizing conditions by adding 1.2 mM selenite. As shown in Figure 2(C), the binding of GR to Trx2 was not affected by the redox conditions ($K_d = 0.4 \pm 0.02$ nM), whereas for Trx1 the affinity to GR was higher under oxidizing conditions ($K_d = 11 \pm 0.2$ nM) (Figure 2D). The interactions of GR with Trx2 were not affected by the presence of DEX or the specific GR antagonist RU-486 [$K_d = 0.6 \pm 0.03$ nM and $K_d = 0.5 \pm 0.01$ nM respectively (results not shown)].

Interaction of Trx2 with the p65 subunit of NF-κB

The p50 subunit of NF-κB is a known Trx1-interacting protein [24–26]. Therefore we examined whether the p50 and

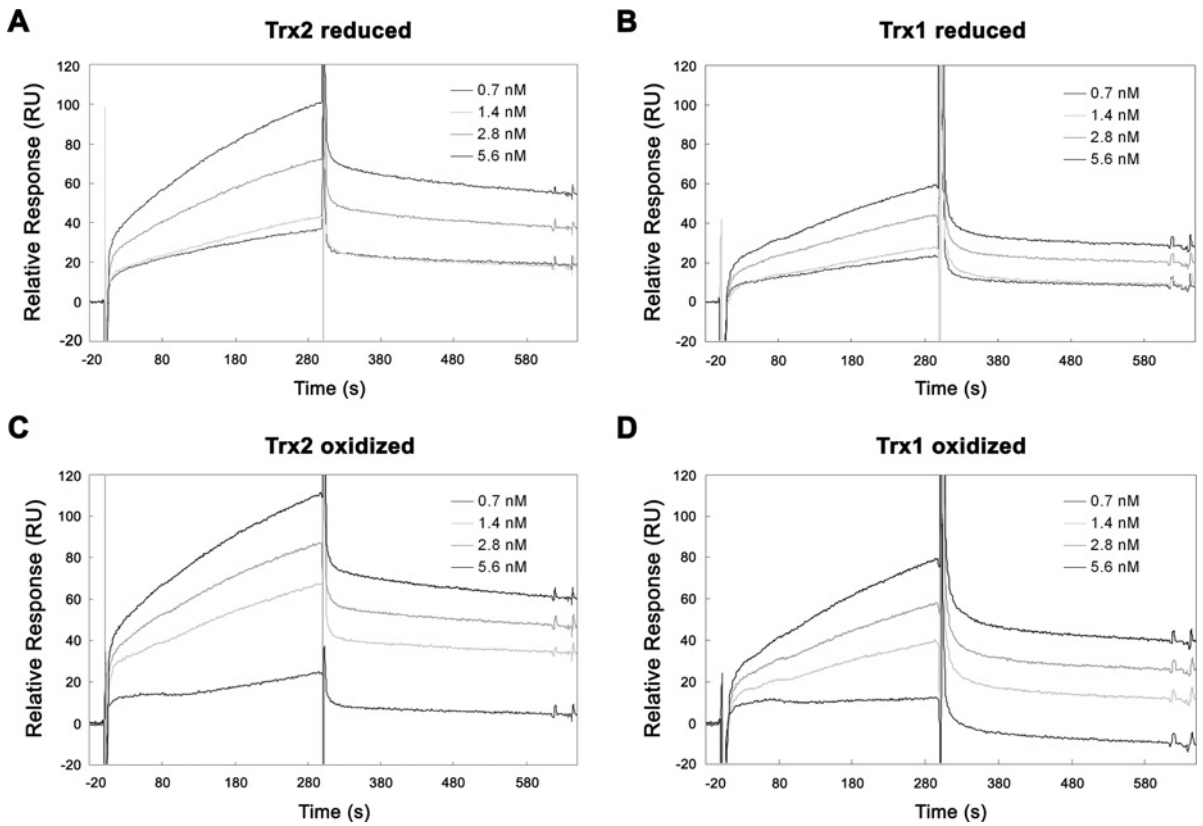

Figure 2 Characterization of Trx and GR interactions by SPR

Human recombinant GR (0.7–5.6 nM) was analysed for interaction with approximately 2500 arbitrary units of (**A** and **C**) immobilized His_6–Trx2 and (**B** and **D**) His_6–Trx1 recombinant proteins in the presence of either 1 mM DTT (reduced) or 1.2 mM selenite (oxidized). The GR–Trx2 interaction is stronger than GR–Trx1 interaction and is independent of redox conditions.

p65 subunits of NF-κB bind to Trx2. Recombinant His_6–Trx2 was immobilized on a Biacore sensor chip and 77 nM recombinant p50 and p65 proteins were subjected to reduction or oxidation with 1 mM DTT or 1.2 mM selenite respectively. The oxidized forms of p50 and p65 were dialysed against 10 mM Hepes, pH 7.4, containing 0.15 M NaCl and 3 mM EDTA and subsequently the reduced or oxidized proteins were analysed for their ability to bind to immobilized and reduced Trx2. As shown in Figure 3, the oxidized but not the reduced form of the p65 subunit of NF-κB interacts with Trx2. Neither the reduced nor the oxidized form of the p50 NF-κB subunit showed any binding to Trx2 in the SPR studies (results not shown).

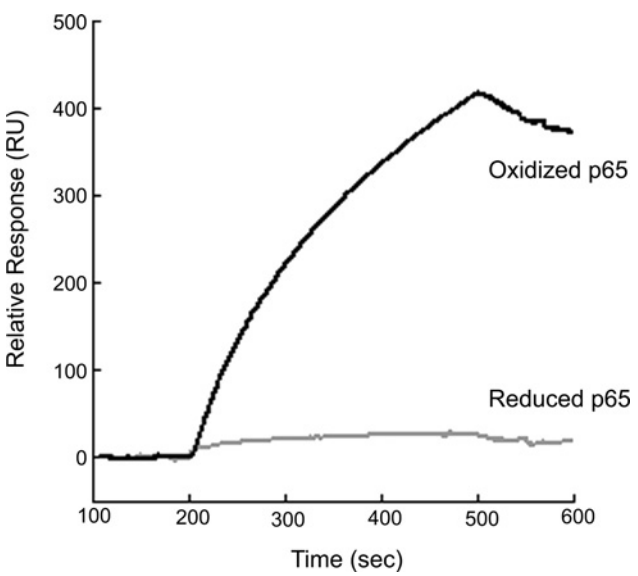

Figure 3 Interaction of Trx2 with the p65 subunit of NF-κB

Equal amounts of recombinant p65 were reduced by 1 mM DTT or oxidized by 1.2 mM selenite for 20 min at room temperature. After dialysis, the oxidized p65 and reduced p65 were analysed for their binding affinity with Trx2 by using SPR. The oxidized, but not the reduced, form of p65 showed binding affinity for Trx2.

Trx2 co-immunoprecipitates with GR and p65

In order to explore the possible interaction of Trx2 with GR and p65 in cells, we immunoprecipitated Trx2 from HEK-Trx2 lysates using either rabbit polyclonal or mouse monoclonal antibodies against human Trx2. The immunoprecipitated proteins were separated by SDS/PAGE (10% gel), Western blotted and analysed for the presence of Trx2 and GR using specific polyclonal antibodies against these molecules. The membranes were subsequently reprobed with antibodies against p65. A representative experiment is shown in Figure 4. The Trx2 immunoprecipitated by either monoclonal (Figures 4A and 4B) or polyclonal anti-Trx2 (Figures 4C and 4D) antibodies interacted and co-immunoprecipitated with GR and p65 in HEK-293 cell

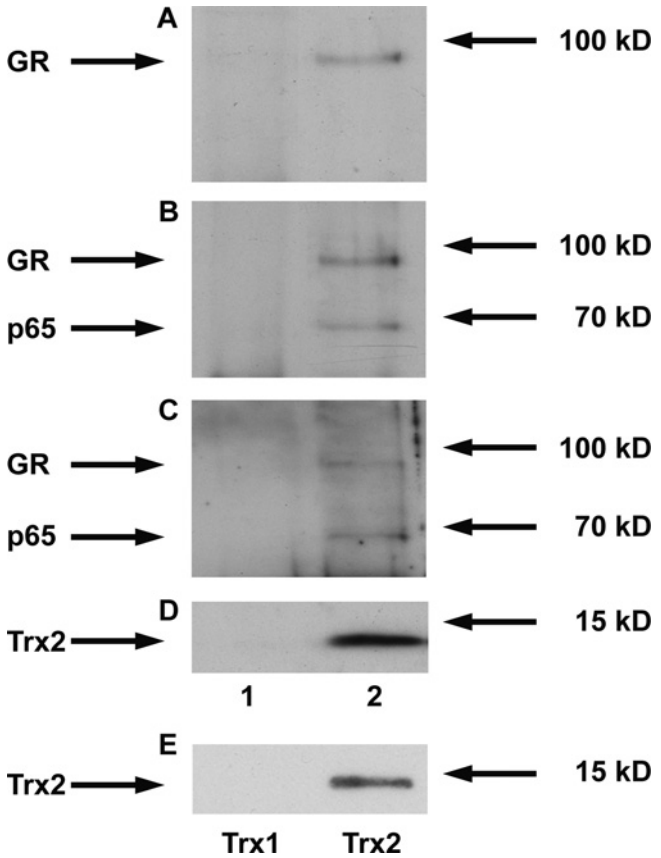

Figure 4 Trx2 interacts with GR and the p65 subunit of NF-κB in HEK-293 cell lysates

Trx2 was immunoprecipitated from HEK-Trx2 cell extracts using either (**A** and **B**) monoclonal or (**C** and **D**) polyclonal antibodies against Trx2. (**D**) The immunoprecipitated Trx2 was detected using rabbit polyclonal anti-Trx2 antibodies. Membranes were probed with (**A**) anti-GR antibodies and subsequently, were reprobed with (**B** and **C**) anti-p65 against the p65 subunit of NF-κB. Lane 1, immunoprecipitation with normal IgG; lane 2, immunoprecipitation with anti-Trx2 antibodies. (**E**) The anti-Trx2 antibody does not show any cross-reactivity. Equal amounts (10 ng) of His_6–Trx1 and His_6–Trx2 recombinant proteins were electrophorised and Western blotted using the anti-Trx2 antibodies. kD, kDa.

lysates (Figures 4A–4C, lane 2). Control non-immune mouse or rabbit IgG did not immunoprecipitate Trx2, GR and p65 (Figures 4A–4C, lane 1). Western blot analysis of equal amounts (10 ng) of recombinant Trx1 and Trx2 proteins using the rabbit anti-Trx2 antibody showed the specificity of the antibody and the absence of any cross-reactivity with Trx1 (Figure 4E).

Generation of HEK-293 cells stably underexpressing or overexpressing Trx2 in an active reduced form

To further investigate the role of Trx2 in mitochondrial functions and especially in transcriptional regulation, we applied the siRNA method to produce HEK-293 cells stably underexpressing Trx2 (HEK-siTrx2). Colonies from HEK-293 cells that were stably transfected with the siRNA Trx2-pSuper vector were selected, expanded and cloned independently. Then total extracts were analysed by Western blotting to estimate the levels of Trx2. Several colonies were found to exhibit 2–4-fold lower levels of Trx2 compared with control cells (Figure 5A), among them the HEK-siTrx2 colony 14 that was selected for further experiments. Trx2-overexpressing HEK-293 cells (HEK-Trx2 cells) showing approx. 20-fold Trx2 overexpression [9] were also used in our studies (Figure 5B). The *in vivo* redox status of Trx2 in both the HEK-siTrx2 and HEK-Trx2 cell lines was analysed using AMS, a high-molecular-mass compound that reacts with free SH groups, leading to a higher molecular mass of the bound protein. Total extracts from HEK-siTrx2 and HEK-Trx2 cells that were subjected to reducing conditions (1 mM DTT), oxidizing conditions (1 mM H_2O_2) or not treated at all were incubated with AMS and analysed by Western blotting. As shown in Figure 5(C), under oxidative conditions AMS did not principally react with Trx2 (Figure 5C, lanes 2 and 5), leading to the detection of a lower molecular-mass band, whereas in either untreated (Figure 5C, lanes 3 and 6) or DTT treated cells (Figure 5C; lanes 1 and 4) Trx2 interacted with AMS leading to a higher molecular mass product. These results indicate that Trx2 predominantly exists in a reduced, active form in both cell lines. Western blot analysis of Trx2 in subcellular fractions of HEK-Trx2 cells, obtained upon homogenization and differential centrifugation, revealed mitochondrial localization of Trx2 in HEK-Trx2 cells (Figure 5D). The exclusive detection of β-actin (a cytosolic marker) and SDH (a mitochondrial marker) in the cytosolic and mitochondrial fraction respectively, apart from their presence in the total extract, verified the purity and the enrichment of the isolated mitochondria (Figure 5D). Moreover, the detection of Trx2 in the mitochondrial fraction and its absence in the cytosolic fraction, where Trx1 is found, further confirmed the absence of any cross-reactivity of the anti-Trx2 antibody with the cytosolic Trx1.

Trx2 stimulates the TNFα-induced NF-κB activation and DEX-induced GR activation of reporter genes

The effect of Trx2 on mitochondrial ROS levels (ROS are known regulators of TNFα-induced NF-κB activation [8,27,28]), prompted us to examine the effect of Trx2 on NF-κB transcriptional activation. For that purpose transfection experiments, using a NF-κB-luc reporter gene construct, were performed. HEK-293, stably overexpressing or underexpressing Trx2 and in the presence or absence of TNFα, were used to measure luciferase activity in a dose- and time-dependent manner. HEK-siTrx2, HEK-Trx2 and HEK-293 control cells stably transfected with the empty vectors (HEK-siC and HEK-C) were analysed for their efficiency to regulate TNFα-induced NF-κB activation. Treatment with TNFα at 10–60 ng/ml rendered the HEK-Trx2 cells more effective in transactivating the NF-κB-luc reporter gene compared with control cells (Figure 6A). A dose of 20 ng/ml of TNFα was sufficient to cause approx. 80 % of the maximum transcriptional activation of NF-κB, achieved by 60 ng/ml of TNFα, and was further used to analyse the effect of Trx2 expression on NF-κB transcriptional activation. After 2 h of TNFα induction, HEK-Trx2 cells were at least 3-fold more efficient in transactivating the NF-κB reporter gene than control cells (Figure 6B, panel a), whereas the down-regulation of Trx2 in HEK-siTrx2 caused an approx. 20 % decrease in the TNFα-induced transactivation of the NF-κB reporter gene compared with the control cells (Figure 6B, panel b).

The role of GR in the regulation of the NF-κB-induced immune response [29] and its physical interaction with mitochondrial thioredoxin suggests a role for Trx2 in the GR-induced transcriptional activation. To test this hypothesis, HEK-Trx2 and HEK-C cells were transfected with a GR-luc reporter gene construct and incubated in the presence or absence of 10^{-6} M DEX. Luciferase activity was evaluated in both cell lines (Figure 6C). Expression of Trx2 resulted in a stimulation of the GR-luc reporter gene transactivation in the nuclear environment, which was significantly increased in the presence of DEX (Figure 6C).

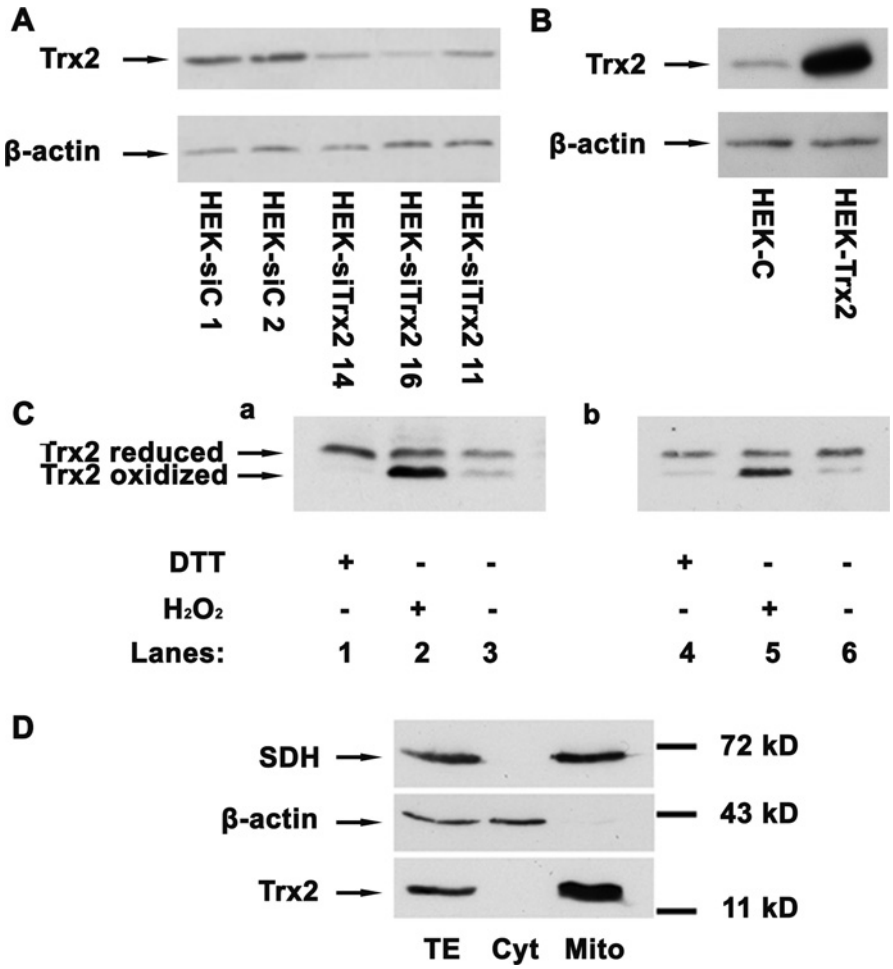

Figure 5 Characterization of HEK-293 cell lines that overexpress and underexpress Trx2

(**A**) By using siRNA-mediated knockdown we generated HEK-293 cells (HEK-siTrx2) that stably underexpressed Trx2 (colonies 14, 16 and 11) by 2–4-fold compared with the control cells (HEK-siC, colonies 1 and 2). Expression of Trx2 in the HEK-siTrx2 colonies was evaluated by Western blotting. (**B**) Overexpression of Trx2 in the HEK-Trx2 cells, was evaluated by Western blot analysis in comparison with actin expression. (**C**) The redox status of Trx2 in (a) Trx2-overexpressing or (b) -underexpressing HEK-293 cells. HEK-293 cells were subjected to oxidation or reduction by treatment with 1 mM H_2O_2 or 1 mM DTT respectively for 30 min at room temperature. AMS treatment was then used to identify the redox status of Trx2. Reduced Trx2 binds to AMS and thus migrates more slowly during SDS/PAGE than oxidized Trx2. (**D**) The localization of Trx2 in HEK-Trx2 cells. Western blot analysis of Trx2, SDH (mitochondrial marker) and β-actin (cytosolic marker) in total extract (TE), the cytosolic faction (Cyt) and the mitochondrial fraction (Mito) of HEK-Trx2 cells.

Effect of Trx2 on mitochondrial gene expression

NF-κB and GR are known transcription regulators of some mitochondrial genes such as COX I, COX III and Cytb (reviewed in [19,20]). The effect of Trx2 on the transcriptional activation of these genes, in the presence or absence of DEX or TNFα, was examined at the mRNA level by real-time PCR, using specific primers for COX I, COX III and Cytb mRNA. GAPDH (Figure 7A) or β-actin (Figure 7B) mRNA measurements were also performed and were used as the reference for the quantification of the results. Trx2 expression increased COX I gene expression up to 2-fold compared with control cells (Figures 7A and 7B, columns 1–3) but had only slight effects on COX III gene expression (Figures 7A and 7B, columns 4–6). Trx2 caused an approx. 50 % increase in Cytb gene expression in the presence of DEX. However, TNFα treatment caused an approx. 40 % decrease in the expression of this gene in the same cells (Figures 7A and 7B, columns 8 and 9).

DISCUSSION

Mitochondria are key organelles as they participate in many cellular processes, apart from their role in energy production [30]. They receive, integrate and transmit cellular signals from hormones, Ca^{2+}, cytochrome *c*, APAF1 (apoptotic peptidase activating factor 1), Diablo, endonuclease G and ROS, leading to survival or apoptosis [19,31]. Mitochondrial ROS are produced as by-products of incomplete oxygen metabolism and may cause severe cellular damage by oxidizing lipids, proteins and mitochondrial DNA [32]. ROS have also been identified as second messengers playing a role in receptor signalling and post-translational modification of signalling molecules [33–35]. The mitochondrion houses a variety of enzymatic antioxidant defence systems, among them the thioredoxin-peroxiredoxin system, utilized for protection from oxidative damage and for the assembly of the organelle [8,35]. Steroid and thyroid hormone receptors, and other transcription factors, are also localized in mitochondria

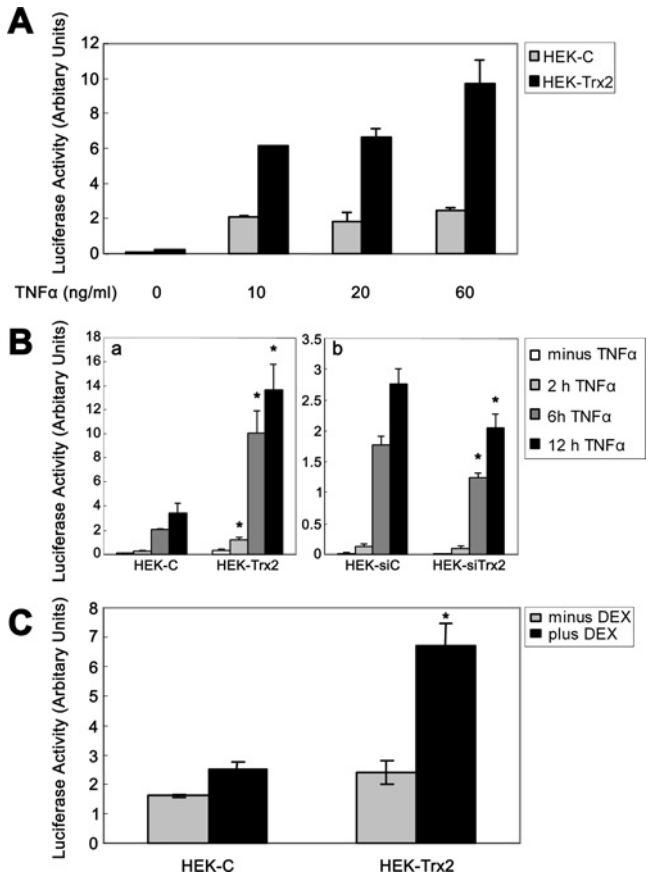

Figure 6 Effect of Trx2 on NF-κB and GR transcriptional activation

(**A**) Dose-dependent TNFα-induced NF-κB transcriptional activation in HEK-Trx2 cells compared with control HEK-C cells. Cells were treated with the indicated amounts of TNFα for 6 h at 37 °C. (**B**) Time course of the effect of 20 ng/ml TNFα on NF-κB transcriptional activation in (a) Trx2-HEK-293 cells or (b) siTrx2-HEK-293 cells. Cells expressing vectors without the Trx2 gene (HEK-C) or the siTrx2 (HEK-siC) were used as controls. An NF-κB-luc reporter gene construct was used in transfection experiments for the quantification of the NF-κB transcriptional activation. (**C**) Effect of stably overexpression of Trx2 on the transcriptional activation of a GR-luc reporter gene in the presence or absence of 10^{-6} M DEX. The transcriptional activation of a β-galactosidase reporter gene was used for the normalization of the results. The results of three independent experiments as means $\pm$ S.D are shown. *$P < 0.05$ compared with similarly treated control cells.

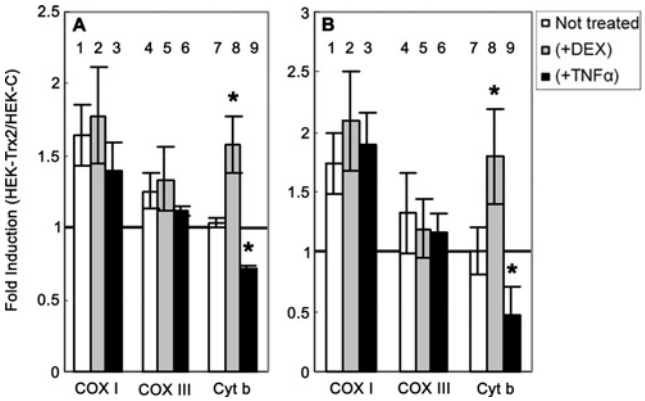

Figure 7 Effect of Trx2 on the transcriptional activation of mitochondrially encoded genes

Real-time PCR was used to determine of the mRNA levels of COX I, COX III and Cytb in cells overexpressing Trx2 compared with control cells in the presence or absence of TNFα or DEX. (**A**) GAPDH or (**B**) β-actin was used as a reference gene. The results of three to five independent experiments as means $\pm$ S.D. is shown. *$P < 0.05$ when compared with cells that were not treated.

and affect their functions (reviewed in [19,20,36]). In these complex mitochondrial processes, the existence of additional regulatory molecules is plausible. Mitochondrial thioredoxin is one such candidate and its role as a regulatory molecule in this respect is being explored.

Accordingly, a direct interaction of Trx2 with GR, as well as with truncated forms of GR carrying either the ligand- or the DNA-binding domains of the molecule, was shown by pull-down experiments. This finding indicates the existence of two binding sites for Trx2 on the GR molecule. However, we cannot exclude the possibility that the conformational state of the entire molecule might be in favour of one binding site. The proposed interaction surface of the Trx2, predicted from its crystal structure [37], as well as the kinetic analysis of the SPR results, showing a 1:1 interaction, are in accordance with the utilization of one GR–Trx2 binding site. These results suggest that thioredoxin may keep the ligand- and the DNA-binding domains of the receptor in a reduced active form, thus allowing it to interact with its ligand and with DNA. Similar results have been demonstrated by Makino et al. [3,4] for the cytosolic thioredoxin. Interestingly, we found that the interaction of GR with Trx2 was stronger than that observed with Trx1. This interaction was not affected by the presence of specific GR agonists or antagonists, indicating that the conformational changes that GR agonists or GR antagonists induce do not affect Trx2–GR interactions. In contrast with what is known [4] and what we also observed for Trx1, Trx2–GR interactions are not affected by the redox state of Trx2. The differential effect of the Trx1 and Trx2 redox status on GR binding could be explained by important differences observed during the transition between the oxidized and the reduced states of human Trx2, compared with human Trx1 [37]. Recently, it has been reported that Trx2 could catalyse protein dinitrosylation [38], which constitutes a regulatory process to control GR–ligand binding [39]. Thus the strong interaction of GR with Trx2 may suggest a potential role of Trx2 not only in the maintenance of the mitochondrial GR in a fully reduced active form, but also in its preservation in a conformational stage that enables GR to interact with other regulatory molecules in the mitochondrial environment. Moreover, the presence of other members of the thioredoxin system, such as TrxR, could ensure the prompt and effective reduction of Trx2, enabling it to reduce and activate GR, being already in close proximity to this protein.

The high affinity of Trx2 with GR indicates a possible Trx2 involvement in GR mitochondrial functions, such as transcriptional regulation, in which mitochondrial GR is proposed to be involved [19,20]. We have recently provided evidence for a mitochondrial GR-dependent regulation of several oxidative phosphorylation enzymes genes, among them COX I, COX III and Cytb [20]. Our results from real-time PCR experiments reveal a Trx2-dependent induction of Cytb expression, under conditions of DEX-induced GR activation, and further support the hypothesis of a Trx2 involvement in mitochondrial GR activation. An opposite Trx2 effect on Cytb expression was observed after pre-incubation of the cells with TNFα, a well-known activator of NF-κB [28]. GR is known to interact with and attenuate the functions of NF-κB in the nucleus [29]. The opposing effects of TNFα and DEX on Cytb expression in the presence of Trx2, not only support and corroborate previously reported observations, showing mitochondrial NF-κB-induced down-regulation of Cytb in HT1080 cells after TNFα treatment [17], but also reveal a crucial role of Trx2 in this process. Moreover, the possibility of opposing functions of GR and NF-κB in mitochondrial

transcription, similar to that observed in the nucleus [29], is also suggested. The expression of COX I and COX III has also been reported to be regulated by GR and NF-κB [17,18,20]. In the present paper we showed induction of COX I mRNA synthesis in the presence of Trx2, indicating involvement of Trx2 in regulation of its expression.

A Trx2-dependent transcriptional regulation of mitochondrial NF-κB is also supported by our observations of direct interactions of Trx2 with the oxidized, but not the reduced, form of the p65 subunit of NF-κB. The redox status of the protein may be used as a signal for recruitment of Trx2 and subsequent reduction and activation of NF-κB. Trx2, as an antioxidant enzyme, could reduce and activate mitochondrial NF-κB and other mitochondrial proteins such as ANT (adenine nucleotide translocase), a molecule that has been identified as a Trx2-interacting protein [40] and that is also known to be involved in mitochondrial NF-κB recruitment and translocation after apoptotic stimulus (including TNFα treatment) [41]. Bottero et al. [16] reported that induction of apoptosis led to IκB–α-NF-κB complex exit from mitochondria, suggesting that this could be a mechanism to increase the number of activated NF-κB molecules susceptible for nuclear translocation. Thus Trx2, as a putative regulator in this process, could control the transcriptional activation of many anti-apoptotic and antioxidant NF-κB-induced genes including Bcl-XL (BCL2-like 1) and MnSOD [42]. The finding that Trx2 interacts with p65 is in agreement with results from a yeast two-hybrid system analysis, revealing the p65 subunit of NF-κB is a Trx2-interacting protein (A. E. Damdimopoulos and G. Spyrou, unpublished work). Trx1 has been reported to interact with the p50 subunit of NF-κB, whereas there are no reports of Trx1 interacting with the p65 subunit [24–26]. The differential preference of Trx1 and Trx2 for the p50 and p65 subunits of NF-κB may reflect differences in the three-dimensional structure of the two proteins and may have important physiological implications. The redox status of Trx2 does not seem to alter the Trx2–GR interaction. However, we cannot exclude the possibility that significant local differences between the structures of the reduced and the oxidized Trx2 [37] could affect the Trx2–p65 interaction, as is the case for the Trx1–p50 interaction [26]. In addition, the possibility of a direct redox interaction between the active thiols of Trx2 and p65 exists. The co-immunoprecipitation of GR and the p65 subunit of the NF-κB protein with Trx2 from HEK-Trx2 cell extracts further supports the concept of a direct involvement of Trx2 in the modulation of GR and NF-κB functions.

A very interesting observation is the ability of Trx2 to control transcription of reporter genes driven by the GR or NF-κB promoters in HEK-293 cells. These results indicate that there is a Trx2-dependent regulation of the GR- and NF-κB-induced transcriptional activation in the nucleus. It has been previously reported [27] that mitochondrial ROS could regulate the anti-apoptotic activation of NF-κB, and evidence has been provided for the involvement of mitochondrial defence systems and mitochondrial-targeted antioxidants in this process [43]. It has also been observed that increased levels of Trx2 leads to reduced mitochondrial ROS levels, even under conditions of oxidative stress [44], whereas decreased levels of Trx2 causes mitochondrial dysfunction, accumulation of intracellular ROS, increased oxidative damage and apoptosis [10,45,46]. Thus Trx2 could also act as a regulator molecule in GR and NF-κB signalling pathways via the regulation of mitochondrial ROS levels and control of cellular redox status. Reducing conditions, either in the cytosol or in the nucleus, facilitates the ligand binding as well as the DNA binding of GR in the respective cellular compartment. Moreover, reducing conditions in the nucleus facilitates NF-κB

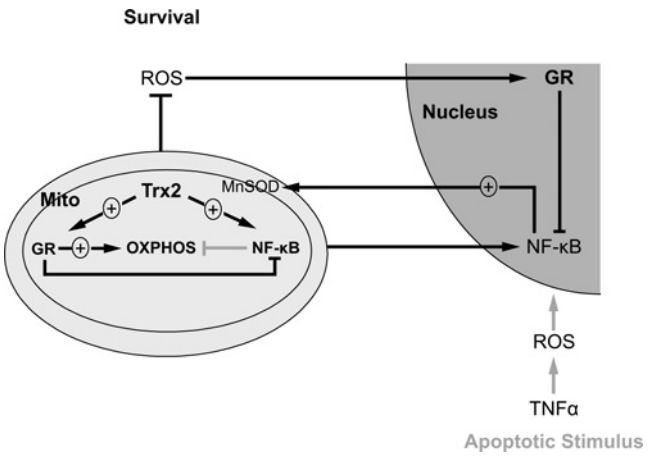

Figure 8 Putative mechanism of Trx2 involvement in NF-κB and GR signalling and mitochondrial functions

Trx2 may act as an anti-apoptotic and antioxidant factor through its direct interaction with GR and NF-κB and by preventing ROS accumulation, leading to nuclear GR and NF-κB activation. Trx2 activates mitochondrial GR and thus increases mitochondrial oxidative phosphorylation enzymes (OXPHOS) gene expression and promotes respiratory chain function. After TNFα treatment NF-κB enters the mitochondria where it is activated by Trx2. Activated NF-κB could then translocate into the cytoplasm and the nucleus to cause anti-apoptotic and antioxidant gene expression. The inhibitory action of NF-κB on OXPHOS gene expression could be suppressed by GR in a similar manner to the mechanism described in the nucleus. Grey lines represent apoptotic processes; black lines represent survival processes.

DNA binding and thus transcriptional activation. The cellular ROS levels and the activity of the antioxidant defence systems, as well as the cell type, seem to be critical for the final signalling effect of ROS on NF-κB activation [34]. This delicate balance and cell-type specificity may be the reason for the contradictory results that arise in the literature regarding the effect of ROS on NF-κB activation [34,47,48]. One example is the observed NF-κB transcriptional inactivation in HeLa cells transiently overexpressing Trx2 [44].

Fluctuations of the Trx2 levels, observed in many physiological and pathological conditions, could also attribute to the fine regulation of GR and NF-κB signalling pathways. We have previously observed elevated levels of Trx2 mRNA after DEX treatment in specific areas of the rat brain [49]. Increased levels of Trx2 have also been observed in many human cancers (http://www.proteinatlas.org). Furthermore, alterations in Trx2 levels have been implicated in many diseases such as ischaemic diseases, hypertension, cardiovascular disease, diseases of the respiratory system, neurodegenerative diseases, immune response and apoptotic or anti-apoptotic functions in which GR and NF-κB have a key role [19,29,50].

A putative mechanism by which Trx2 exerts its anti-apoptotic and antioxidant activity is summarized in Figure 8. According to our hypothesis, Trx2 mitochondrial thioredoxin could affect mitochondrial and cellular functions not only by protecting against ROS generation, but also by interacting with steroid receptors and other transcription factors, such as GR and NF-κB, present in mitochondria and possibly involved in differential regulation of mitochondrial gene expression. Thus Trx2 could directly or indirectly affect the respiratory chain action and the generation of mitochondrial signals, such as ROS, that subsequently act as second messengers and also control the nuclear transcription activation of GR and NF-κB. In the mitochondrial context, Trx2, probably through direct interaction with GR and NF-κB, could control respiratory chain action by promoting glucocorticoid- or TNFα-regulated mitochondrial

gene expression. Moreover, as the mitochondrial translocation of NF-κB is increased after apoptotic stimulus, Trx2 may promote an increase in activated NF-κB available for mitochondrial exit and subsequent nuclear translocation, which then acts to induce antioxidant or anti-apototic gene expression. Therefore Trx2 mitochondrial thioredoxin could act as a pro-survival or pro-apoptotic factor, affecting mitochondria enzyme synthesis and function not only by reducing ROS, but also by acting as a regulator of NF-κB and GR signalling.

AUTHOR CONTRIBUTION

Anna-Maria G. Psarra designed, performed and analysed the molecular, cellular and biochemical experiments and wrote the paper. Stefan Hermann performed and analysed the SPR experiments. George Panayotou performed and analysed the SPR experiments and assisted in the design of the experiments. Giannis Spyrou provided analytic tools, analysed data and gave conceptual advice. All authors read and approved the final manuscript.

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