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Nucleoredoxin, Glutaredoxin, and Thioredoxin Differentially Regulate NF-κB, AP-1, and CREB Activation in HEK293 Cells

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Well-established mechanisms for regulation of protein activity include thiol-mediated oxidoreduction in addition to protein–protein interactions and phosphorylation. Nucleoredoxin (NRX), glutaredoxin (GRX), and thioredoxin (TRX) have been shown to act as a potent thiol reductase and reactive oxygen species regulator. They constitute a oxidoreductase superfamily and have been suggested as a candidate operating in the redox regulation of gene expression. We demonstrated here that intracellular localization of these redox molecules differ from each other and that the redox molecules differentially regulate NF-κB, AP-1, and CREB activation induced by TNFα, PMA, and forskolin and by expression of signaling intermediate kinases, NIK, MEKK, and PKA in HEK293 cells. This is a first report that describes involvement of NRX and GRX and differences from TRX in transcriptional regulation of NF-κB, AP-1, and CREB in living cells.

The concept of redox regulation is becoming increasingly important at diverse levels of cellular functions (1, 2). The delicate interplay inside cells between oxidants and antioxidants ultimately determines the activity profile for many transcription factors.

Glutaredoxin (GRX) (also known as thioltranferase) and thioredoxin (TRX) are small disulfide reducing enzymes (3). These enzymes have the conserved consensus sequences "-CXXC-" at their active sites and exert reducing activity for the disulfide bonds on protein disulfides through the redox active cysteines. In addition, a novel nuclear protein, nucleoredoxin (NRX) was recently cloned (4). Recombinant NRX protein has oxidoreductase activity on the insulin disulfide bonds as TRX.

These disulfide reducing enzymes have been reported to be involved in various cellular functions, including redox regulation of certain enzyme activities (2). Thiol groups of GRX and TRX were reported to regenerate several enzymes in addition to their antioxidant roles (5). Moreover, the consensus sequences "-CXXC-" have been found in TRX-like domains of several larger proteins such as protein disulfide isomerase and phoshoinositide-specific phospholipase C (2).

TRX has been shown to be a candidate endogenous molecule operating in the redox-regulation of gene expression via modulation of many transcription factors (6–9). In contrast, reports describing transcriptional regulation by GRX in eukaryotic cells are few and the role of NRX in transcriptional regulation is not known. This prompted us to compare the transcriptional regulation of molecules belonging to the TRX superfamily using reporter genes driven by nuclear factor kappa-B (NF-κB), activator factor-1 (AP-1), and cyclic AMP-response element binding protein (CREB) in living cells.

MATERIALS AND METHODS

Cell lines and reagents. A human embryo kidney cell-derived cell line HEK293 and a murine embryonic fibroblast cell line NIH3T3

Abbreviations used: NRX; nucleoredoxin, GRX; glutaredoxin; TRX, thioredoxin; NF-κB, nuclear factor κ B; AP-1, activating protein 1; CREB, CRE binding protein; NIK, NF-κB inducible factor; MEKK, MAP/Erk kinase kinase kinase; DMEM; Dulbecco's modified Eagle's medium.

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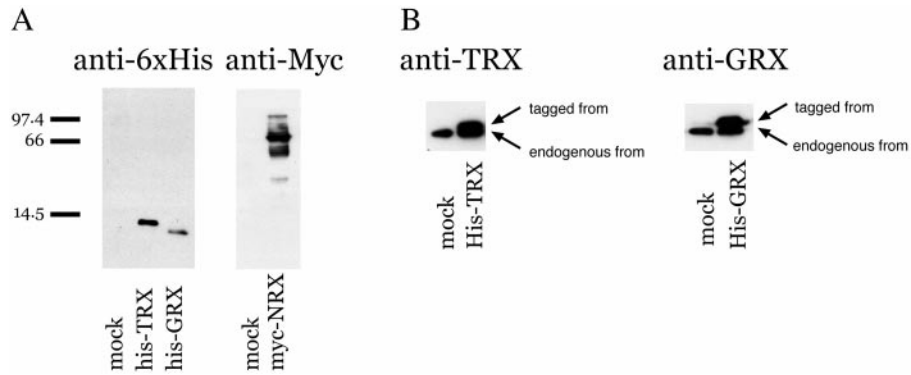

FIG. 1. Expression 6×His-human TRX and GRX, and myc-NRX in HEK293 cells. One microgram of pcDNA3-his-TRX-wt, pcDNA3-his-GRX-wt, or pEF-myc-NRX plasmid was introduced into HEK293 cells (1×10^6/60-mm dish) using Fugene 6 reagent. After 24 h, cells were harvested and 20 μg of lysates were submitted to Western blotting analysis using monoclonal antibodies against His × 6 epitope (1:1000) or Myc epitope (1:1000) (A) and antibodies against human TRX and GRX (B).

were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were maintained in DMEM supplemented with 10% FBS and antibiotics (50 U/ml penicillin and 100 μg/ml streptomycin) at 37°C under a humidified atmosphere of 5% CO_2. Recombinant human tumor necrosis factor α (TNFα) was purchased from Boehringer-Mannheim GmbH. 12-*O*-Tetradecanoylphorbol 13-acetate (PMA) and forskolin (FK) were purchased from Sigma Chemicals Co. Monoclonal antibody raised against hexahistidine (6xhis)-tag and C-Myc-tag (9E10, 0.4 μg/μl) was from Qiagen and Boehringer-Mannheim, respectively, and anti-phospho-IκBα rabbit polyclonal antibody was from New England Biolabs. Anti-human TRX monoclonal antibody (11-mAb) and anti-human GRX polyclonal antibody were described previously.

Expression plasmids. The plasmids, pcDNA3-his-GRX and pcDNA3-his-TRX were made by inserting *Eco*RI fragment from pQE-GRX and pQE-TRX into *Eco*RI-cut pcDNA3, respectively (7, 10). pEF-myc-NRX was kindly provided by Drs. Kurooka and Honjo and described elsewhere (4). The expression vector for NF-κB inducing kinase (NIK) was kindly provided by Drs. Shinkura and Kitada and described previously (11). Expression vectors for constitutively active mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK) or protein kinase A (PKA) were purchased from Stratagene.

Western blotting. Western blotting analysis was performed following the protocol described previously (7).

Indirect immunofluorescence cell staining. Indirect immunofluorescence cell staining was performed following the protocol described previously (12).

Reporter gene assay. Three reporter constructs (pNF-κB-Luc, pAP-1-Luc, and pCRE-Luc) were purchased from Stratagene. pGAL4(1–147)-NF-κBp65 (268–551) was made following the protocol described elsewhere (13). pFC-c-Jun (1–223) was from Stratagene. pGAL4-CREB (14) was kindly provided by Dr. Greenberg. A reporter construct containing five copies of a GAL4-binding site upstream of a minimal promotor (pG5E1-bLuc) (15) was kindly provided by Dr. Han.

HEK293 cells were plated in 24-well plates at a density of 5×10^4 cells per well. The Fugene 6 reagent (Boehringer-Mannheim GmbH) was used for the transfection procedure. pEGFP-N3 was used for monitoring transfection efficiency in each transfection and we found that efficiency were more than 80% (data not shown). In each transfection, each amount of an expression plasmid (for TRX, GRX, NRX, NIK, MEKK, or PKA), 250 ng of reporter construct (pNF-kB-Luc, pAP-1-Luc, or pCRE-Luc) and 100 ng of internal control plasmid (pCMV-β-galactosidase, Clontech) as an internal control were premixed and used. Total amount of DNA were adjusted equally. For transactivation assay, 500 ng of test plasmids, 200 ng of each GAL4-fusion construct, 100 ng of pG5E1-bLuc, and 50 ng of pCMV-β-galactosidase were used. In each assay, after incubation for 12 h, cells were treated by each reagent (TNFα, PMA, or FK) for 6 h. Then the cells were harvested and the luciferase activity was determined using an assay system (Promega Corp.) with a luminometer, Lumat LB9507 (Berthold) (7). The relative fold induction of luciferase activity was calculated after normalization dividing the luciferase activity by β-galactosidase activity.

Statistical analysis. Statistical significance between two groups was tested using the unpaired Student *t* test. Differences were considered statistically significant at a value of $P < 0.05$.

RESULTS

Differential Regulation of NF-κB, AP-1, and CREB Activation by TRX, GRX, and NRX

To investigate effect of overexpression of the redox molecules on the transcription, we prepared expression vectors for human TRX, human GRX, and mouse NRX. Introduction of each expression plasmid caused significant expression of each tagged protein (Fig. 1A). In the case of TRX and GRX, the amount of exogenously expressed protein are more than 2-fold of endogenous one (Fig. 1B) although we do not have any experimental evidence on the endogenous expression level of NRX. In this paper we exclusively used transient transfection protocol because we do not have any HEK293 cells stably expressing TRX, GRX, and NRX at this moment. In fact, as described by Gallegos *et al.*, it is not easy to get stable transfectant with high expression levels of TRX (16).

We examined the effect of overexpression of redox molecules on TNFα-induced NF-κB activation using a luciferase gene reporter. Expression of the redox molecules alone had only marginal effects on the activation of the transcription factors in the case of NF-κB. In Fig. 2A, expression of TRX suppressed TNFα-induced NF-κB activation In sharp contrast, GRX and NRX significantly enhanced the activation of NF-κB by

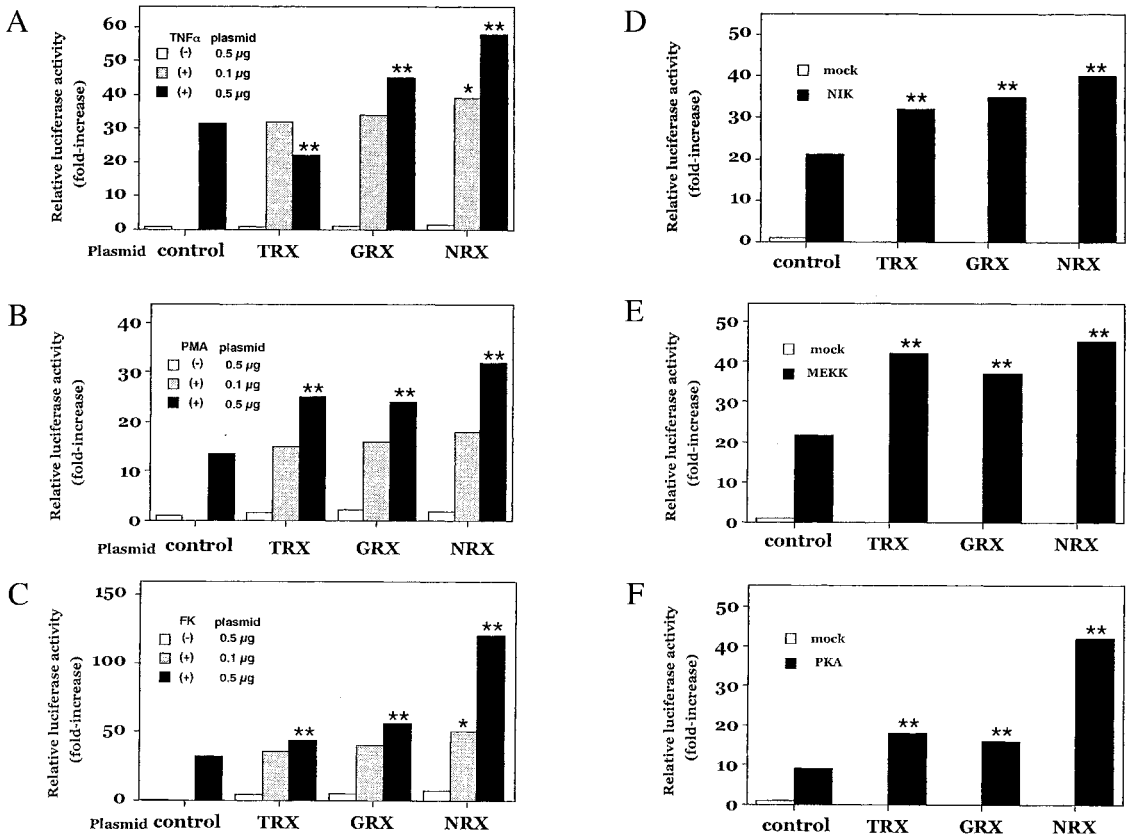

FIG. 2. Modulation of NF-κB, AP-1, and CREB activation by the redox molecules. HEK293 cells grown on 24-well plates were allowed to recover for 8–12 h. As reporter plasmids, pNF-κB-Luc for (A) and (D), pAP-1-Luc for (B) and (E), or pCRE-Luc for (C) and (F) was used. After transfection, cells were incubated for 12 h and then treated by TNFα (100 ng/ml) (A), PMA (50 ng/ml) (B), or FK (10 μM) (C). After 6 h, cells were harvested and subjected to the luciferase assay (A–C). Cells were transfected with NIK (D), MEKK (E), or PKA (F) and corresponding reporter, and harvested and subjected to the luciferase assay after 24 h. The results are representative of two independent experiments (each done in triplicate) and presented as fold increases in luciferase activity over the baseline seen with the mock transfectant without treatment. The SDs were within more than ±10% of the means. In A, B, and C, ** represents $P < 0.001$ and * represents $P < 0.05$ vs control (mock transfectant with the treatment).

TNFα compared with mock transfection. PMA-induced NF-κB activation was enhanced by NRX and GRX but suppressed by TRX (data not shown).

Next we investigated the influence of these redox molecules on PMA-induced AP-1 activation and FK-induced CREB activation. The activities of AP-1 and CREB were significantly enhanced by GRX or NRX as well as TRX (Figs. 2B and 2C). The effects were dose-dependent. Interestingly, the basal AP-1 and CREB activities were enhanced by all the redox molecules.

In addition, we examined the influence of the redox molecules on gene activation elicited by overexpression of the intermediate kinases; NIK, MEKK, and PKA. NRX and GRX enhanced the activation of NF-κB by NIK. TRX did not suppressed but rather enhanced this activation (Fig. 2D). Moreover, MEKK-induced NF-κB activation was also enhanced by expression of these molecules (data not shown). The activation of AP-1 by MEKK (Fig. 2E) and CREB by PKA (Fig. 2F) were enhanced by overexpression of each redox molecule.

Differential Regulation of GAL4-Transcription Factors Fusion Protein Activity by TRX, GRX, and NRX

The activation of *cis*-acting elements often reflects the activation of more than one transcriptional factor. It is known that many signal transduction pathways converge at a protein kinase, which phosphorylates and activates a specific transcriptional factor. Therefore, the activation of a GAL4-fused transcriptional factor will give a more precise assessment of the involvement of a protein or compound of interest in a specific signal transduction. As shown in Fig. 3, expression of GRX suppressed TNFα-induced GAL4-p65 activity as well as TRX. On the other hand, NRX had only a marginal effect in this process. In contrast, all the redox molecules tested enhanced PMA-induced GAL4-c-Jun and FK-induced GAL4-CREB activity. Because exogenous protein expression by introduction of plasmids is dependent on various factors; strength of the

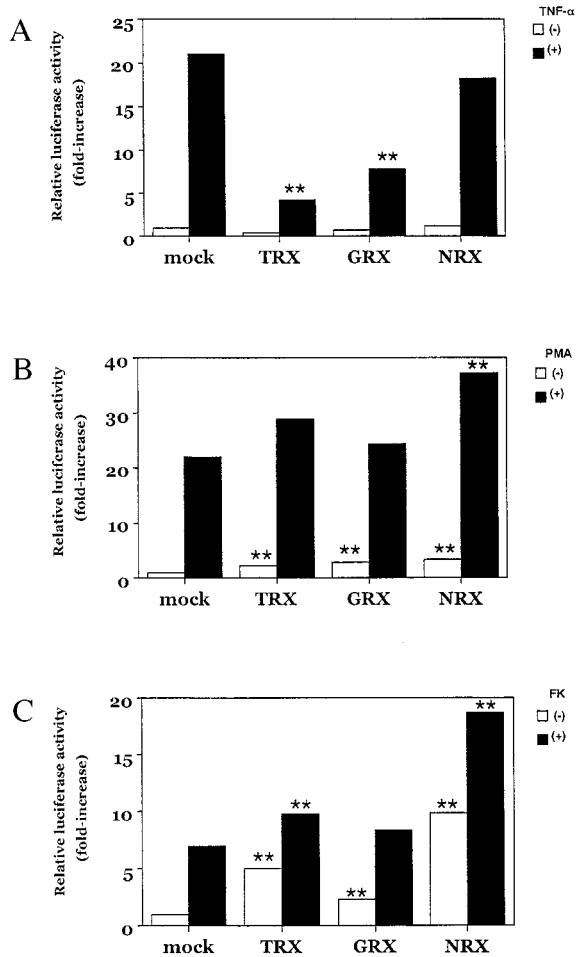

FIG. 3. Differential regulation of GAL4-transcription factors fusion protein activity by the redox molecules. HEK293 cells grown on 24-well plates were allowed to recover for 8–12 h. As *trans* plasmids, pGAL4-p65 (A), pGAL4-cJun for (B), or pGAL4-CREB for (C) was used with a reporter, pG5E1-bLuc plasmid. After transfection, cells were incubated for 12 h and then treated by TNFα (100 ng/ml) (A), PMA (50 ng/ml) (B) or FK (10 μM) (C). After 6 h, cells were harvested and subjected to the luciferase assay. The results are representative of two independent experiments (each done in triplicate) and presented as fold-increases in luciferase activity over the baseline seen with the mock transfectant without treatment. The SDs were within more than $\pm$ 10% of the means. In A, B, and C, ** represents $P < 0.001$ and * represents $P < 0.05$ vs control (mock transfectant with the treatment).

promoters, stability of mRNA, and efficiency of translation, we cannot simply compare potency of GRX, TRX, and NRX on the transcriptions. However, taken together with results from Figs. 2 and 3, it is strongly suggested that forced expression of NRX preferentially augments CREB-dependent luciferase activity compared to NF-κB- or AP-1-dependent activity.

Differential Intracellular Localization of NRX, GRX, and TRX

Because the subcellular localization of proteins, especially proteins relevant to signal transduction is very important component for their functions, we investigated the intracellular distributions of TRX, GRX and NRX before and after TNFα treatment. Expression plasmid for His-TRX, His-GRX or Myc-NRX was introduced into NIH3T3 cells and intracellular protein localization was investigated by an indirect immunofluorescence method. As shown in Fig. 4, TRX which was present mainly in the cytoplasm (Fig. 4A) but translocated into the nucleus (Fig. 4B) with TNFα treatment. In contrast, GRX is distributed in the cytoplasm and the nucleus before the treatment (Fig. 4C) and no significant translocation was observed after the treatment (Fig. 4D). NRX dominantly localized in the nucleus and localization was not affected by TNFα treatment. Not only TNFα but also PMA and FK treatment did not change intracellular localization of NRX and GRX significantly (data not shown).

Differential Regulation of IκBα Phosphorylation in Response to TNFα by the Redox Molecules

To understand mechanism of differential redox regulation by the three molecules better, we examined the phosphorylation state of IκBα in response to TNFα treatment in HEK293 cells transfected by the plasmids. TNFα elicited IκBα phosphorylation in HEK293 cells (Fig. 5, lane 1). This is consistent to the results of transactivation assay using pGAL4-p65 plasmid. NRX

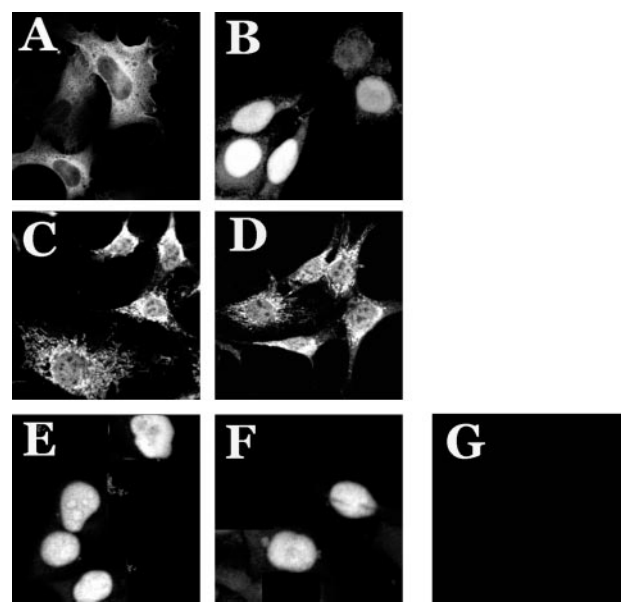

FIG. 4. Micrographs of his-TRX, his-GRX, and myc-NRX expressed in NIH3T3 cells. Plasmid pcDNA3-his-TRX-wt (A and B), pcDNA3-his-GRX-wt (C, D, and G), or pEF-myc-NRX (E and F) was introduced into NIH3T3 cells. 12 h after transfection, cells were treated by TNFα (100 ng/ml for 1 h at 37°C) (B, D, E, and G) or not (A, C, and F). Then cells were fixed and stained. In G, mouse IgG was used as 1 st Ab.

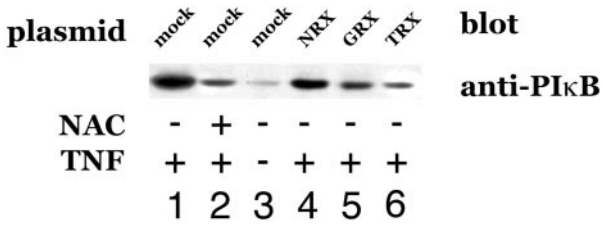

FIG. 5. Differential regulation of IκBα phosphorylation in response to TNFα by the redox molecules. HEK293 cells were transfected by 1 μg of each expression plasmid indicated and allowed to grow for 24 h. Cells were treated with TNFα (100 ng/ml) for 15 min and then harvested. In lane 2, cells were treated with NAC (20 mM) for 4 h before TNFα treatment. Thirty micrograms of total lysate was subjected to Western blot analysis using anti-phospho-IκBα rabbit polyclonal antibody.

had almost no effect (lane 4) and GRX had only a marginal effect on IκBα phosphorylation (lane 5). In contrast, NAC or TRX significantly suppressed the phosphorylation of IκBα (lanes 2 and 6).

DISCUSSION

The oxidoreductases of TRX family commonly have two active cysteine residues, separated by two amino acids (-CXXC-) in a similar folding structure (3, 17). Considerable experimental and theoretical efforts have been focused on the role of the residues between the active site cysteines and it has been shown that they determine the standard state redox potential of the TRX superfamily of proteins (18). In fact, the redox potential of *E. coli* TRX and that of *E. coli* GRX are reported to be −270 mV and −235 mV, respectively. GRX does not scavenge hydrogen peroxide (H_2O_2), which is a potent signaling intermediate of cytokines, by itself, although it couples to glutathione peroxidase (19). In contrast, TRX by itself (20) or via other proteins such as peroxiredoxin (Prx) (21, 22) and glutathione peroxidase can reduce H_2O_2. NRX, which has been identified as a nuclear protein, is similar to TRX not only structurally but also functionally (4). NRX has a TRX-consensus sequence and functions as oxidoreductase exemplified in insulin reducing activity. These differences may explain the discrepancy of competency as an antioxidant and reducing enzyme and as a regulator of ROI generation system.

In general, transcription factors consist of DNA-binding region (DBD) and transactivating domain (TAD) which interacts with core transcription factors. In addition to redox regulation of cysteine residues on the DBD, transcription factors undergo other type of posttranslational modification on the TAD. As shown in Figs. 2A and 2D, although TNFα-induced activation of NF-κB was suppressed by TRX, NIK-induced activation was rather enhanced. These results suggest that the mode and targets of redox regulation of NF-κB may not be unique but multiple. Some reducing factors vary in their subcellular localization; overexpressed TRX translocated from the cytoplasm to the nucleus in response to TNFα treatment and this is in accordance to our previous reports on endogenous TRX in HeLa cells, in which PMA, UV irradiation, H_2O_2, or hypoxia induced nuclear translocation of TRX (7, 9, 12). On the other hand, GRX exists in both of the cytoplasm and the nucleus and significant localization change was not observed even after TNFα treatment. NRX resides in the nucleus and TNFα treatment did not change the localization. We have shown that distinct role of TRX in the cytoplasms and the nucleus. In fact, nuclear-targeted TRX, which is GAL4-DBD fused form, does not have any effects on TNFα-induced degradation of IκBα as well as NRX (12). It seems that GRX also have distinct roles in the TNF-induced NF-κB-dependent gene induction. In HEK293 cells overexpression of GRX have enhanced the NF-κB-dependent gene induction although it partially inhibited phosphorylation of IκBα and the reporter activity using pGAL4-p65 plasmid elicited by TNF. As well as TRX, the character of regulation by GRX may be finally determined by the elaborate balance of distinct effects in the cytoplasm and in the nucleus. On the other hand, TADs of c-Jun and CREB seem to undergo another type of redox regulation. The redox molecules enhanced their activities even without stimulation. This mode of regulation is similar to the case of redox regulation of hypoxia-inducible factor 1α (HIF1α). We have demonstrated that interaction between hypoxia-inducible factor 1α and CPB/p300 is under redox regulation (23). Conserved SH groups of CPB/p300-binding interface on the TAD may be another target of the redox molecules. Marked enhancement of CREB-dependent transcription by NRX may be explained by this mechanism. Moreover, differential modulation of IκBα in Fig. 5 strongly suggest that activities of some signaling intermediates are also under differential control by the redox active enzymes. Many kinases and phosphatases have been shown to be involved in these process as signaling intermediates. Among them, PKC (24) and Ask1 (25) have been identified as TRX-binding proteins and shown to be negatively regulated by interaction with TRX. However, we have shown that Ask1 does not play an essential role in TNFα-elicited NF-κB activation in HEK293 cells (26).

This is a first report which demonstrates involvement of NRX in redox regulation of transcription to the best of our knowledge. This diversity of redox regulation may be derived from diversity of enzymatic activities and intracellular localizations in response to stimuli. further studies on three-dimensional protein structure and intra-cellular associated molecules may facilitate us to understand the redox regulation of cellular signaling.

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