Distinct Roles of Thioredoxin in the Cytoplasm and in the Nucleus

A TWO-STEP MECHANISM OF REDOX REGULATION OF TRANSCRIPTION FACTOR NF-κB

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Oxidative stresses such as UV irradiation to mammalian cells triggers a variety of oxistress responses including activation of transcription factors. Recently, activation of nuclear factor-κB (NF-κB) has been shown to be under oxidoreduction (redox) regulation controlled by thioredoxin (TRX), which is one of major endogenous redox-regulating molecules with thiol reducing activity. In order to elucidate where in the cellular compartment TRX participates in NF-κB regulation, we investigated the intracellular localization of TRX. UVB irradiation induced translocation of TRX from the cytoplasm into the nucleus. In our in vitro diamide-induced cross-linking study, we showed that TRX can associate directly with NF-κB p50. Overexpression of wild-type TRX suppressed induction of luciferase activity under NF-κB-binding sites in response to UV irradiation compared with the mock transfectant. In contrast, overexpression of nuclear-targeted TRX enhanced the luciferase activity. Thus, TRX seems to play dual and opposing roles in the regulation of NF-κB. In the cytoplasm, it interferes with the signals to IκB kinases and blocks the degradation of IκB. In the nucleus, however, TRX enhances NF-κB transcriptional activities by enhancing its ability to bind DNA. This two-step TRX-dependent regulation of the NF-κB complex may be a novel activation mechanism of redox-sensitive transcription factors.

Exposure of mammalian cells to UV light induces various responses in cells (1, 2). Many of the cellular responses can be triggered by a change in intracellular redox state resulting in cytotoxicity or stress to cells (2, 3). Oxidative stress caused by exposure to UV light, hydrogen peroxide, heavy metals, and inflammatory cytokines can elicit not only “negative” responses such as cell death but also “positive” responses including cell proliferation and activation (1, 4). There is a growing body of evidence that shows that the intracellular redox state can modulate various kinds of gene expression. TRX is a ubiquitous protein with two redox-active half-cysteine residues in its catalytic active center, having the consensus amino acid sequence -Cys-Gly-Pro-Cys- (9). The protein exists either in a reduced form with a dithiol or in an oxidized form, in which the half-cystine residues form an intramolecular disulfide bridge. TRX participates in redox reactions by reversible oxidation of its active center dithiol to a disulfide and catalyzes dithiol-disulfide exchange reactions (10) involving many thiol-dependent cellular processes, including gene expression and signal transduction. Human TRX was originally cloned as a soluble factor named adult T-cell leukemia-derived factor, which was purified from the conditioned medium of human T-cell lymphotrophic virus-I-transformed CD4+ T-cell line, ATL-2 (11–14).

The transcription factors, NF-κB, as well as activating factor 1 (AP-1), execute important roles in immune function, inflammatory response, cell adhesion, and growth control (15–17). AP-1 was shown to be an antioxidant-responsive transcription factor (1, 18, 19). Transient overexpression of TRX can stimulate AP-1-dependent transcription (8, 20, 21). We showed that TRX, which is mainly in the cytoplasm, quickly translocates into the nucleus in response to phorbol 12-myristate 13-acetate (PMA) and activates AP-1 transcription activity by direct association with an intranuclear redox factor, Ref-1 (8). In the case of NF-κB, however, there seems to be some controversy on the redox regulation of its activation. NF-κB is stored as an inactive cytoplasmic complex in association with IκB. When activation signals reach the IκB kinase complex (22–27), IκB are phosphorylated by IκB kinases to be degraded and dissociated from NF-κB, and this free NF-κB complex translocates from the cytoplasm into the nucleus (17, 28). It is suggested that reactive oxygen intermediates (ROIs), which are generated in the cells, act as second messenger molecules as follows: cytokines such as TNFα binding its specific receptor causes oxidative stress or an increase in the ROI concentration in cells (1). Treatment of cells with radical scavengers, such as N-acetyl-l-cysteine and pyrrolidine dithiocarbamate, as well as transient overexpression of TRX inhibited nuclear translocation and transactivation of NF-κB (20, 29).

Once translocated into the intranuclear compartment, however, the redox environment seems to be quite important for NF-κB activity. DNA binding activity of NF-κB is shown to be under redox regulation through the modulation of cysteines (5, 20). Like c-Jun, c-Fos (30), Myb (31), p53 (32), PEBP2 (6), and nuclear receptors such as glucocorticoid receptor (7, 33) and estrogen receptor (34), NF-κB component, p50, and p65/Rel-A have a well conserved cysteine (Cys) in its DNA-binding loops (35, 36). An in vitro study (37) showed that an alkylating agent, mortal necrosis factor-α, PAGE, polyacrylamide gel electrophoresis; wt, wild type; mAb, monoclonal antibody.
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*N*-ethylmaleimide, irreversibly inhibited the DNA binding activity of NF-κB; NF-κB was inhibited by modulating its conserved Cys residue, using diamide, a sulfhydryl (SH)-oxidizing agent. This inhibition was reversed by treatment with reducing agents such as 2-mercaptoethanol, dithiothreitol, and recombinant human TRX (5, 38). Moreover, there is a report that transient overexpression of human TRX in COS1 and Jurkat T-cell augments gene expression from human immunodeficiency virus, type I-long terminal repeat (39). TRX, thus, is suggested to be a candidate for the endogenous molecules that play in redox regulation of gene expression of transcription factor NF-κB. The main aim of this paper is to clarify where and how TRX regulates a transcription factor, NF-κB complex, which translocates from cytosol to nucleus, and to elucidate a dynamic role of TRX on redox regulation of transcription in cells. For this purpose, we investigated the subcellular localization of TRX after stimulation including UVB irradiation and TNFα treatment and a possibility of direct association between NF-κB and TRX in the nucleus. Our data indicate that TRX translocates from the cytoplasm into the nucleus and translocated TRX potentiates NF-κB-dependent transcription. Moreover, direct association between TRX and NF-κB in the nucleus was suggested by *in vitro* cross-linking assay.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Transformed human keratinocytes, established from human squamous cell carcinoma of skin, HSC-1, were a gift from Dr. Y. Kondo (40). HSC-1, NIH3T3, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 95% humidity in 5% CO2 in air at 37 °C.

**Reagents**—Human tumor necrosis factor-α (TNFα) was from Life Technologies, Inc. Anti-human TRX monoclonal antibodies, 11-mAb and 21-mAb (41), were established and provided by FujiRebio, Inc. (Tokyo, Japan). Anti-NF-κB p50 (NLS), anti-NF-κB p65 (40), and anti-IκBα (C21) polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**UV Light Irradiation**—Cells were plated on chamber slides (Nunc Inc., Naperville, IL). The culture media were replaced with prewarmed phosphate-buffered saline (PBS), and the cells were exposed to an UVB source (UVP Inc., Upland, CA). After exposure, cells were incubated with culture media at 37 °C.

**Plasmids**—Mutagenesis of human TRX was performed by a polymerase chain reaction-based technique (8) and subcloned into BamHI-SalI-cut pBluescript II SK+ plasmid (pBSII-TRX). Expression vectors for TRX, pcDNA3-TRX-wt and pcDNA3-TRX-dm, were made by inserting *Bam*HI- or *Hin*dIII-cut pBluescript II SK+ fragment from pBSII-TRX plasmids into *Bam*HI- or *Hin*dIII-cut pcDNA3 (Invitrogen Corp., San Diego, CA). An reporter vector, pcMX-GAL4-TRX-wt, was described previously (8). pcDNA3-anti-TRX was made by inserting *Bam*HI-HindIII fragment from pcRSor-TRX plasmid (8) into *Bam*HI-HindIII-cut pcDNA3.

**Transfection**—The expression plasmids were introduced into the cells by the use of LipofectAMINE reagent (Life Technologies, Inc.), following the protocol previously described (8).

**Indirect Immunofluorescence Cell Staining**—Indirect immunofluorescence cell staining was performed following the protocol previously described. Briefly, cells were fixed with 3.7% paraformaldehyde in PBS containing 10% fetal calf serum for 20 min at room temperature, followed by permeabilization for 10 min using 0.2% (w/v) Triton X-100 in PBS. After incubation with primary antibodies (for TRX, monoclonal 11-mAb; for NF-κB p50 or p65, polyclonal antibody) for 1 h at room temperature, the slides then were incubated for 1 h with a secondary antibody as follows: fluorescent isothiocyanate-conjugated sheep anti-mouse IgG (Amersham Pharmacia Biotech) for TRX, or tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) for NF-κB p50 or p65. Slides with stained cells were mounted in 90% glycerol with 1 mg/ml p-phenylene diamine and examined with a confocal microscope, MRC-1024 (BioRad).

**Expression of Recombinant Proteins in Bacteria**—Human TRX recombinant proteins were expressed in *Escherichia coli* as the N-terminal fused form with hexahistidine (6His) tag using the pQE30 expression plasmid (Qiagen) essentially following the protocol described previously (8). Truncated human NF-κB p50 DNA-binding domain (residues 35–381) was expressed based on the pQE-p50 as a 6His-tagged protein by use of the same protocol as that used for the expression of TRX.

**Western Blot Analysis of Nuclear Fraction of HSC-1**—Crude nuclear cell extracts were prepared as described (42). Each fraction was applied to 15% SDS-PAGE and electrophoresed. After electrophoresing, the polyvinylidene difluoride membrane (Millipore, Bedford, MA) was treated with 5% (v/v) skim milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.5% Tween 20) and incubated with antigen-specific antibodies against human TRX, followed by incubation with peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech). The epoxide was visualized with an ECL Western blot detection kit (Amersham Pharmacia Biotech). The amount of TRX in each sample was estimated by analysis of the density of each band, using a computerized densitometer (PDI Analyzer; ToYoBo, Tokyo, Japan).

**In Vitro Disulfide Cross-linking Interaction Assay**—In the cross-linking assay, 6xHis-TRXs (100 ng) and 6xHis-p50 (200 ng) were incubated for 30 min at room temperature with 1 μM dithiothreitol (8). Then they were mixed and incubated in the presence of 10 mM dithiole for 30 min at room temperature in 20 μl of PBS, pH 7.3. The reaction mixture was denatured for 5 min at 90 °C in dissociation buffer with or without 2-mercaptoethanol (1%). Each mixture was applied to a 15% SDS-PAGE and electrophoresed. Epitopes were detected by Western blot.

**Insulin-reducing Assay**—To estimate the reducing activity of TRX, an insulin-reducing assay was performed according to a previous report (43). A yeast TRX reducing enzyme was provided by Sigma Co., Ltd. (Tokyo, Japan) and can reduce mammalian TRX. The decrease in absorbance at 340 nm was recorded by use of a THERMOMAX microplate reader (Molecular Devices) to detect maximal NADPH consumption rate (Vmax, millioptical density at 340 nm/min). As a control, samples were incubated with the mixture without insulin. Each value was calculated according to a method previously reported. The calculated values were compared with the standard curve for rTRX (100–1200 ng/ml) to obtain quantitative determinations of the absolute amounts of TRX.

**RESULTS**

**UVB-induced Translocation of TRX from the Cytoplasm into the Nucleus in Association with NF-κB**—As a step toward dissecting the roles of TRX in early stage of UV responses, we examined intracellular locations of TRX after UV exposure. Transformed human keratinocytes, HSC-1 cells, were stained using an immunofluorescence method with 11-mAb raised against human TRX. In control cells without UVB irradiation, TRX was stained mainly in the cytoplasm (Fig. 1A), whereas after 1 h exposure to 400 J/m2 UVB, most of the fluorescence translocated into the nucleus (Fig. 1B). Under the same conditions, nuclear translocation of NF-κB p65/RelA was also observed (Fig. 1, C and D). In contrast to the case of RelA subunit, NF-κB p50 was localized in the cytoplasm and remained there even after UVB treatment (Fig. 1, E and F). The UVB-induced translocation of TRX was observed also in HSC-1 cells (Fig. 1, G and H). Similar translocation pattern was detected by use of another monoclonal antibody, 21-mAb, which may recognize a different epitope on human TRX (data not shown).

To quantify the subcellular TRX after UVB irradiation, cytoplasmic and nuclear extracts of HSC-1 cells were fractionated and analyzed by Western blot analysis. In agreement with the results of the immunofluorescence staining, the amount of TRX...
in the nuclear fraction extracted 1 h after UVB irradiation was increased by about 4-fold (Fig. 2). The total amount of intracellular TRX was unchanged 1 h after irradiation (data not shown).

**TNFα Treatment Also Translocates TRX into the Nucleus**—Next, we examined whether stimulation or treatment other than UV irradiation could translocate TRX into the nucleus. TNFα is one of pro-inflammatory cytokines and is known to be a potent NF-κB activator. It is reported that formation of ROIs is crucial in this process (43). As shown in Fig. 3, TNFα treatment (100 ng/ml) resulted in the translocation of TRX from the cytoplasm into the nucleus in HeLa cells no later than 1 h after the treatment. As reported previously PMA also translocated TRX (8).

**Direct Association between TRX and the NF-κB p50 Component by in Vitro Disulfide Cross-linking**—As we and others (8, 9, 44, 45) have shown previously, an oxidoreductase, TRX, forms an intermediate with various target molecules through disulfide linkage. To dissect further the detail of redox regulation of NF-κB, we examined direct association between TRX and NF-κB in *in vitro* systems. We reasoned that it would be possible to trap the transient physical association using cross-linking reagent(s) such as diimide, which converts free sulfhydryls to disulfides by cysteine oxidation (8, 9). In order to determine which of the five cysteines in TRX is responsible for intermolecular disulfide bond formation, TRX mutants, which contained cysteines-to-serines or cysteine-to-alanine substitution(s), were purified as the hexahistidine (6xHis)-tagged form. A series of experiments was performed with the 6xHis-tagged NF-κB p50 and mutants of TRX. It is known that TRX-(C62S/C69S/C73S) retains its reducing activity (46). Wild (wt) and mutant recombinant TRX proteins were incubated with recombinant NF-κB p50 DNA-binding domain, and then their migration patterns were analyzed. Antibodies against NF-κB p50 (Fig. 4, 4th and 8th lanes) and TRX (data not shown) recognized the same extra bands migrating around 50 kDa in the lanes of TRX-wt and TRX-(C62/69/73S) only under oxidizing conditions.

**Establishment of Nuclear-targeted TRX**—In order to understand intranuclear roles of TRX, we constructed nuclear-targeted expression vector of TRX (8). TRX lacks an authentic nuclear localization signal. Therefore, we constructed nuclear-targeted TRX by fusing the yeast GAL4 DNA-binding domain (amino acids 1–147), which has a strong nuclear localization signal, to C-terminus of TRX (8). To prepare nuclear-targeted TRX, we made a fusion construct with yeast GAL4 DNA-binding domain (amino acids 1–147), which has a strong nuclear localization signal, and human TRX as pCMX-GAL4-TRX-wt. Because anti-human TRX monoclonal antibody, 11-mAb, does not recognize mouse TRX (41), it is possible to trace the intracellular localization of the overexpressed TRX in NIH3T3 cells with this antibody by immunofluorescent method. At 24 h after transfection of plasmids, immunofluorescent staining showed that human wild-type TRX dominantly existed in the cytoplasm (Fig. 5A) and translocated into the nucleus after UVB irradiation (Fig. 5B); in contrast, GAL4-TRX-wt existed in the nucleus...
without any treatment (Fig. 5C) and remained in the nucleus after irradiation (data not shown). To evaluate the function of fusion construct of GAL4-TRX, the TRX activity in transfected cells was analyzed by insulin degradation assay. GAL4-TRX-wt-transfected NIH3T3 cells and TRX-wt showed 1.76- and 1.83-fold increase in TRX activity over mock-transfected cells (Table I).

NF-κB Activation by Intranuclear Overexpression of TRX in UVB-irradiated HeLa Cells—Next we examined the intracellular role of TRX on transactivation of NF-κB. HeLa cells were transiently cotransfected with a 5x-κB-luciferase reporter gene and various TRX expression plasmids. In Fig. 6, overexpression of wild-type TRX suppressed NF-κB-dependent luciferase gene activity elicited by UVB irradiation compared with UVB transfection in dose-dependent manner of pcDNA3-TRX-wt. In sharp contrast, overexpression of nuclear-targeted GAL4-TRX-wt enhanced the luciferase activity. Overexpression of Ref-1 also enhanced the luciferase activity although this enhancement was not significant. TRX-dm, which does not have reducing activity, had only a marginal effect.

Requirement of TRX in NF-κB-mediated Transcription—Although a targeted disruption of mouse TRX gene has recently been reported by us (49), homozygous mutants died shortly after implantation, and the cells derived from pre-implantation embryos failed to grow. TRX(−/−) cells are not available at this moment. As alternatives, we attempted to inactivate endogenous TRX by treatment of cells with either an antisense plasmid of human TRX or a specific inhibitor of TRX reductase, DNCB (50). In Fig. 7A, we showed that introduction of antisense TRX significantly reduced the amount of endogenous TRX. In Fig. 7B, introduction of anti-TRX plasmid or 1-chloro-2,4-dinitrobenzene, DNCB treatment (50 μM) suppressed NF-κB-dependent luciferase gene activity by TNFα. We next investigated influence of knock-down treatment of TRX system on IκBα degradation. As shown in Fig. 7C, knock-down treatment of TRX cause the enhancement of TNFα-induced IκBα degradation. This is a very good contrast to the reporter assay data.

**Discussion**

In this paper, we demonstrate that TRX quickly translocates from cytoplasm into nucleus in response to NF-κB activation stimuli such as UVB irradiation and TNFα treatment, and TRX plays distinct roles in the cytoplasm and in the nucleus on the signaling pathway to the NF-κB-dependent transcription. In cytoplasm, overexpression of TRX suppresses the degradation of IκB in the 11-mAb at 16 h after transfection. A and C, without any treatment; B, 1 h after UVB irradiation.

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**Table I**

| TRX activity | μg/mg protein |
|--------------|---------------|
| Mock         | 9.08          |
| pcDNA3-TRX-wt| 16.61         |
| pcMX-GALA-TRX-wt | 15.96    |

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**Fig. 6** Intracellular overexpression of TRX induces NF-κB-dependent reporter gene activity. A, influence of overexpression of various endogenous redox molecules on the NF-κB-mediated transcription. NAC, N-acetyl-l-cysteine. HeLa cells were transiently cotransfected with a 5x-κB-luciferase reporter gene plasmid (1 μg) and expression vectors (1 μg) or pcDNA3 plasmid as control (1 μg) with 0.5 μg of pSV-β-galactosidase. Cells were harvested at 12 h after irradiation. Luciferase activities were determined and normalized on the basis of β-galactosidase expression (8). The results are the means (mean ± S.D.) of three experiments (each done in duplicate) and presented as fold increases in luciferase activity over the base line seen with the mock transfectant without treatment. B, dose-dependent effect of TRX on the NF-κB-mediated reporter gene expression. Total amount of plasmid was adjusted 3.5 μg with pcDNA3 plasmid.

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are crucial components of signal transduction. For example, in most cell types, NF-κB is present as a heterodimer comprising p50 and p65 that are sequestered in the cytoplasm and are associated with a member of the IκB family of inhibitory proteins. When activation signals reach the IκB kinase complex, IκBs are phosphorylated by IκB kinases to be degraded and dissociated from NF-κB, and this free NF-κB complex translocates from the cytoplasm into the nucleus. Also in the case of mitogen-activated protein kinase signaling, mitogen-activated
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Fig. 7. Requirement of TRX for the NF-κB transcriptional activity. HeLa cells were plated and allowed to recover for 8–12 h. After transfection, cells were incubated for 12 h and then treated by TNFα (100 ng/ml). After 30 min for IκBα degradation assay or 12 h for reporter assay, cell were harvested. In the case of DNCB treatment, cells were treated for 30 min before TNFα application. A, amount of TRX of HeLa cells after introduction of sense or antisense TRX expression vector. HeLa cells were transfected the mock expression plasmid (lane 1), pcDNA3-TRX-wt, which means the expression plasmid of wild type TRX (lane 2), or pcDNA3-anti-TRX which means the expression plasmid of antisense TRX (lane 3). Cell lysates (10 μg per lane) were resolved on a 15% SDS-PAGE and analyzed. TRX protein were visualized using an antibody raised against IκBα protein. B, relative luciferase activity. HeLa cells were transiently transfected with a 5×κB-luciferase reporter gene plasmid (1 μg) and expression vectors (1 μg) or pcDNA3 plasmid as control (1 μg) with 0.5 μg of βSV-β-galactosidase. The results are the means (mean ± S.D.) of three experiments (each done in duplicate) and presented as fold increases in luciferase activity over the base line seen with the mock transfectant without treatment. C, regulation of TNFα-elicited degradation of IκBα by cellular TRX levels. Lane 1, pcDNA3 without TNFα treatment; lane 2, pcDNA3; lane 3, pcDNA3-ωTRX; lane 4, pcDNA3-anti-TRX; lane 5, DCNB with TNFα treatment. Cell lysate (50 μg per lane) were resolved on a 10% SDS-PAGE and analyzed by the protocol described. IκBα protein were visualized using an antibody raised against IκBα antibody (C21).

NF-κB is an important molecule, TRX translocated into the nuclear compartment in response to generation of ROI by Fe-nitrilotriacetic acid (51), and also in HeLa cells H$_2$O$_2$ treatment induced TRX translocation (7). As UV irradiation and TNFα and PMA treatment are shown to generate ROIs in the cytoplasm (1, 2, 4), ROIs might trigger the nuclear translocation of this redox-acting molecule, TRX. Indeed, pretreatment with an antioxidant, N-acetyl-cysteine suppressed TNFα-elicited TRX translocation. Although TRX has no authentic nuclear localization signal sequence and details of the translocation mechanism are under investigation, mutants of TRX, TRX-C62S/C69C/C73S and TRX-(C62S/C69S/C73S), can locate in the nucleus (data not shown). These data suggest that the redox status of TRX itself is not the primary determinant of the translocation. Recently we (52) and others (53) identified some proteins as TRX-binding proteins. Regulation of association between TRX and these proteins may play an important role in determining intracellular localization of TRX.

According to the multidimensional NMR study by Qin et al. (44, 45), the enzymatically active center of TRX consists of a “groove,” and the oligopeptide from DNA-binding loop of NF-κB p50 fits the groove. As shown in Fig. 4, we demonstrated that TRX could directly associate NF-κB p50 DNA-binding domain through its cysteines in the catalytic domain in vitro. Structural analysis using NMR technique and our in vitro experiment, using whole recombinant molecules, demonstrates that TRX interplays directly with NF-κB. Thus, the specificity of the molecular reaction between TRX and NF-κB is an important issue to be discussed. GSH is the most abundant small nonprotein thiol concentrated in the nucleus of mammalian cells. However, in electrophoretic morbidity shift assay, TRX as a reducing molecule of NF-κB cannot be replaced by the glutathione system containing GSH, GSH reductase, and NADPH.3 Thus, there seems to be distinctions in the interaction between transcription factors and reducing enzymes; NF-κB is a direct target of the TRX system but not of the GSH system. Redox factor-1 (Ref-1), which is known as a nuclear protein with augmenting DNA binding activity of AP-1 and recently shown by us (8) as one of the target proteins of TRX, also has enhancing activity of binding between NF-κB and DNA (54). At this moment, we cannot neglect a possibility that TRX acts on NF-κB function via other nuclear proteins such as Ref-1 and nucleoredoxin (55).

We can consider NF-κB transcriptional activation as two distinct steps with respect to the cellular compartment. One step is IκBα degradation in the cytoplasm and following nuclear translocation of the complex. Another step is DNA binding of the complex and transactivation in the nucleus. Fig. 6 shows that overexpression of wild-type TRX, which has catalase-like peroxide activity (56) and is also an electron donor to TRX-dependent peroxide family (57, 58), in the cytoplasm suppressed transcription by NF-κB system by scavenging ROI as a second messenger of UVB and TNFα treatment. Indeed, as shown in Fig. 7C, overexpression of TRX-wt suppressed degradation of IκBα elicited by TNFα. Moreover, knock-down treatment of endogenous TRX system using antisense plasmid against human TRX and DNCB augmented IκBα degradation, whereas this treatment suppressed κB-dependent transcription. These

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1. TRX remains in the cytoplasm, quickly translocates in the nucleus after UV irradiation (Fig. 1B), PMA (Fig. 3A), or TNFα treatment (Fig. 3B). As this phenomenon can be observed in HSC-1 cells from human skin cancer, HeLa cells from human cervical cancer, and NIH3T3 cells from mouse fibroblast, nuclear translocation of TRX seems to be a common phenomenon at least in adhesive cell types. In addition, we reported that in mouse Fe-nitrotetracetic acid-induced renal tubular damage models, TRX translocated into the nuclear compartment in response to generation of ROI by Fe-nitrotetracetic acid (51), and also in HeLa cells H$_2$O$_2$ treatment induced TRX translocation (7). As UV irradiation and TNFα and PMA treatment are shown to generate ROIs in the cytoplasm (1, 2, 4), ROIs might trigger the nuclear translocation of this redox-acting molecule, TRX. Indeed, pretreatment with an antioxidant, N-acetyl-cysteine suppressed TNFα-elicited TRX translocation. Although TRX has no authentic nuclear localization signal sequence and details of the translocation mechanism are under investigation, mutants of TRX, TRX-C62S/C69C/C73S and TRX-(C62S/C69S/C73S), can locate in the nucleus (data not shown). These data suggest that the redox status of TRX itself is not the primary determinant of the translocation. Recently we (52) and others (53) identified some proteins as TRX-binding proteins. Regulation of association between TRX and these proteins may play an important role in determining intracellular localization of TRX.

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2. K. Hirota, unpublished observations.

3. T. Kawabe and J. Yodoi, unpublished observations.
evidences suggest that signaling pathway to IkB degradation or phosphorylation are redox-sensitive. It is very interesting to know which molecule(s) is (are) redox-sensitive, and we have data suggesting that TRX or N-acetyl-l-cysteine-sensitive step is downstream of TNF receptor associated factors and upstream of NF-kB-inducing kinase. Thus, redox regulation of NF-kB transcription by TRX has two distinct components. One is cytoplasmic and the other is nuclear. If ROIs are generated and act on the NF-kB system, ROIs might serve as part of messengers mediating the release of the inhibitory subunit IkB from NF-kB. Under such oxidizing conditions, reactive SH groups on NF-kB should be oxidized. NF-kB or Rel family proteins share a characteristic sequence motif with a cysteine and three arginine residues in the DNA-binding loop, and these cysteines are susceptible to oxidation (59–61). Droge et al. (62) showed that oxidized glutathione is important in the NF-kB activation signal cascade, and elevated oxidized glutathione easily modulates the cysteine(s) of NF-kB in vivo. Thus, the NF-kB complex liberated from IkB may not be fully activated. It is necessary for oxidized cysteine(s) of NF-kB to be reduced for DNA binding.

We previously reported that various oxidative stresses including UVB irradiation markedly induce TRX expression in cells (63–66). In addition, we have data that TNFα treatment also induced TRX in HeLa cells (data not shown). Induction of TRX can be observed no later than 6 h after oxidative stress. Induced TRX protein, which may modulate redox state in the cytoplasm, could work as an effector of negative feedback of NF-kB transcription. In contrast to the induction, nuclear translocation of TRX is detected within 1 h of cell response to stress. At the early phase of UV response, therefore, endogenously expressed TRX could play an important role as a positive regulator of NF-kB as well as AP-1.

In this paper, we showed that human TRX translocates into the nucleus from the cytoplasm quickly in response to oxidative stress such as UV irradiation and TNFα treatment. Thus, nuclear accumulation of TRX might be an important process in the function of certain transcription factors. In addition to the alteration of expression levels induced by different kinds of cellular stress, differential subcellular localization in response to the extracellular stimuli may constitute a mechanism of the pleiotropic action of TRX in cells. Recently, it was reported that E330, a quinone derivative, selectively inhibited NF-kB-mediated gene expression by suppressing NF-kB DNA binding activity in the nucleus, without affecting any degradation of IkBα and translocation of NF-kB into the nucleus (67). This evidence strongly suggests the existence of distinct regulation of NF-kB function in the cytoplasm and in the nucleus.

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