Thioredoxin-dependent Redox Regulation of p53-mediated p21 Activation*

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Thioredoxin (TRX) is a dithiol-reducing enzyme that is induced by various oxidative stresses. TRX regulates the activity of DNA-binding proteins, including Jun/Fos and nuclear factor-κB. TRX also interacts with an intranuclear reducing molecule, redox factor 1 (Ref-1), which enhances the activity of Jun/Fos. Here, we have investigated the role of TRX in the regulation of p53 activity. Electrophoretic mobility shift assay showed that TRX augmented the DNA binding activity of p53 and also further potentiated Ref-1-enhanced p53 activity. Luciferase assay revealed that transfection of TRX enhanced p53-dependent expression of p21 and further intensified Ref-1-mediated p53 activation. Furthermore, Western blot analysis revealed that p53-dependent induction of p21 protein was also facilitated by transfection with TRX. Overexpression of dominant negative mutant TRX (mTRX) suppressed the effects of TRX or Ref-1, showing a functional interaction between TRX and Ref-1. cis-Diaminedichloroplatinum (II) (CDDP) induced p53 activation and p21 transactivation. The p53-dependent p21 transactivation induced by CDDP was inhibited by mTRX overexpression, suggesting that TRX-dependent redox regulation is physiologically involved in p53 regulation. CDDP also stimulated translocation of TRX from the cytosol into the nucleus. Hence, TRX-dependent redox regulation of p53 activity indicates coupling of the oxidative stress response and p53-dependent repair mechanism.

Thioredoxin (TRX)1 was originally identified in Escherichia coli and has become known as a dithiol hydrogen donor for a variety of target proteins, including ribonucleotide reductase, an enzyme essential for DNA synthesis. TRX has a highly conserved amino acid sequence, Trp-Cys-Gly-Pro-Cys-Lys, at its active site. The two cysteine residues at the active site, Cys-32 and Cys-35, undergo reversible oxidation-reduction reactions catalyzed by a NADPH-dependent enzyme thioredoxin reductase. TRX and glutathione constitute the major cellular reducing system (1). Human TRX was cloned as an adult T cell leukemia-derived factor, produced by human T cell leukemia virus I-transformed T cells (2). TRX is induced by various kinds of stress, such as viral infection, and secreted from the cells (3, 4). Recently, TRX-dependent peroxidase (peroxiredoxin) families have been cloned (5). TRX is important in cytoprotection against oxidative stresses (6, 7). TRX is induced by various kinds of oxidative stress, including UV, x-ray irradiation, or CDDP treatment. TRX modulates the DNA binding activity of transcription factors, including nuclear factor-κB, polyoma virus enhancer-binding protein 2, glucocorticoid and estrogen receptors, and hypoxia-inducing factor 1-α (8–12).

The tumor suppressor protein p53 is induced by various kinds of oxidative stress. Its overexpression arrests cell cycle progression in the G1 phase and suppresses cell proliferation after p21 induction (13, 14). p53 exerts its tumor suppressor effect by controlling the expression of cell cycle-related genes after DNA damage (15, 16). p53 is induced by oxidative stresses, such as x-ray or UV irradiation or CDDP treatment. Enhanced phosphorylation of p53 by ataxia-telangiectasia gene product or DNA-dependent protein kinase appears to be an important regulatory mechanism of p53 in response to DNA damage (17–19). Besides the mechanism, involvement of redox regulation in the p53 gene has been reported. The site-specific DNA binding activity of p53 is dependent upon its highly conserved central DNA binding domain that contains a zinc ion (20–22) and cysteine residues (23). Mutation of these cysteine residues in murine p53 markedly decreased sequence-specific DNA binding activity in vitro (24). Agents such as dithiothreitol (DTT) and metal chelators modulate the DNA binding capacity of p53 (25).

Recently, redox factor 1 (Ref-1) was purified as an activator of p53 and was shown to transactivate p53 in vivo (26). Ref-1 was originally identified as a DNA repair enzyme exhibiting apurinic/apyrimidinic endonuclease activity (27, 28). Ref-1 is a ubiquitous nuclear protein and purified as an activator of activator protein-1 (AP-1) that is a heterodimer of Fos/Jun. Ref-1 activates AP-1 DNA binding activity by reducing cysteine residues in Fos and Jun (29, 30). TRX activates the AP-1 Fos/Jun (heterodimer) through the nuclear redox protein Ref-1 (31). In addition, we previously reported that TRX is able to interact physically with Ref-1 (31). These findings raise the possibility that TRX may, directly or indirectly, regulate p53 activity. Thus, in this study, we tried to elucidate the role of TRX, an endogenous reducing agent, in p53 activity.

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† The abbreviations used are: TRX, thioredoxin; mTRX, mutant TRX; Ref-1, redox factor 1; CDDP, cis-diaminedichloroplatinum (II); AP-1, activator protein-1; DTT, dithiothreitol; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline.
FIG. 1. Influence of redox agents and factors on the sequence-specific DNA binding of p53. A, effects of redox modulating agents, such as DTT or diamide, on the DNA binding activity of wild-type p53. 600 ng of recombinant wild-type p53 (rP53) was added to the reaction (except lane 1, control), in the absence (lane 2) or presence of DTT (lane 3, 10 mM; lanes 4 and 6–9, 50 mM) or with diamide (lane 5, 1 mM) for 20 min on ice and then analyzed by the EMSA as described under "Experimental Procedures." A 100-fold excess of wild-type oligonucleotide encompassing the p53-responsive element (wt) (lane 6) or mutated oligonucleotides (m) (lane 7), 300 ng of anti-p53 antibody (DO-1) (lane 8), or 300 ng of MOPC21 used as a control antibody (cont.) (lane 9) were used for monitoring p53 specificity.

B, effects of TRX and Ref-1 on the DNA binding activity of wild-type p53. 600 ng of recombinant wild-type p53 (rP53) was incubated with 0.01 or 0.1 mM recombinant TRX in the presence of TRX reductase and NADPH for 20 min on ice. Recombinant p53 without treatment (lane 2) or with treatment was subjected to the EMSA. In lanes 5 and 6, 0.01 and 0.1 mM recombinant Ref-1, respectively, was added to the reaction mixture. Anti-p53 antibody (300 ng of DO-1) (lane 7) or MOPC21 (300 ng) (lane 8) used as a control antibody (cont.) (lane 8) were used for monitoring specificity in the presence of 50 mM DTT.

C, effects of TRX and Ref-1 on the DNA binding activity of wild-type p53. Recombinant wild-type p53 (rP53) was incubated with 0.1 or 1 mM recombinant TRX in the presence of TRX reductase and NADPH for 20 min on ice. rP53 was incubated with 0.1 mM (lane 3) or 1 mM (lane 6) recombinant TRX in the presence of TRX reductase and NADPH for 20 min on ice. rP53 was incubated with 0.1 mM (lane 4) or 1 mM (lane 7) recombinant Ref-1 for 20 min on ice. rP53 without treatment (lane 2) and with treatment (lanes 3–10) were subjected to the EMSA. Anti-p53 antibody (300 ng of DO-1) (lane 9) or MOPC21 (300 ng) (lane 10) as a control antibody (cont.) was used for monitoring specificity in the presence of 50 mM DTT.
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EXPERIMENTAL PROCEDURES

**Cell Lines and Culture**—Human WiDr colon cancer cells were kindly provided by Dr Takahashi and maintained in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (FBS) under 5% CO₂ at 37 °C. MG63 human osteosarcoma cells (obtained from RIKEN cell bank, Tsukuba, Japan) and HeLa human cervical carcinoma cells (obtained from RIKEN cell bank, Tokyo, Japan), MCF-7 cells (obtained from the cell bank) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% FBS at 5% CO₂ in air at 37 °C. p53 was mutated in WiDr cells and deleted in MG63 cells.

**Oligonucleotides and Electrophoretic Mobility Shift Assay (EMSA)**—Aliquots of recombinant p53 (p53; residues 1–393) glutathione S-transferase fusion protein purchased from Santa Cruz Biotechnology (Santa-Cruz, CA) were incubated in 10-fold diluted DNA-binding buffer (200 μM Tris-Cl, pH 7.9, 1 mM EDTA, 10% glycerol, 1 μg of poly(dI-dC), 100 mM KCl, 150 mg/ml bovine serum albumin). Samples were incubated with 32P end-labeled double stranded oligonucleotide 5'-TACGAGCATGTCTAAGCATGGGG-3' (wild-type) for 20 min at 25 °C. Reaction products were analyzed by electrophoresis at 200 V onto 4% polyacrylamide gels containing 0.25% Tris borate-EDTA buffer at 4 °C for 3.5 h. DNA-protein complexes were identified by autoradiography. The changes in the p53 DNA binding activity were quantitated and analyzed by a densitometer with the National Institutes of Health Image program (Research Service Branch, National Institutes of Health). The specificity of p53 in the assay was tested by adding 100-fold excess of either wild-type or mutant competitors (5'-TACGAGCATGTCTAAGCATGGGG-3'), as well as the supershift caused by the addition of two times of the anti-p53 antibody (DO-1, Santa Cruz Biotechnology). Recombinant TRX was provided by Ajinomoto Co. Ltd (Kawasaki, Japan). Recombinant Ref-1 was prepared as described previously (9).

**Transfection and Luciferase Assay**—WiDr cells were seeded at 7 × 10⁵ cells per 60-mm dish prior to transfection. In each transfection, the pcDNA3-wild-type TRX expression plasmid, pcDNA3-mutant type TRX expression plasmid (mTRX, C32S/C35S), which lacks reducing activity, with pcCMV-Ref-1 expression vector (Ref-1) was co-transfected with 1.0 μg of wild-type p53 (WT P53) or mutant-type p53 (MUT P53) expression plasmids together with 2.5 μg of either the reporter construct (WT P21 LUC) or mutant construct (MUT P21 LUC), which lacked the p53 binding site. The total amount of DNA was normalized to equivalent amounts by control parental plasmids (pcDNA3). Luciferase activities, which were normalized by assaying Renilla luciferase activity, were determined at 20 h. Fold activation represents the mean of triplicate samples. The results are means ± S.D. of triplicate wells. Effects of transient overexpression of wild-type TRX on p53-dependent transactivation of wild-type p21 promoter in WiDr cells. pcDNA3-wild-type TRX (TRX) (2.0 μg) was co-transfected with wild-type p53 (WT P53) and wild-type p21 luciferase construct (WT P21 LUC). These results are representative of three independent experiments.

**Immunofluorescence Cell Staining**—HeLa cells were seeded prior to staining at 2.5 × 10⁴ cells/well in culture slides (Falcon). The cells were
tated oligonucleotide (Fig. 1). They were competed with an excess of wild-type oligonucleotide encompassing the p53 binding site but not with excess of mutant oligonucleotide. The total amount of DNA was normalized to equivalent amounts by control parental plasmids (pcDNA3). p21 expression was determined by Western blot analysis. The top panel shows \( \beta \)-actin expression analyzed by reprobing the same membrane with anti-\( \beta \)-actin monoclonal antibody.

RESULTS

Recombinant TRX and/or Ref-1 Enhance Sequence-specific DNA Binding of p53—We first tested the influence of DTT (an SH-dependent chemical reducing agent) or diamide (a chemical oxidizing agent) on p53-specific DNA binding activity, using an EMSA. Pretreatment of recombinant p53 (rP53) with 50 mM DTT resulted in an increase in DNA binding activity (Fig. 1A, compare lanes 2–4). These bound bands were specific, because they were supershifted upon the addition of the human monoclonal antibody against p53 (DO-1; Fig. 1A, lane 8). In addition, they were competed with an excess of wild-type oligonucleotide encompassing the p53 DNA binding site but not with an excess of mutated oligonucleotide (Fig. 1A, lanes 6 and 7). Densitometrical analysis demonstrated that the relative intensities of the specific bands of lanes 2–7 in Fig. 1A are 1, 1.2, 12, 0, 2.5, and 11, respectively. These results show that the binding activity of p53 depends on the redox condition and that a reducing environment is essential for the formation of p53-DNA complexes.

TRX is an endogenous thiol-reducing molecule that modulates the DNA binding of various transcriptional factors. Therefore, we next examined the effect of recombinant human TRX on the wild-type p53 binding activity using EMSA. We compared the binding activity of p53 in the absence and presence of recombinant human TRX. As seen in Fig. 1B (compare lanes 2–4), recombinant TRX significantly enhanced the sequence-specific DNA binding activity of wild-type p53 in the presence of TRX reductase and NADPH. The effect of TRX was dose-dependent and was visible at a concentration as low as 0.01 \( \mu \)M. The effect of 1 \( \mu \)M recombinant TRX was equivalent to that of 50 mM DTT in enhancing the activity. Ref-1 also significantly enhanced the DNA binding (Fig. 1B, compare lanes 2, 5, and 6), as reported previously (26). Densitometrical analysis showed that the relative intensities of the specific bands of lanes 2–6 in Fig. 1B are 1, 3.4, 5.5, 3.1, and 4.9, respectively. The effect of Ref-1 was seen at a concentration as low as 0.01 \( \mu \)M and reached a plateau level at 0.1 \( \mu \)M (Fig. 1C, lanes 4 and 7). In addition to this result, TRX significantly enhanced Ref-1-mediated p53 binding activity (i.e., Ref-1-mediated p53 binding activity was enhanced by TRX) (Fig. 1C, compare lanes 3–8). TRX (0.1 \( \mu \)M) was capable of enhancing Ref-1-mediated p53 binding activity to a greater extent than that of 0.1 \( \mu \)M Ref-1 alone. Densitometrical analysis showed that the intensities of the specific bands of lanes 2–8 in Fig. 1C are 1, 5.8, 4.9, 11.9, 6.5, 5.2, and 20, respectively. These bound complexes were specific as they were supershifted with a p53-specific antibody (DO-1) (Fig. 1C, lane 9).

Transient Expression of TRX or Ref-1 Activates the Function of Wild-type p53—We next examined whether TRX and/or Ref-1 could enhance the transcriptional activity of p53 in vivo. The expression vector pcDNA3/CMV-wild-type p53 transactivated the reporter gene that contained the wild-type p53-responsive element of the p21 gene but not the reporter gene that lacked the wild-type p53-responsive element. In contrast, the pcDNA3/CMV-mutant-type p53 was unable to transactivate the wild-type p21 luciferase reporter gene (Fig. 2A). Using this p53-dependent reporter assay for the p21 promoter, we tested the effect of TRX and/or Ref-1 on the transcriptional activity of p53. The reporter gene including the p21 promoter was transfected in colon cancer cells (WiDr cells harboring mutant-type p53) or osteosarcoma cells (MG63 cells lacking wild-type p53). 5.8-fold induction of luciferase activity was observed by overexpression of 1 \( \mu \)g of wild-type TRX (Fig. 2B) compared with the control. 7.6-fold induction was observed by overexpression of 1 \( \mu \)g of Ref-1 (Fig. 2C). In MG63 cells, the same enhancing effect by TRX or Ref-1 was observed (data not shown). Neither TRX nor Ref-1 affected the basal transcriptional activity (data not shown).

Effect of TRX on the Expression of p21 Induced by p53—We then performed Western blot analysis to test whether TRX or Ref-1 overexpression can modulate the expression level of p21. Transfection of either wild-type TRX (Fig. 3, lane 5) or Ref-1 (Fig. 3, lane 6) with wild-type p53 increased the p21 expression more than that of the control vector (Fig. 3, lane 3). TRX alone or Ref-1 alone did not significantly change p21 expression level (compare lanes 1–3). The amount of \( \beta \)-actin in each lysate was almost equivalent. These results suggest that either TRX or Ref-1 augments p53-dependent p21 expression in vivo.

Transient Co-expression of TRX and Ref-1 Activates the Function of Wild-type p53—We then tested the enhancing effect when TRX and Ref-1 were co-transfected. When subopti-
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Mutant TRX Suppresses the Up-regulated Function of Wild-type p53 by TRX or Ref-1—Because TRX and Ref-1 are reported to interact functionally and physiologically in the AP-1 system (29, 31), we further analyzed the interaction of TRX and Ref-1 in p53 activation. As mutant TRX in which active site cysteines are replaced by serine residues (TRX (Cys-32/Cys-35)) competitively inhibits thioredoxin reductase activity (35), we examined the effect of TRX (C32S/C35S) overexpression on the p53 activation. Transfection of a mutant TRX (C32S/C35S) expression vector repressed the effect of TRX in p53 activation (Fig. 5). Upon co-transfection of this expression vector, the p53 activation by Ref-1 was significantly suppressed, whereas basal transcriptional activity was unchanged by the transfection of mutant TRX (C32S/C35S) (Fig. 5B). These data suggest that at least part of the Ref-1 mediation of p53 activation is modulated by the redox activity of TRX.

We next examined whether the redox regulation by TRX is involved in physiological p53 function. p53 transactivates p21 promoter upon oxidative stress, such as treatment with CDDP. We tested the effect of mutant TRX (C32S/C35S) overexpression in the transactivation of the p21 promoter by CDDP treatment in MCF-7 cells harboring wild-type p53. The p21 promoter activity in transfectants with a mutant TRX (C32S/C35S) expression vector was suppressed to 50%, compared with that in transfectants with control vector (Fig. 6). The result indicates a role of TRX-dependent redox regulation in p53 activity.

Nuclear Translocation of TRX by CDDP—TRX is known to exist in cytoplasm without oxidative stress. On the other hand, p53 translocates from cytoplasm to nucleus upon oxidative stress for example treatment with UV or CDDP. We studied subcellular localization of TRX on CDDP treatment. In the absence of CDDP, TRX mainly remained in cytoplasm. In contrast, after 60 min of incubation with 2 μM CDDP, cells with nuclear staining were seen (Fig. 7). These data suggest that TRX translocates from cytosol to nucleus upon oxidative stress.

DISCUSSION

We have demonstrated here that TRX enhances the sequence-specific DNA binding activity of p53 both directly (when applied alone) or indirectly (by enhancing Ref-1 mediation), and we have provided clear evidence that TRX is an important factor in the regulation of p53 DNA binding activity.

In the EMSA, recombinant TRX or Ref-1 augmented the sequence-specific DNA binding of p53. The effect of TRX and Ref-1 was seen at almost the same concentration. The enhancing effect of TRX on Ref-1-dependent p53 activation was marked in the EMSA. The effect of Ref-1 was reached to a plateau level at a concentration of 0.1 μM. However, the effect was further augmented by the addition of TRX.

In the luciferase assay, our results showed that either TRX or Ref-1 augments p53-dependent p21 expression in vivo. As seen in the EMSA, TRX enhanced Ref-1-mediated p53 activation in WI DR cells harboring mutant p53 or MCF-7 cells lacking p53. These results further indicate a functional coupling between TRX and Ref-1 in the p53 activation system. The interaction between TRX and Ref-1 has also been observed in other
systems. TRX can activate AP-1 DNA binding activity (29), acting as a factor in a redox cascade involving Ref-1. We have previously demonstrated that TRX and Ref-1 enhanced PMA-induced activation of AP-1 (31). The DNA binding activity of transcriptional factor polyoma virus enhancer-binding protein 2 is regulated by the redox mechanism with TRX and Ref-1 (9). The interaction of co-activators with hypoxia-inducing factor 1-α or hypoxia-inducing factor 1-α-like factor has been regulated by TRX/Ref-1 system (12). The overexpression of the mTRX C32S/C35S, which lacks reducing activity, decreased the effect of the transfected Ref-1, as well as TRX, indicating that at least part of the Ref-1 mediation of p53 activation is modulated by the redox activity of TRX. In the presence of a reducing agent, Ref-1 was reported to be a potent stimulator of wild-type p53 (26). Our previous report also showed the direct interaction of Ref-1 and TRX through the cysteine residues of TRX (31). Therefore, besides its direct action on p53, TRX seems to be able to act indirectly on p53 via the redox regulation of Ref-1. Mutant TRX (C23S/C35S) overexpression suppressed the transactivation of the p21 promoter by CDDP treatment in MCF-7 cells, indicating that the redox regulation of TRX plays a role in physiological p53 activity. In a yeast system, the importance of the TRX system in p53 activity has also been reported, in studies in which deletion of the thioredoxin reductase gene inhibited p53-dependent reporter gene expression (36). In addition, we have shown here that TRX translocates from cytosol to nucleus upon treatment with CDDP. The translocation was also induced by oxidative stresses, including UV irradiation (37), or hydrogen peroxide (10). Therefore, it is presumed that TRX translocates to the nuclear compartment upon oxidative stress to interact with Ref-1. It might be possible that TRX is involved in the mechanism of translocation of p53 upon oxidative stress. Both the direct interaction between p53 and TRX and the mechanism of the dynamic regulation of Ref-1 by TRX in the nuclear compartment require further analysis.

Higher levels of TRX are detected in cancerous tissues, such as adult T cell leukemia, hepatocellular carcinomas, and cervical neoplastic squamous epithelial cells of the uterus (38–40). Several groups analyzed p53 status in adult T cell leukemia patients and human T cell leukemia virus I-infected cells. Inhibition of p53 transactivation function by the human T-cell lymphotropic virus type 1 Tax protein was reported (41). On the other hand, the mutations of p53 are frequent in cancer tissues. Therefore, this abnormal expression of TRX might be explained as a compensation mechanism for p53 dysfunction. Mutation of the cysteine residues in p53 could function as an escape mechanism from redox regulation. This possibility should be further investigated in the cancerous predisposition or cancerous tissues.

In this study, p53-dependent p21 expression was up-regulated by the TRX-Ref-1 cascade. p53 is thought to be a gatekeeper against DNA damage, and it induces G1 arrest to afford cells time to repair damaged DNA (15, 16). TRX is induced and translocated into cell upon oxidative stress. Thus, our results suggest that TRX interacts with p53 in response to oxidative stress and plays a role in stimulating p53 function.

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