c-Jun NH2-terminal Kinase-mediated Redox-dependent Degradation of IκB

ROLE OF THIOREDOXIN IN NF-κB ACTIVATION*

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NF-κB is a redox-sensitive transcription factor known to be activated by oxidative stress as well as chemical and biological reductants. Its DNA binding activity requires reduced cysteines present in the p65 subunit of the dimer. Thioredoxin (Trx) is an endogenous disulfide oxidoreductase known to modulate several redox-dependent functions in the cell. NF-κB was activated by addition of *Escherichia coli* thioredoxin in a redox-dependent manner in A549 cells. Such activation was accompanied by degradation of IκB in the cytosol. In addition, only the reduced form of thioredoxin activated NF-κB, whereas the oxidized form was without any effect. Overexpression of human thioredoxin also caused activation of NF-κB and degradation of IκB. On the contrary, dominant-negative redox inactive mutant thioredoxin expression did not activate NF-κB, further confirming the redox-dependent activation of NF-κB. We also investigated the mechanism of activation of NF-κB by thioredoxin. We demonstrate that thioredoxin activates c-Jun NH2-terminal kinase (JNK)-signaling cascade, and dominant-negative expression of mitogen-activated protein kinase kinase kinase 1 (MEKK1), JNK kinase, or JNK inhibits NF-κB activation by thioredoxin. In contrast, wild-type MEKK1 or JNK kinase induced NF-κB activation alone or in combination with thioredoxin expression plasmid. These findings were also confirmed by NF-κB-dependent luciferase reporter gene transcription.

Nuclear transcription factor κB (NF-κB) is a multi-subunit factor that can rapidly activate the expression of genes involved in inflammatory, immune, and acute phase responses (1). Although multiple forms can exist, the principal active form appears to be a heterodimer consisting of 50- and 65-kDa subunits (2). The heterodimer remains bound to an inhibitor protein IκB in the cytoplasm. In response to a variety of stimuli, IκB is phosphorylated and ubiquitinated, followed by degradation by the 26 S proteasome (3). This process exposes the nuclear localization signal, allowing the heterodimeric complex to interact with the nuclear transport machinery and to translocate to the nucleus (2). A characteristic of NF-κB is that many different agents can induce its DNA binding activity (2). Oxidants (4) as well as reductants (5–7) are known to activate NF-κB. Although redox-dependent activation of NF-κB is widely recognized, little is known about how cellular redox status could modulate the signaling events that are associated with the activation of NF-κB.

Thioredoxin (Trx) is a potent protein disulfide reductase that catalyzes protein reduction using reducing equivalents from NADPH in conjunction with thioredoxin reductase (TR). Remarkably low concentrations of thioredoxin are effective in reducing disulfides in insulin, fibrinogen, human chorionic gonadotropin, nitric-oxide synthase, ribonucleotide reductase, glucocorticoid receptors, and other proteins (8–11). The rate of reduction of insulin disulfide by thioredoxin was found to be 10,000 times higher than that by dithiothreitol (8). Thus, reduced thioredoxin is an extremely potent protein disulfide reductase. Intracellurally, most of this ubiquitous low molecular mass (12 kDa) protein remains reduced (12–13). Thioredoxin has two critical cysteine residues at the active site, which in the oxidized protein, form a disulfide bridge located in a protrusion from the three-dimensional structure of the protein (8). The flavoprotein thioredoxin reductase catalyzes the NADPH-dependent reduction of this disulfide (8). Small increases in thioredoxin can cause profound changes in sulfhydryl-disulfide redox status in protein (8). Additionally, thioredoxin was shown to restore DNA binding activity of NF-κB in a cell-free system (14). In the same report, it was shown that redox regulation of NF-κB activity appeared to be exerted after dissociation of IκB from the NF-κB complex. Moreover, thioredoxin was shown to form a complex with p50 subunit of NF-κB (15). In addition, other reports have shown that critical cysteine 62 in NF-κB is required to be reduced by thioredoxin for its activation (16). We reported earlier that reducing thiolic can activate NF-κB in intact cells (7). We also showed that sulfhydryl oxidation or alkylation can inhibit tumor necrosis factor-α- or interleukin-1-induced NF-κB activation (17).

Mitogen-activated protein (MAP) kinases are serine/threonine kinases activated by dual phosphorylation on both a tyrosine and a threonine (18). These enzymes are important components of signaling pathways that transduce extracellular stimuli into intracellular responses. There are three major forms of MAP kinases; extracellular signal-regulating kinase, c-Jun-NH2-terminal Kinase (JNK) or stress-activated protein kinase), and p38 MAP kinase. Extracellular signal-regulating kinase pathway is activated by growth factors and phorbol esters (18). JNK/stress-activated protein kinase pathway is activated in response to cellular stresses such as heat shock, UV irradiation, or inflammatory cytokines (19). Inflammatory...
cysteines as well as environmental stresses such as osmotic shock activate p38 MAP kinase (20).

The JNK-signaling cascade functions through the activation of an initiating kinase such as MAP kinase kinase kinase (MEKK1), which in turn phosphorylates the MAP kinase kinase (MKK4/SEK1), and MKK4 finally activates the JNK by phosphorylating the serine and threonine residues on it (reviewed in Ref. 19). Although the signal transduction cascade leading to the activation of JNK is relatively well defined, the steps leading to the phosphorylation of IκBα are poorly understood. Recent studies demonstrate that IκBα can be phosphorylated by MEKK1, an upstream kinase of the JNK pathway (21). Moreover, many of the stimuli that induce NF-κB activation, such as tumor necrosis factor-α, UV radiation, and lipopolysaccharide, also activate the JNK signaling cascade. Since phosphorylation of, IκBα is required for its degradation and Trx can activate NF-κB in intact cells, we hypothesized a potential role of JNK in Trx-mediated IκBα degradation and activation of NF-κB.

In this report, we demonstrate that thioredoxin activates NF-κB and causes degradation of IκBα. Additionally, we have shown that MEKK1 is the initiating kinase of the JNK pathway that mediates the NF-κB activation by thioredoxin. Moreover, we also demonstrate that JNK subgroup of MAP kinases is activated by redox-active thioredoxin. Furthermore, we have also shown that thioredoxin can induce NF-κB-dependent reporter gene expression, and such transcription can be abrogated by inhibition of JNK-signaling intermediates using dominant-negative constructs.

**EXPERIMENTAL PROCEDURES**

**Materials—Escherichia coli** thioredoxin was obtained from Promega Corp. (Madison, WI). Thioredoxin reductase and human thioredoxin were obtained from American Diagnostica, Inc. (Greenwich, CT). Anti-p50, anti-p65, pJNK, JNK, and anti-IκB antibodies were obtained from American Diagnostica, Inc. (Greenwich, CT). Anti-p50, anti-p65, pJNK, JNK, and anti-IκB antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other materials were obtained in the highest available grade. A549 cells (adenocarcinoma cells) were obtained from ATCC. Lactacystin was obtained from Sigma. Human thioredoxin reductase and human thioredoxin were obtained in the highest available grade. A549 cells (adenocarcinoma cells) were obtained from ATCC. Lactacystin was obtained from Sigma.

**Cell Culture and Transfections—**A549 cells were cultured in Kaigan’s modified F-12K medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 100 units of penicillin/streptomycin. Confluent monolayers were treated with various concentrations of thioredoxin for different time periods as indicated in the figure legends. Cell viability was determined by the trypsin blue exclusion method. Human pulmonary artery endothelial cells were obtained from Clonetics Corp. and propagated in endothelial growth medium (Clonetics, CA). MEKK1 (pCMV-SV40-MEKK1) was a gift from the cell-line division of Dr. Tom Maniatis (Harvard University, Boston) and have been described (21). The dominant-negative JNKK expression plasmid (pRAdnJNKK) and dominant-negative JNK (pSRA-AFP/JNK) expression plasmid was generous gifts of Dr. Gary L. Johnson (National Jewish Medical Center, Denver, CO) and have been described (22). Transfection of various expression plasmids into A549 cells was carried out using Transfectam reagent (Promega) or Geneporter reagent (Gene Therapy Systems Inc. San Diego, CA) as per manufacturer’s protocol.

**Site-directed Mutagenesis—**Site-directed mutagenesis of the redox-active Cys-32 and Cys-35 to serine was performed by a synthesized oligonucleotide (5’TACATTTTGGAGGCCAGACAGCAAGTGGCC-3’) and quick-change mutagenesis kit (Stratagene, La Jolla, CA) as per manufacturer’s protocol. Briefly, double-stranded mutagenic oligonucleotide was synthesized (Genosys) and purified by polyacrylamide gel electrophoresis. Mutagenic oligonucleotide was added to the double-stranded plasmid pcDNA3-Trx. The plasmid was denatured, and the mutagenic oligonucleotide was annealed by temperature cycling using Pfu Turbo DNA polymerase. After temperature cycling, the methylenated nonmutated parental template DNA was digested with DpnI. XL1-Blue supercompetent cells (Stratagene) were transformed with plasmid. Base substitution in the mutagenic thioredoxin open reading frame was verified by sequencing (University of Texas Medical Branch, Galveston, TX). The mutagenic plasmid (pcDNA3-dnTrx) was amplified for transfection experiments.

**Nuclear Extract Preparation—**Nuclear extracts were prepared as described previously (23). Briefly, 10° cells were washed in 10 ml of phosphate-buffered saline and centrifuged (1,500 × g for 5 min). The pellet was resuspended in phosphate-buffered saline (1 ml), transferred into an Eppendorf tube, and centrifuged again (16,000 × g; 15 s). Phosphate-buffered saline was removed, and the cell pellet was resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 2 μM diithiothreitol, 0.1 μM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol), and the tube was rocked gently at 4 °C for 30 min on a shaking platform. The nuclear extract was centrifuged for 10 min in a microcentrifuge at 4 °C, and the supernatant was frozen at −70 °C in aliquots until the electrophoretic mobility shift assay (EMSA) was performed. Protein was quantified by Bradford protein assay (Bio-Rad; Ref. 24).

**Electrophoretic Mobility Shift Assay—**For the EMSA, the NF-κB specific oligonucleotide was obtained from Promega Corp. Oligonucleotide was end-labeled using T4 polynucleotide kinase (Promega) and [γ-32P]ATP (NEN) in 10× kinase buffer (0.5 mM Tris-HCl, pH 7.5, 0.1 mM MgCl2, 50 mM diithiothreitol, 1 mM spermine, and 1 mM EDTA). For competition studies, 3.5 pmol of unlabeled oligonucleotide was used. Nuclear extract without labeled oligonucleotide was preincubated for 15 min at 4 °C followed by a 20-min incubation at room temperature followed by the addition of labeled oligonucleotide. The binding reaction contained 10 μg of sample protein, 5 μl of 5× incubation buffer (20% glycerol, 5 mM MgCl2, 5 mM EDTA, 5 mM diithiothreitol, 500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.4 mg/ml calf thymus DNA). In some of the binding reactions poly(dI-dC) (Amersham Pharmacia Biotech) was added to a final concentration of 2 μg. The nuclear protein-32P-oligonucleotide complex was separated from free 32P-labeled oligonucleotide by electrophoresis through a 6% native polyacrylamide gel in a running buffer of 0.25× TBE (5× TBE = 500 mM Tris, pH 8.0, 450 mM borate, 5 mM EDTA).

**Supershift Assay—**For the supershift assay, some of the binding reactions contained 200 ng of anti-p50 or anti-p65 antibody (Santa Cruz) along with 2 μg of poly(dI-dC) (Amersham Pharmacia Biotech).

**Western Blotting of IκB—**Post-nuclear supernatant was treated as the cytosolic extract and quantified with Bradford assay (Bio-Rad, Ref. 24). Equal amounts of protein were resolved on a 10% or 12% SDS-polyacrylamide gel electrophoresis. After electrophoresis, protein was transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech) or polyvinylidene difluoride membrane (Bio-Rad), immunoblotted with anti-IκB (Santa Cruz), and visualized by the ECL system (Amersham Pharmacia Biotech) using anti-rabbit-HRP IgG (Santa Cruz).

**JNK Activity Assay—**The activity of JNK was assayed by a nonradioactive assay kit as per the manufacturer’s protocol (New England Biolabs, Beverly, MA). Briefly, stress-activated protein kinase/IκB kinase was precipitated from the cell lysate and c-Jun fusion protein was fused glutathione-Sepharose beads. c-Jun contains a high affinity binding site for stress-activated protein kinase/IκB, NH2-terminal to the two phosphorylation sites, Ser-63 and Ser-73. After selectively pull-down JNK using c-Jun fusion protein beads, the beads were extensively washed, and the kinase reaction was carried out in the presence of cold ATP in a final volume of 25 μl. The reaction was stopped with 25 μl of 2× SDS sample buffer and loaded onto a 10% polyacrylamide gel. Protein was transferred to nitrocellulose by electroblotting, and c-Jun phosphorylation was selectively measured using phospho-c-Jun antibodies (New England Biolabs, Beverly, MA) as per manufacturer’s protocol (New England Biolabs).
RESULTS

Redox-dependent Activation of NF-κB by E. coli Thioredoxin in A549 Cells—A549 cells were incubated with various amounts of E. coli thioredoxin as shown in Fig. 1A. After incubation, nuclear extract was prepared, and EMSA was performed using a consensus oligonucleotide for NF-κB. As demonstrated in Fig. 1A, the oxidized form of E. coli thioredoxin did not activate NF-κB. On the contrary, incubation of cells with a thioredoxin-reducing system (thioredoxin, TR, and NADPH) activated NF-κB in a time-dependent manner. Maximal activation of NF-κB occurred at about 2 h in A549 cells (Fig. 1A). In the dose-response study, oxidized thioredoxin at a concentration of 1–5 μM did not activate NF-κB. On the other hand, a thioredoxin-reducing system activated NF-κB in a dose-dependent manner (Fig. 1B). Thioredoxin reductase or NADPH individually or in combination did not activate NF-κB, suggesting the activation of NF-κB by reduced Trx.

NF-κB Activated by Thioredoxin Is a p50/p65 Heterodimer—NF-κB complex is formed by p50/p50 heterodimer, cRel/p50 heterodimer, or p50 homodimer. Thus, to determine the type of NF-κB complex that is formed by thioredoxin, we performed gel-supershift assay using anti-p50 or anti-p65 antibodies. As demonstrated in Fig. 2, the use of anti-p50 supershifted the NF-κB band, confirming a p50 subunit in the complex. The NF-κB band was abolished by the use of p65 antibody, indicating that the DNA contact is exclusively a function of p65 subunit. Use of both antibodies lessened the intensity of the band, suggesting inhibition of DNA binding.

Redox-active E. coli Thioredoxin Activates NF-κB in Human Pulmonary Artery Endothelial Cells (HPAEC)—A549 cells are derived from pulmonary epithelial cells and are highly dedifferentiated. Hence, to demonstrate the activation of NF-κB by thioredoxin in primary cell cultures, we used pulmonary artery endothelial cells (HPAEC). HPAEC were cultured as described under “Experimental Procedures.” First lane, untreated A549 cells; second lane, cells exposed to 5 μM Trx-SH; third lane, nuclear extracts incubated with 200 ng of anti-p65; fourth lane, nuclear extracts incubated with 200 ng of anti-p50; fifth lane, nuclear extracts incubated with 200 ng of anti-p65 plus 200 ng of anti-p50; sixth lane, competition reaction with cold NF-κB consensus nucleotide.

Redox-dependent Degradation of IκB by Thioredoxin—Thioredoxin has been shown to activate NF-κB in a cell-free system (14). Activation of NF-κB by thioredoxin was also shown to
occurred after the dissociation of IκB complex form NF-κB. Recent studies have also demonstrated the activation of NF-κB-dependent reporter in MCF-7 cells stably expressing thioredoxin (25). However, the mechanism of such activation has not been elucidated. In this study, we have shown that externally added E. coli thioredoxin could activate NF-κB in intact cells. Thus, to demonstrate whether NF-κB is activated as a consequence of degradation of IκB by thioredoxin redox, we assayed for IκB in the cytoplasmic extracts. As shown in the Fig. 4A, IκB was degraded in the cytoplasm at 2 h when the A549 cells were incubated with a thioredoxin-reducing system (lane 6). Additionally, thioredoxin treatment caused IκB degradation in HPAEC after 3 h (Fig. 4B, lane 7). These time points correlate to the EMSA studies, demonstrating that degradation of IκB and NF-κB DNA binding occurring simultaneously.

**Redox-active Human Thioredoxin Activates NF-κB—**Human thioredoxin is similar to E. coli thioredoxin in its redox function (8). However, the human thioredoxin contains two catalytic cysteines at positions 32 and 35 and three other structural cysteines (8). In addition, human thioredoxin is unable to enter the cell (26) unlike the bacterial thioredoxin. Thus, to investigate whether human thioredoxin is similar to E. coli thioredoxin in causing the activation of NF-κB, we overexpressed human thioredoxin by transfecting an expression vector containing Trx open reading frame (pcDNA3-Trx) as described under “Experimental Procedures.” Overexpression of thioredoxin induced the activation of NF-κB, as demonstrated in Fig. 5A (lane 2). IκB was also degraded in response to Trx overexpression (Fig. 5B, third lane). To further verify the role of redox-active cysteines of thioredoxin in NF-κB activation, we mutated the Cys-32 and Cys-35 by site-directed mutagenesis as described under “Experimental Procedures.” The redox-inactive thioredoxin was produced in a dominant-negative manner when such mutagenic cDNA was cloned to an overexpression vector (dnTrx) and transfected to A549 cells. Transfection of A549 cells with dnTrx did not activate NF-κB (Fig. 5A, third lane) or degrade IκB (Fig. 5B, second lane), confirming that the redox activity of Trx is required for the activation of NF-κB (Fig. 5B) in intact cells.

**Protein Kinase C (PKC) Does Not Mediate IκB Degradation by Thioredoxin—**Thioredoxin has been shown to activate PKC (27). Recent reports also indicate that PKC can mediate NF-κB activation in a variety of cell types (28). Therefore, we hypothesized that PKC may mediate the NF-κB activation by thioredoxin. We incubated cells with specific inhibitors of PKC, calphostin C (29) or GF109203X (30), and then stimulated the cells with E. coli thioredoxin-reducing system. In this experiment, we specifically sought to determine the degradation of IκB by thioredoxin and the inhibition of thioredoxin-mediated phosphorylation of JNK by E. coli Thioredoxin—Since JNK pathway is activated by cytokines or UV radiation, which also activates NF-κB, we hypothesized that the external addition of E. coli thioredoxin may activate JNK, and such activation may prevent the IκB complex from NF-κB. Recent studies have also demonstrated the activation of NF-κB-dependent reporter in MCF-7 cells stably expressing thioredoxin (25). However, the mechanism of such activation has not been elucidated. In this study, we have shown that externally added E. coli thioredoxin could activate NF-κB in intact cells. Thus, to demonstrate whether NF-κB is activated as a consequence of degradation of IκB by thioredoxin redox, we assayed for IκB in the cytoplasmic extracts. As shown in the Fig. 4A, IκB was degraded in the cytoplasm at 2 h when the A549 cells were incubated with a thioredoxin-reducing system (lane 6). Additionally, thioredoxin treatment caused IκB degradation in HPAEC after 3 h (Fig. 4B, lane 7). These time points correlate to the EMSA studies, demonstrating that degradation of IκB and NF-κB DNA binding occurring simultaneously.

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**Fig. 3.** Thioredoxin activates NF-κB in HPAEC. HPAEC were maintained as described under “Experimental Procedures.” HPAEC were exposed to E. coli thioredoxin, both oxidized and the reduced system (with NADPH and TR as described in Fig. 1) for 1 to 4 h. First lane, untreated HPAEC; second through fifth lanes, cells treated with 2 μM E. coli Trx-S2 and incubated for 1–4 h; sixth through ninth lanes, cells treated with E. coli reducing system (2 μM) for 1–4 h.

**Fig. 4.** Degradation of IκB by thioredoxin in A549 cells. A, A549 cells were incubated with 2 μM E. coli oxidized thioredoxin or 2 μM thioredoxin-reducing system for various time periods. Post-nuclear supernatants were subjected to Western blot analysis using anti-IκB antibodies, as described under “Experimental Procedures.” First lane, untreated A549 cells; second through fourth lanes, cells incubated with oxidized thioredoxin for 1, 2, or 4 h; fifth through seventh lanes, cells incubated with thioredoxin reducing system for 1, 2, or 4 h. B, degradation of IκB by thioredoxin in HPAEC. HPAEC were exposed to oxidized or reduced thioredoxin as mentioned in Fig. 1. Post-nuclear supernatants were subjected to Western blot analysis using anti-IκB antibodies, as described under “Experimental Procedures.” First through fourth lanes, Trx-S2, 2 μM; fifth through eighth lanes, Trx-(SH)2, 2 μM. As seen in the seventh and eighth lanes, IκB was degraded by reduced E. coli thioredoxin.

**Fig. 5.** Activation of NF-κB by overexpression of human thioredoxin. A, A549 cells were transfected with either pcDNA3, pcDNA3-Trx, or pcDNA3-dnTrx as described under “Experimental Procedures.” Nuclear extracts were prepared, and EMSA was performed as described under “Experimental Procedures.” First lane, pcDNA3 only; second lane, 4 μg of pcDNA3-Trx; third lane, 4 μg of pcDNA3-dnTrx. B, degradation of IκB by redox-active human thioredoxin. Post-nuclear supernatant of cells treated with various constructs (as described for Fig. 5A) was immunoblotted for IκB as described under “Experimental Procedures.” First lane, cells transfected with pcDNA3 only; second lane, cells transfected with pcDNA3-dnTrx; third lane, cells transfected with pcDNA3-Trx.
mediated NF-κB activation. The cytosolic extracts of *E. coli* thioredoxin-treated cells were subjected to phospho-JNK detection using phospho-specific antibodies from Santa Cruz. As demonstrated in the Fig. 7A (lanes 2–4), oxidized *E. coli* thioredoxin did not activate the JNK; however, the thioredoxin-reducing system activated JNK after 2 h of incubation (lane 6), a time point similar to the activation of NF-κB (Fig. 1A). Although we detected phospho-JNK in thioredoxin-treated cells, there is reason to believe that total JNK protein may increase due to thioredoxin treatment, and the phospho-specific antibody may lose its specificity at higher JNK levels. To investigate these possibilities, we detected total JNK level by using a polyclonal antibody to JNK (Santa Cruz). As demonstrated in Fig. 7B, there was no change in the total JNK levels, confirming the specific JNK phosphorylation in response to thioredoxin treatment. To determine the specificity of JNK activation by thioredoxin, we also determined the activation of extracellular signal-regulating kinase or p38 MAP kinases by thioredoxin using phospho-specific antibodies. We did not detect phosphoextracellular signal-regulating kinase or phospho-p38 in the thioredoxin-treated cells (data not shown). These results suggest that thioredoxin specifically activates JNK-signaling cascade.

**Redox-active Thioredoxin Increases JNK Activity**—Since reduced *E. coli* thioredoxin activated JNK phosphorylation, we sought to determine whether the overexpression of human thioredoxin could also activate JNK. Moreover, since human thioredoxin is not permeable into the cells, overexpression of thioredoxin in the cell is an appropriate method to study the effect of human thioredoxin on JNK activity. We have demonstrated that only the redox-active thioredoxin is able to activate NF-κB. Hence, we also sought to determine the effect of redox-inactive thioredoxin on JNK activity. *A549* cells were transfected with pcDNA3-Trx or pcDNA3-dnTrx for 24 h followed by cell lysis, and JNK activity assay was performed as described under “Experimental Procedures.” Redox-active thioredoxin potently activated JNK, as determined by its ability to phosphorylate c-Jun (Fig. 7C, lane 3). On the other hand, redox-inactive mutant thioredoxin was unable to activate JNK (Fig. 7C, lane 4). Thus, the data suggest a redox-control of JNK activation by thioredoxin.

**dnJNKK or dnJNK Inhibit Thioredoxin-induced NF-κB Activation**—If JNK activation is responsible for NF-κB activation by thioredoxin, then blocking the JNK activation by dominant-negative expression of JNK or dominant-negative expression of JNK kinase should inhibit NF-κB activation by thioredoxin. Therefore, to delineate the role of the JNK-signaling pathway in NF-κB activation by thioredoxin, we cotransfected pcDNA3-Trx and dnJNKK (MKK4/SEK1) or dnJNK expression plasmids into *A549* cells. As demonstrated in Fig. 8A, both dnJNKK and dnJNK inhibited NF-κB activation by thioredoxin.

![Fig. 6. Protein kinase C does not mediate IκB degradation by thioredoxin.](image)

*A549* cells were pre-incubated with GF109203X (15 μM) or calphostin C (1 μM) for 1 h. After incubation, Trx-(SH)₂ (*E. coli* system) was added, and cells were incubated for another period of 2 h, after which nuclear extract was prepared. Post-nuclear extract was treated as cytosolic extract. The cytosolic extract was subjected to Western blotting as described under “Experimental Procedures.” First lane, untreated *A549* cells; second lane, 5 μM Trx-SH₂; third lane, calphostin C, 1 μM; fourth lane, calphostin C + Trx-SH₂; fifth lane, GF109203X, 15 μM; sixth lane, GF109203X + Trx-SH₂, 5 μM.

![Fig. 7. Activation of JNK by reduced *E. coli* thioredoxin in *A549* cells.](image)

**A**. *A549* cells were treated with oxidized or reduced *E. coli* thioredoxin (2 μM) as described in Fig. 1. Cytosolic extracts were subjected to Western blotting for phospho-JNK. Immunoreactive bands were visualized by ECL detection. **B**. Stress-activated protein kinase/JNK was precipitated from *A549* cells (as described in Fig. 1) were probed with anti-JNK antibody in a Western blotting experiment. First lane, unstimulated *A549* cells; second through fourth lanes, cells treated with *E. coli* thioredoxin and incubated for 1, 2, or 3 h; fifth through seventh lanes, cells treated with 2 μM reducing *E. coli* system and incubated for 1, 2, or 3 h. B, total cellular JNK protein level does not change in response to thioredoxin treatment. **C**. Activation of JNK by overexpression of redox-active human thioredoxin in *A549* cells. *A549* cells in 60-mm² dishes were transfected with pcDNA3, pcDNA3-Trx, or pcDNA3-dnTrx as described under “Experimental Procedures.” Stress-activated protein kinase/JNK was precipitated from the cell lysates by c-Jun fusion protein bound to glutathione-Sepharose beads. After selectively pulling down JNK using c-Jun fusion protein beads, the beads were extensively washed, and the kinase reaction was carried out in the presence of cold ATP in a final volume of 25 μL. The reaction was stopped with 25 μL of 2× SDS sample buffer and loaded onto a 10% polyacrylamide gel. First lane, cells transfected with pcDNA3 only; second lane, cells transfected with 2 μg of pcDNA3-Trx; third lane, cells transfected with 4 μg of pcDNA3-Trx; fourth lane, cells transfected with 4 μg of pcDNA3-dnTrx.

**Dominant-negative Kinase-dead MEKK1 Inhibits NF-κB Activation by Thioredoxin—MEKK1** is an initiating kinase that activates the JNK cascade through phosphorylation of MKK4 or the p38 MAP kinase cascade by phosphorylating MKK3/MKK6 (19). Additionally, MEKK1 can directly phosphorylate IκBα kinase, which can cause the phosphorylation of IκBα and the activation of NF-κB (21). Therefore, activation of MEKK1 can directly cause NF-κB activation by phosphorylation of IκBα kinase without involving the JNKK or JNK intermediate signaling steps. Since the JNK pathway is activated by thioredoxin, we sought to determine the effect of inhibition of JNK cascade by dominant-negative MEKK1. When pcDNA3-Trx and pcDNA3-dnMEKK1 were cotransfected, we observed inhibition of NF-κB binding (Fig. 8A).

**Thioredoxin-mediated IκB Degradation Is Inhibited by dnMEKK1, dnJNKK, or dnJNK—** We have shown that overexpression of human thioredoxin causes degradation of IκB. Hence, if dnMEKK1 inhibits NF-κB activation by thioredoxin, we expect to observe inhibition of IκB degradation by cotransfection of pcDNA3-Trx and pcDNA3-dnMEKK1. Additionally, if dnJNK or dnJNKK also inhibits NF-κB activation by thioro-
As described under “Experimental Procedures.” First lane, A549 cells transfected with pcDNA3-Trx; second lane, cells transfected with pcDNA3-Trx and pcDNA3-dnMEKK1; third lane, cells transfected with pcDNA3-Trx and dnMEKK1; fourth lane, cells transfected with pcDNA3-Trx and dnJNKK; fifth lane, cells transfected with pcDNA3-Trx and dnJNK. B, degradation of IxB by thioredoxin was prevented by dnMEKK1, dnJNKK, or dnJNK. A549 cells were transfected with various expression plasmids as described under “Experimental Procedures.” Nuclear and cytosolic extracts were prepared 24 h post-transfection. IxB bands were detected by Western blotting as described under “Experimental Procedures.” First lane, A549 cells transfected with pcDNA3 empty vector; second lane, A549 cells transfected with pcDNA3-Trx; third lane, cells transfected with pcDNA3-dnMEKK1; fourth lane, cells transfected with dnJNKK plasmid; fifth lane, cells transfected with dnJNK plasmid.

NF-κB-mediated luciferase reporter gene transcription was inhibited when cells were cotransfected with dnMEKK1, dnJNKK, or dnJNK, demonstrating that the activation of JNK cascade is required in thioredoxin-induced, NF-κB-mediated gene transcription.

Wild-type MKK4 or MEKK1 Expression Increases Thioredoxin-mediated NF-κB Activation and NF-κB-dependent Luciferase Reporter Gene Expression—To further confirm the role of the JNK pathway in thioredoxin-mediated NF-κB activation, we cotransfected pcDNA3-Trx and wild-type MKK4 or MEKK1 expression plasmid into A549 cells and assayed for NF-κB activation by EMSA. Since dnMKK4 or dnMEKK1 inhibited NF-κB activation and NF-κB-dependent luciferase expression, we expected an increase in NF-κB activation with wild-type MKK4 or MEKK1 cotransfection with thioredoxin expression plasmid. Indeed, there was a significant increase in NF-κB-dependent luciferase expression (Fig. 10A). In addition, transfection of wild-type MKK4 or MEKK1 alone or in combination with trx expression plasmid induced NF-κB activation (Fig. 10B).

Lactacystin Inhibited IxB Degradation and p65 Translocation—We further explored the role of IxB degradation by thioredoxin in NF-κB activation. Lactacystin inhibits NF-κB by preventing degradation of IxB due to inhibition of the 26 S proteasome (31). Hence, if Trx activates NF-κB by degradation of IxB, inhibiting the 26 S proteasome by lactacystin should inhibit NF-κB in the presence of Trx.
inhibit NF-κB activation and IκB degradation by thioredoxin. Therefore, we incubated Trx-transfected cells with lactacystin (4 h after transfection) and determined NF-κB activation by EMSA after 24 h of treatment. As demonstrated, lactacystin inhibited NF-κB activation (Fig. 11A, lane 3) and IκB degradation (Fig. 11B, lane 2) in Trx-transfected cells, further supporting the involvement of IκB degradation in Trx-mediated NF-κB activation. Additionally, we also evaluated the effect of Trx expression on p65 translocation. We probed the nuclear extracts of cells transfected with Trx (as described for Fig. 11A) with anti-p65 antibody (Santa Cruz) by Western blotting. As demonstrated in Fig. 11B, Trx-induced p65 translocation to the nucleus, and such translocation was inhibited by lactacystin.

**DISCUSSION**

In the present report, we have demonstrated that the addition of *E. coli* thioredoxin to the culture medium activates NF-κB. We have further provided evidence that oxidized *E. coli* thioredoxin was unable to activate NF-κB, whereas the thioredoxin-reducing system (Trx + NADPH + TR) could activate NF-κB and degrade IκB in A549 cells. Furthermore, we have shown that overexpression of human thioredoxin activates NF-κB. In contrast, dominant-negative redox-inactive human thioredoxin failed to activate NF-κB. Additionally, we also demonstrate that the JNK-signaling cascade is activated by thioredoxin. Cotransfection of cells with pcDNA3-Trx and dominant-negative kinase-dead MEKK1 also inhibited NF-κB activation by thioredoxin, demonstrating a potential role of MEKK1 in the activation of NF-κB by thioredoxin. In addition, cotransfection of cells with pcDNA3-Trx and dnJNKK or dnJNK inhibited Trx-induced NF-κB activation. Thus, in this report we have demonstrated, employing two different systems (the external addition of *E. coli* thioredoxin and overexpression of human thioredoxin or its mutant form) that redox-active thioredoxin activates NF-κB, which is mediated by the initiating kinase of the JNK pathway, MEKK1.

It is possible that thioredoxin that enters the cell could be reduced by endogenous thioredoxin reductase and NADPH. However, *E. coli* thioredoxin is not as effective a substrate as the mammalian thioredoxin for mammalian thioredoxin reductase (8), and because of the oxidizing environment of cell culture medium, it was considered possible that the effect of thioredoxin could be mediated by its oxidized species. More-
over, we have earlier demonstrated that most of the oxidized *E. coli* thioredoxin that enters the cell remains in the oxidized form (32). Additionally, for the mammalian TR, the \( K_m \) of *E. coli* thioredoxin is 35 \( \mu \)M, 14-fold higher than the \( K_m \) for mammalian thioredoxin (8). Therefore, using a 2–5 \( \mu \)M concentration of *E. coli* thioredoxin, we effectively prevented the reduction of *E. coli* thioredoxin by cellular thioredoxin reductase.

In a recent study, we demonstrate that by using an enzymatic reducing system for reduction of thioredoxin, 85% of thioredoxin that entered the cell was in fully reduced form (32). Thus, the use of *E. coli* thioredoxin was an excellent experimental paradigm in differentiating the redox effect of *E. coli* thioredoxin from that of endogenous thioredoxin. We further demonstrated that *E. coli* thioredoxin can also induce NF-\( \kappa \)B activation in primary cultures of pulmonary artery endothelial cells. Since primary cultures are considered to represent normal in vivo conditions, our results indicate that activation of NF-\( \kappa \)B by *E. coli* thioredoxin is not limited to transformed cell lines.

Mammalian thioredoxin does not enter cultured cells (27). Therefore, to determine the effect of human thioredoxin on NF-\( \kappa \)B activation, we transiently overexpressed thioredoxin in A549 cells. Thioredoxin overexpression activated NF-\( \kappa \)B 24 h post-transfection (Fig. 5A). However, in the reducing environment of the cell, most of the overexpressed thioredoxin is expected to be in the reduced state, and therefore, activation of NF-\( \kappa \)B by overexpressed thioredoxin could be attributed to its reduced form. To determine the effect of thioredoxin redox status on NF-\( \kappa \)B activation, we mutated the catalytic Cys-32 and Cys-35 by site-directed mutagenesis. When inserted into a pCMV-directed expression vector, this mutated thioredoxin was expressed in a dominant-negative manner. Overexpression of dominant-negative thioredoxin did not induce NF-\( \kappa \)B activation in A549 cells, indicating the requirement of redox-active cysteines in the activation of NF-\( \kappa \)B.

Previous reports have shown that thioredoxin activates NF-\( \kappa \)B after I\( \kappa \)B dissociates from the NF-\( \kappa \)B complex, indicating only a reducing function of thioredoxin (14). Therefore, to understand the mechanism of activation of NF-\( \kappa \)B by thioredoxin, we immunobotted I\( \kappa \)B in the cytosolic extract. We have shown that the I\( \kappa \)B inhibitory protein is degraded as a result of treatment of cells with 2 \( \mu \)M *E. coli* thioredoxin-reducing system. There was no degradation of I\( \kappa \)B when cells were incubated with 2 \( \mu \)M oxidized *E. coli* thioredoxin. Similar observations were made with respect to primary cultures of endothelial cells (Fig. 3).

Overexpression of human thioredoxin also degraded I\( \kappa \)B (Fig. 5B) and induced translocation of p65 to the nucleus (Fig. 11B). Lactacystin, a 26 S proteosome inhibitor, inhibited thioredoxin-mediated NF-\( \kappa \)B activation and degradation of I\( \kappa \)B, further supporting the role of I\( \kappa \)B degradation in NF-\( \kappa \)B activation (Fig. 11). However, expression of dominant-negative redox-inactive thioredoxin did not degrade I\( \kappa \)B. Therefore, it is likely that thioredoxin redox status modulates an upstream signaling event, resulting in NF-\( \kappa \)B activation.

Since the JNK-signaling cascade is activated by many stimuli that also activates NF-\( \kappa \)B, we hypothesized that JNK or its upstream kinase JNKK may mediate NF-\( \kappa \)B activation by thioredoxin. If this is true, then dominant-negative JNKK or JNK should inhibit NF-\( \kappa \)B activation by thioredoxin. Indeed, in cotransfection experiments, dnJNKK and dnJNK inhibited NF-\( \kappa \)B activation and prevented degradation of I\( \kappa \)B, indicating a potential role of the JNK pathway in NF-\( \kappa \)B activation. MEKK1 is an initiating kinase of the JNK pathway and activates MKK4/SEK1, which activates the JNK. Since MEKK1 can directly phosphorylate I\( \kappa \)B, causing its ubiquitination and subsequent degradation, we reasoned a likely role of MEKK1 in the activation of NF-\( \kappa \)B by thioredoxin. MEKK1 can directly phosphorylate I\( \kappa \)B, causing its activation and subsequent degradation of I\( \kappa \)B, or it can activate MKK4, which in turn can activate JNK. When cells were cotransfected with pcDNA3-Trx and pcDNA3-dnMEKK1, there was inhibition of NF-\( \kappa \)B activation. However, cotransfection of pcDNA3-Trx and dnJNKK or dnJNK also inhibited NF-\( \kappa \)B activation and prevented degradation of I\( \kappa \)B. Therefore direct phosphorylation of I\( \kappa \)B kinase by MEKK1 in thioredoxin-stimulated cells is not a likely possibility. Together, these results demonstrate that thioredoxin activates NF-\( \kappa \)B by degradation of I\( \kappa \)B, which is mediated by the JNK-signaling pathway.

Both NF-\( \kappa \)B and MAP kinases, particularly JNKs and p38, are activated by similar agents. A connection between NF-\( \kappa \)B and JNK activation has recently been suggested based on the observation that overexpression of MEKK1-stimulated NF-\( \kappa \)B activation (33–34). Common precipitation assays further demonstrated an interaction between JNK and the NF-\( \kappa \)B subunit c-Rel that could be confirmed by two-hybrid assays (34). Additionally, studies have demonstrated that dominant-negative kinase-dead MEKK1 expression was able to inhibit tumor necrosis factor-\( \alpha \)-induced activation of NF-\( \kappa \)B (21). However, the exact mechanism of activation of NF-\( \kappa \)B by MEKK1 or the JNK has not been elucidated. Clearly, further studies are required to delineate the mechanisms of NF-\( \kappa \)B activation by the JNK pathway. Our present data demonstrate that redox-active thioredoxin was able to activate JNK pathway, which is initiated at the MEKK1 level. Redox control of MEKK1 activation is rather a novel finding. However, the mechanism of activation of
MEKK1 by thioredoxin awaits further investigation.

Recently, thioredoxin has been shown to migrate to the nucleus upon stimulation of cells with oxidative stress or other stresses (35). Since oxidative stress is known to induce and translocate thioredoxin to the nucleus (35), it is likely that translocated thioredoxin may modulate the transcription factor by facilitating its DNA binding due to reduction of sulfhydryls on the DNA binding domain. However, since IkBα kinase must be phosphorylated to allow the ubiquitin-mediated degradation of IkB may precede the thiol-reducing function of thioredoxin. Therefore, thioredoxin-mediated MEKK1-dependent phosphorylation of IkB may precede the thiol-reducing function of thioredoxin. Additionally, thioredoxin may facilitate the NF-κB DNA binding in the nucleus by its protein-reducing function after the translocation of the p50/p65 heterodimer.

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