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Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-κB and AP-1

HEIKE SCHENK*, MATTHIAS KLEIN*, WILHELM ERDBRÜGGER†, WULF DRÖGE*, AND KLAUS SCHULZE-OSTHOFF*‡

*Division of Immunochemistry, Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany; and †Department of Medicine, University of Essen, Essen, Federal Republic of Germany

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ABSTRACT **The transcription factors NF-κB and AP-1 have been implicated in the inducible expression of a variety of genes involved in responses to oxidative stress and cellular defense mechanisms. Here, we report that thioredoxin, an important cellular protein oxidoreductase with antioxidant activity, exerts different effects on the activation of NF-κB and AP-1. Transient expression or exogenous application of thioredoxin resulted in a dose-dependent inhibition of NF-κB activity, as demonstrated in gel shift and transactivation experiments. AP-1-dependent transactivation, in contrast, was strongly enhanced by thioredoxin. A similar increase of AP-1 activity was also observed with other, structurally unrelated antioxidants such as pyrrolidine dithiocarbamate and butylated hydroxyanisole, indicating that the thioredoxin-induced increase of AP-1 activation was indeed based on an antioxidant effect. Moreover, the stimulatory effect on AP-1 activity was found to involve *de novo* transcription of the c-*jun* and c-*fos* components but to be independent of protein kinase C activation. These results suggest that thioredoxin plays an important role in the regulation of transcriptional processes and oppositely affects NF-κB and AP-1 activation.**

Reactive oxygen intermediates (ROI) are produced during various electron-transfer reactions (1). When generated in excess, ROI can damage cells by peroxidizing lipids and disrupting proteins and nucleic acids. At moderate concentrations, however, ROI may exert signaling functions and regulate gene expression. ROI have been shown to activate the NF-κB transcription factor (2–4), among others. NF-κB controls the inducible expression of a variety of genes involved in inflammatory and immune responses (5). A characteristic feature of NF-κB is its activation by posttranslational mechanisms involving dissociation of the inhibitory protein IκB. Agents known to induce NF-κB activation include various cytokines, phorbol esters, and viral transactivators. It has been proposed that the formation of ROI may be a common denominator of the diverse NF-κB-inducing signals. This integrative role of ROI has been suggested because NF-κB can be induced by hydrogen peroxide and activation by various stimuli is commonly inhibited by antioxidants (2–4, 6–8). The redox regulation of NF-κB has received increased attention because the factor is an essential activator of human immunodeficiency virus replication and because redox disorders of the immune system may exert crucial roles in AIDS pathogenesis (9).

Another wide mediator of immediate-early gene expression is the transcription factor AP-1, which couples extracellular signals to gene-activating events associated with growth, differentiation, and cellular stress (10, 11). AP-1 is composed of the *jun* and *fos* gene products which form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes. Interestingly, several prooxidant conditions, such as hydrogen peroxide and UV irradiation, can induce AP-1 activation (12–14). The activation of AP-1 is regulated by complex mechanisms consisting of posttranscriptional events acting on preexisting AP-1 molecules and transcriptional activation leading to increased amounts of AP-1-binding proteins. In addition, redox modification of a conserved cysteine residue in the DNA-binding domain of Fos and Jun may be another mechanism of controlling DNA binding of AP-1 (15, 16). In the present study we examined the effects of thioredoxin and other antioxidants on the activation of AP-1 and NF-κB in intact cells. Thioredoxin has a strong reducing activity for thiols and functions as a major protein oxidoreductase inside the cell (17). Surprisingly, thioredoxin exerted quite opposite effects on the transactivation activity of the two transcription factors. Whereas the induction of NF-κB activity was inhibited by thioredoxin and other antioxidants, the activity of AP-1 was strongly promoted under reducing conditions.

MATERIAL AND METHODS

Cells and Reagents. The murine fibrosarcoma cell line L929 and the human cervical carcinoma cell line HeLa were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Phorbol 12-myristate 13-acetate ("tetradecanoylphorbol acetate," TPA), pyrrolidine dithiocarbamate (PDTC), butylated hydroxyanisole (BHA), and dithiothreitol were purchased from Sigma. Acetyl-CoA and poly(dI-dC) were obtained from Boehringer Mannheim; [^{14}C]chloramphenicol was from Amersham.

Expression of Recombinant Human Thioredoxin. Thioredoxin was expressed as a fusion protein containing six histidine residues plus another six-amino acid linker attached to its N terminus (pQE9 vector, Qiagen, Chatsworth, CA). Expression was induced in logarithmic cultures of *Escherichia coli* by the addition of 1.5 mM isopropyl β-D-thiogalactopyranoside. After 3 hr, bacteria were centrifuged and lysed by sonication under nondenaturing conditions. Thioredoxin was purified by using a nickel-chelate affinity resin (Qiagen). The purified protein fraction was reduced by 2 mM dithiothreitol and dialyzed against 35 mM NaCl/2.5 mM phosphate, pH 7.2 (0.25× PBS). The recombinant thioredoxin had dithiol-dependent reducing activity in the insulin reduction assay (18).

Plasmids. A chloramphenicol acetyltransferase (CAT) reporter plasmid, TRE2CAT, was obtained by insertion of two spaced TPA response elements (TREs) 5′ to the thymidine kinase promoter of pBLCAT2 (19). The NF-κB-dependent

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Abbreviations: BHA, butylated hydroxyanisole; CAT, chloramphenicol acetyltransferase; PDTC, pyrrolidine dithiocarbamate; PKC, protein kinase C; ROI, reactive oxygen intermediates; TPA, "tetradecanoylphorbol acetate" (phorbol 12-myristate 13-acetate); TRE, TPA response element.

‡To whom reprint requests should be addressed.

CAT reporter plasmid NFκB4CAT has been described (6). The eukaryotic expression vector pCMV-ADF encoding human thioredoxin (ADF, ref. 20) was a gift from P. A. Baeuerle.

Transfections and CAT Assays. Transient transfections were performed by the DEAE-dextran method. Cells were seeded in 60-mm^3 Petri dishes at 3×10^6 the day before transfection. Eight micrograms of CAT plasmid and 0.25–10 μg of thioredoxin expression vector pCMV-ADF were used. Forty-eight hours after transfection, cells were stimulated with TPA (25 ng/ml) and subjected to CAT assays 15–18 hr later. Stably transfected L929 cells harboring the TRE2CAT construct were generated with an improved DNA/calcium phosphate coprecipitation technique (21). For CAT assays, cells were seeded in six-well plates at 10^6 per well and treated with the indicated concentrations of thioredoxin, PDTC, or BHA. Two hours later, the cells were stimulated with TPA and extracted after another 15–18 hr. CAT activity was measured in the extracts as described (22).

Electrophoretic Mobility-Shift Assays. Subconfluent cell cultures were treated with the inhibitors and then stimulated with TPA (25 ng/ml) after 2 hr. After incubation for a further 2 hr, cells were rinsed in ice-cold PBS, scraped off and collected by centrifugation. Extracts were prepared essentially as described (4) and incubated with NF-κB- and AP-1-specific ^{32}P-labeled oligodeoxynucleotides. Binding reactions and electrophoresis were performed as described (4). For specificity controls a 100-fold excess of unlabeled probe was applied. The sequences of the NF-κB- and AP-1-specific probes (binding sites are underlined) were 5′-AGCTTCAGAGGGGATTTCCGAGAGG-3′ and 5′-AGCTTGATGAGTCAGCCGGATC-3′, respectively.

Other Methods. For Northern blot analysis, poly(A)$^+$ RNA was isolated with the Micro Fast Track kit (Invitrogen). Equal amounts of RNA were electrophoresed in 1% agarose/formaldehyde gels and transferred to a nylon membrane. After prehybridization, blots were hybridized with [^{32}P]dCTP-labeled probes for human c-*jun* and c-*fos*. Hybridization was performed in 150 mM NaCl/10 mM phosphate, pH 7.4/1 mM EDTA/10% dextran sulfate/50% formamide at 42°C. Protein kinase C (PKC) activity was measured as described (23). After partial purification on DEAE-cellulose, PKC-specific kinase activity was determined in cytoplasmic and membrane fractions by phosphorylation of the myelin basic protein-(4–14) undecapeptide.

RESULTS

Overexpression of Thioredoxin Inhibits NF-κB Activation but Increases AP-1-Dependent Transactivation. HeLa cells were transiently transfected with pCMV-ADF, an expression plasmid for human thioredoxin, and CAT reporter plasmids under control of either four NF-κB binding sites (NFκB4CAT) or two AP-1 binding sequences (TRE2CAT). Forty-eight hours after transfection, cells were stimulated with TPA for 15 hr and harvested, and the cell lysates were subjected to CAT assays. When cells were transfected with increasing amounts of the thioredoxin-encoding plasmid pCMV-ADF, TPA-stimulated NF-κB activation was inhibited in a dose-dependent way (Fig. 1*A*). A similar decrease of CAT activity was observed in unstimulated cells. The highest doses of pCMV-ADF (5–10 μg) caused a 20- to 30-fold inhibition of CAT activity in TPA-stimulated cultures. In contrast, graded amounts of the transfected thioredoxin plasmid led to a dose-dependent increase of AP-1-dependent transactivation (Fig. 1*B*). An optimal dose of pCMV-ADF (2 μg) increased CAT activity of TPA-stimulated cells about 10-fold. Higher amounts, however, showed less effect. A similar dose dependency was seen in cells without prior TPA stimulation.

Treatment with Thioredoxin or Antioxidants Induces AP-1 Transactivation. The profound increase in AP-1-dependent transactivation that accompanied transient expression of thioredoxin prompted us to examine the effects of treatment with exogenous thioredoxin. Recombinant human thioredoxin was expressed as a hexahistidine fusion protein and purified by nickel-chelate affinity chromatography. When L929 cells stably transfected with the AP-1-driven CAT gene were treated with increasing concentrations of thioredoxin, similar effects on AP-1 transactivation were detected as in the previous cotransfection experiments (Fig. 2). A thioredoxin concentration as low as 20 μg/ml (≈1.4 μM) resulted in a significant increase of AP-1 activity, irrespective of cell stimulation. At 50 μg/ml, thioredoxin even induced similar levels of CAT activity in TPA-stimulated and unstimulated cultures.

Since thioredoxin is a thiol molecule with potent antioxidant and radical-scavenging activity, we extended our experiments to other scavenging compounds. PDTC, a radical scavenger and iron chelator, was described as a strong inhibitor of NF-κB activation (2, 3). We found that AP-1-

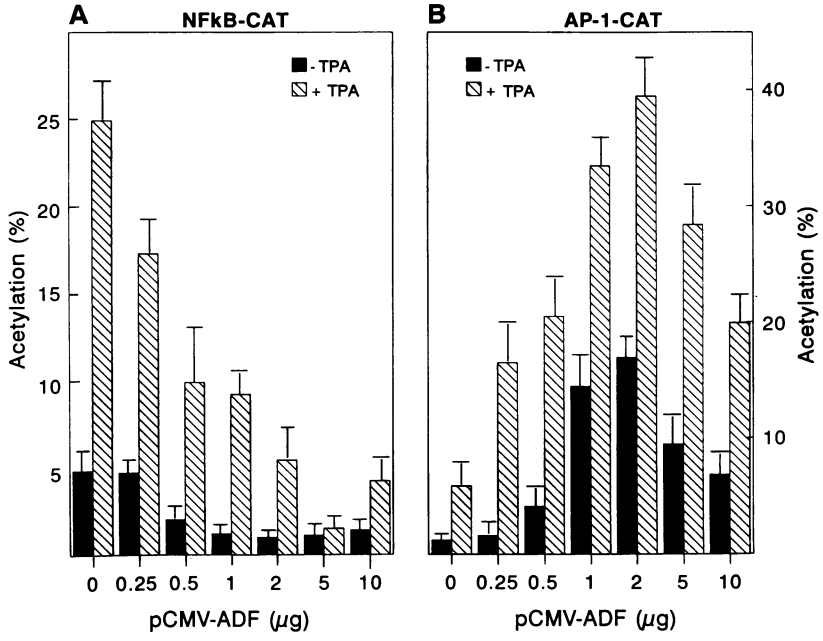

FIG. 1. Transient expression of thioredoxin differentially affects NF-κB- and AP-1-dependent transactivation. HeLa cells were cotransfected with the indicated amounts of pCMV-ADF together with either the NF-κB-dependent CAT reporter construct NFκB4CAT (*A*) or the AP-1-dependent construct TRE2CAT (*B*). TPA stimulation of cells was performed 48 hr after transfection. Fifteen hours later, cell extracts were prepared and assayed for CAT activity. Hatched bars, TPA-stimulated cells; filled bars, unstimulated cells. Values represent the mean ± SEM from one triplicate experiment out of three independent experiments.

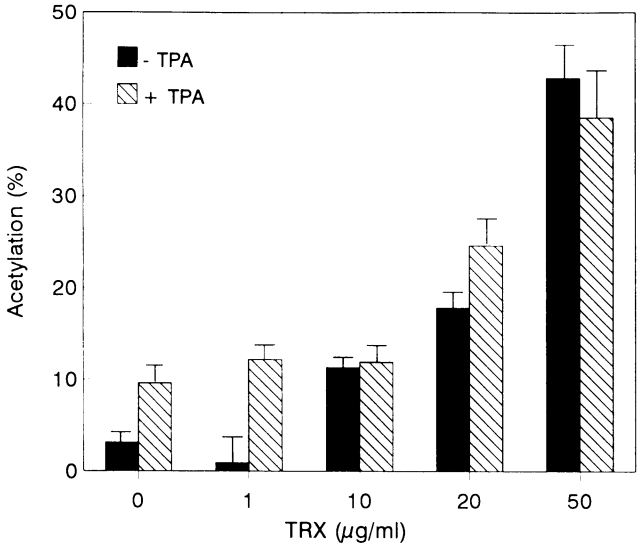

FIG. 2. AP-1-dependent transactivation is increased by exogenous thioredoxin. L929 cells stably transfected with TRE2CAT were treated with the indicated concentrations of recombinant thioredoxin (TRX) 2 hr before TPA stimulation. Cells were harvested after another 15 hr and assayed for CAT activity. Hatched bars, TPA-stimulated cells; filled bars, unstimulated cells.

dependent transactivation, in contrast, was markedly enhanced by PDTC. The addition of 20 μM PDTC caused a nearly 5-fold increase of AP-1 activity in TPA-stimulated cells (Fig. 3*A*). Also, treatment with BHA, a potent phenolic antioxidant and inhibitor of NF-κB activation (4, 8, 24), resulted in strong enhancement (Fig. 3*B*). BHA at 100–300 μM increased CAT activity >10-fold. A strong increase was also seen with 2-mercaptoethanol (data not shown). This suggests that, indeed, diverse antioxidant conditions stimulate AP-1 activity.

Effects of Thioredoxin and PDTC on NF-κB and AP-1 DNA-Binding Activities. Electrophoretic mobility-shift assays were applied to investigate early effects of thioredoxin and other antioxidants on NF-κB and AP-1 activation. HeLa

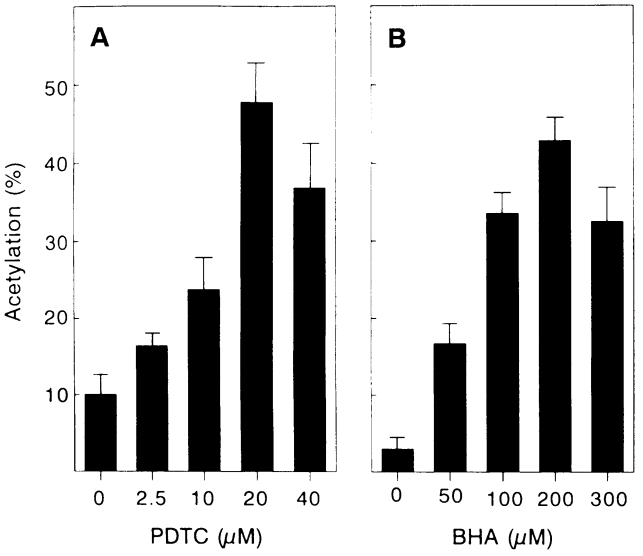

FIG. 3. Antioxidants enhance TPA-triggered AP-1-dependent gene expression. L929 cells stably transfected with TRE2CAT were incubated with the indicated concentrations of PDTC (*A*) or BHA (*B*). Two hours later, cells were stimulated with TPA. After further incubation for 15 hr, cell extracts were prepared and analyzed in CAT assays.

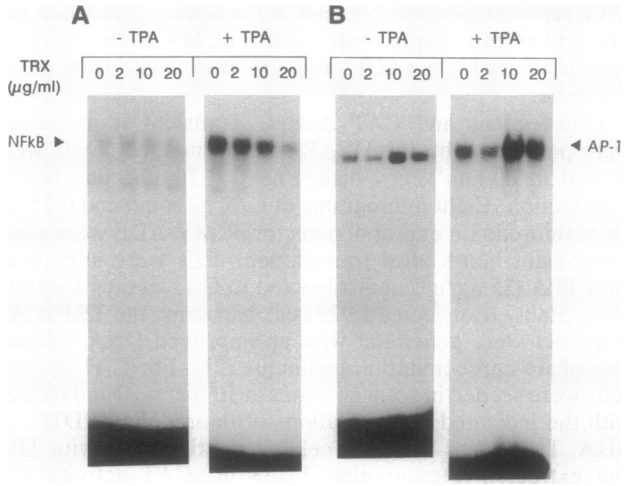

FIG. 4. Effects of thioredoxin on the DNA-binding activities of NF-κB (*A*) and AP-1 (*B*). HeLa cells were treated for 2 hr with the indicated concentrations of thioredoxin (TRX) and then stimulated with TPA for 2 hr. Identical cell extracts were incubated with ^{32}P-labeled NF-κB- and AP-1-specific oligonucleotides and analyzed in electrophoretic mobility-shift assays. Positions of the specific DNA-protein complexes are indicated by arrowheads.

cells were preincubated with thioredoxin for 2 hr and then stimulated with TPA. After another 2 hr of incubation, cell extracts were harvested and analyzed in mobility-shift assays with AP-1- and NF-κB-specific oligonucleotides. In agreement with the transactivation experiments, thioredoxin caused a dose-dependent decrease of TPA-triggered NF-κB binding activity (Fig. 4*A*). Maximal inhibition was obtained with thioredoxin at 20 μg/ml. In contrast, the DNA-binding activity of AP-1 was strongly enhanced by thioredoxin (Fig. 4*B*). This increase of AP-1 activity was found in unstimulated and TPA-stimulated cultures. Maximal AP-1 DNA binding was observed when cells were treated with a combination of TPA and thioredoxin at 10 μg/ml, whereas higher doses of thioredoxin had a weaker effect. Similar effects on NF-κB and AP-1 DNA binding were obtained by treatment of cells with PDTC (Fig. 5). The low concentration of 2.5 μM PDTC strongly inhibited NF-κB activation. AP-1 DNA binding, in contrast, was strongly enhanced by PDTC treatment. Whereas NF-κB activation was nearly completely sup-

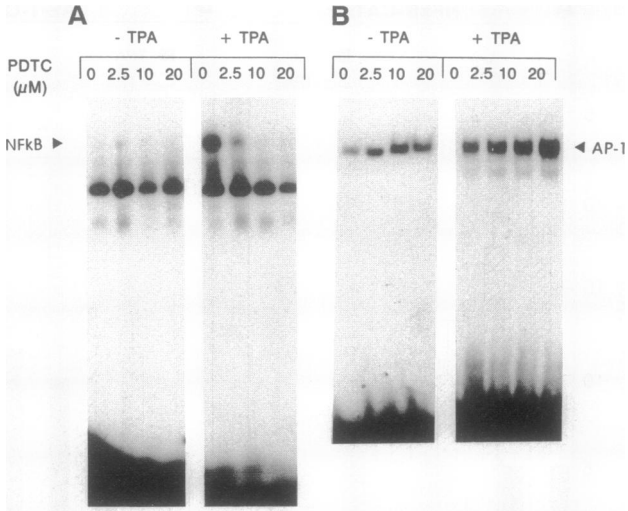

FIG. 5. Effects of PDTC on the DNA-binding activities of NF-κB (*A*) and AP-1 (*B*). HeLa cells were treated with the indicated concentrations of PDTC as described in Fig. 4, and cell extracts were analyzed in mobility-shift assays.

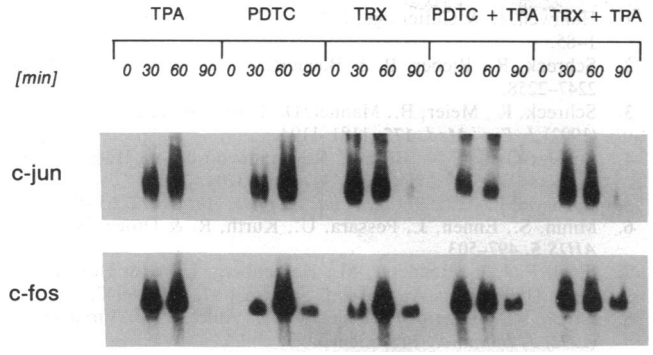

FIG. 6. Effect of thioredoxin (TRX) and PDTC on c-*jun* and c-*fos* transcription. L929 cells were stimulated for the indicated times with TPA (25 ng/ml), PDTC (40 μM), TRX (50 μg/ml), or combinations thereof. Equal amounts of poly(A)$^+$ RNA were electrophoresed in an agarose/formaldehyde gel and hybridized with ^{32}P-labeled oligonucleotides specific for c-*jun* and c-*fos*.

pressed by 2.5 μM PDTC, higher concentrations (20 μM) were required to maximally enhance TPA-triggered DNA binding of AP-1.

Mechanism of Antioxidant-Increased AP-1 Activity. To determine whether the strong effects of thioredoxin and PDTC on AP-1-dependent transactivation were due to *de novo* synthesis of its subunits, expression of c-*jun* and c-*fos* mRNA was analyzed. When L929 cells were stimulated with thioredoxin or PDTC, with or without TPA, a rapid and transient increase of c-*jun* and c-*fos* transcripts was observed in all cases (Fig. 6). Transcriptional induction of c-*jun* could be detected after 30–60 min, following similar kinetics in cells stimulated either with antioxidants or with TPA. A similar pattern of mRNA synthesis was detectable for c-*fos*. However, in comparison to TPA stimulation, transcriptional activation of c-*fos* was slightly delayed and more persistent in cells treated with thioredoxin or PDTC. Antioxidant-induced transcription was maximal after 60 min and still detectable after 90 min. Since TPA is known to stimulate AP-1 activity through PKC, we further studied the role of PKC in thioredoxin- or PDTC-mediated stimulation of AP-1 activity. PKC is activated by its translocation from the cytosol to the cell membrane. Indeed, TPA-treated cells revealed a strong increase in membrane-associated PKC activity (Fig. 7). In contrast, translocation of PKC was not observed following stimulation with either thioredoxin or PDTC. A lack of involvement of PKC was also noticed when PKC was depleted in L929 cells by prolonged incubation with bryostatin. TPA did not increase CAT activity in PKC-depleted cells, whereas PDTC and thioredoxin still had stimulatory effects (data not shown). Therefore, antioxidant-induced AP-1 transactivation apparently does not follow the classical signaling pathway via PKC.

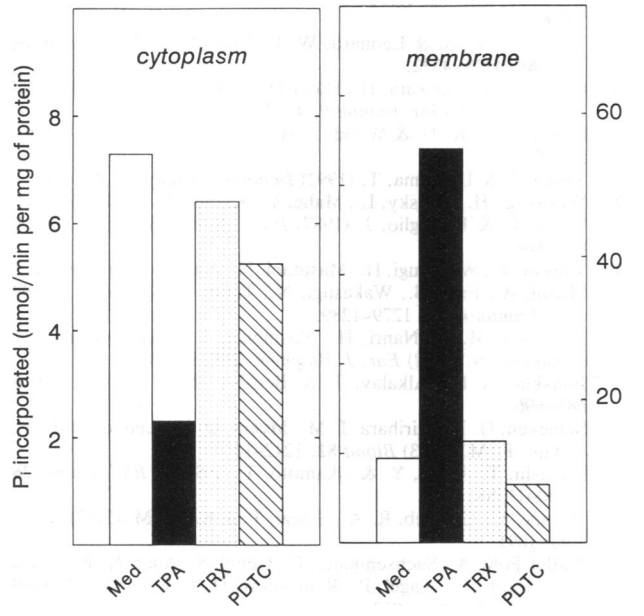

FIG. 7. Effect of TPA, thioredoxin (TRX), and PDTC on PKC activation. L929 cells were stimulated for 15 min with TPA (50 ng/ml), TRX (50 μg/ml), or PDTC (50 μM). PKC activity was determined in cytoplasmic and membrane fractions by a peptide phosphorylation assay. Med, medium-only control.

DISCUSSION

A number of recent studies support the idea that ROI and the redox state of certain proteins play an important role in gene regulation. Different modes of redox regulation have been described for a variety of mammalian transcription factors, including steroid receptors (25), c-Myb protein (26), TFIIIC (27), and the transcriptional activators NF-κB (2–4, 6–8, 28, 29) and AP-1 (15, 16). In the present study, we have analyzed the effects of thioredoxin and other antioxidants on the activation of NF-κB and AP-1. The results suggest thioredoxin may play an important role in the regulation of gene expression. Further, thioredoxin and some other antioxidants have strikingly different effects on the regulation of NF-κB and AP-1 activation.

Inhibitory Effect of Thioredoxin on NF-κB Activation. Thioredoxin, originally identified as a dithiol hydrogen donor for ribonucleotide reductase, has been implicated in a variety of cellular redox processes (17, 30). In the epidermis, thioredoxin is assumed to exert important functions in defense to UV irradiation and oxidative stress (31). Thioredoxin has further been reported to exert a number of interleukin 1-like activities including the stimulation of interleukin 2 secretion, interleukin 2 receptor expression, and cell proliferation (32, 33). In this report we show that thioredoxin may play a role in the regulation of transcriptional activation. Transient expression of thioredoxin resulted in a pronounced inhibition of NF-κB-dependent transactivation in CAT assays. Similar inhibitory effects were detected at the level of DNA binding in mobility-shift assays. These observations suggest that thioredoxin most probably interferes with the release of IκB as the major regulatory step of NF-κB activation. Thioredoxin functions as an efficient protein disulfide reductase, and most thioredoxin has been detected in the reduced state inside the cell (34). The inhibitory effects of thioredoxin, therefore, agree with earlier reports about the inhibition of NF-κB activation by various low molecular weight thiol compounds. However, whereas inhibition of NF-κB activation by these thiols required relatively high concentrations (e.g., 30 mM *N*-acetylcysteine), inhibition by thioredoxin was obtained in the micromolar range. Therefore, it seems unlikely that the inhibition is based on a radical-scavenging effect. A more likely possibility is that ROI may modify an intermediate oxidative stress-sensitive messenger molecule which may act similarly to the redox-regulated LTK kinase or Src tyrosine kinases (35, 36). A signal generated by these molecules may be reversed by thioredoxin, presumably by the reduction of a critical disulfide bond in the protein. In apparent contrast to the inhibition of NF-κB activation in the cytoplasm, it has been reported that direct NF-κB/DNA interaction is promoted by thioredoxin and antioxidants through the reduction of a conserved cysteine residue in the DNA-binding domain (16, 28). This redox modification could represent a secondary physiological control mechanism exerted by thioredoxin inside the nucleus (37).

The Increase of AP-1 Activity by Thioredoxin and Other Antioxidants. In contrast to NF-κB, AP-1-dependent transactivation was markedly increased upon transient expression or addition of thioredoxin. This finding was unexpected, since AP-1 has been reported to be rapidly activated under a variety of prooxidant conditions, such as hydrogen peroxide or UV irradiation (12–14). To exclude the possibility that thioredoxin might have undergone oxidation inside the cell, the effects of other antioxidants such as PDTC and BHA were also evaluated. The finding that stimulation of AP-1-dependent transactivation could be also obtained with these structurally unrelated compounds strongly indicates that the increase in AP-1 activity was based on an antioxidant effect. At first, it seems paradoxical that both oxidative and reducing signals can activate AP-1. However, activation of AP-1 is regulated by complex mechanisms which consist of distinct effects on the preexisting AP-1 complex or on the *de novo* synthesis of AP-1 subunits. Our results favor the interpretation that distinct mechanisms may be responsible for induction of AP-1 activity under pro- and antioxidant conditions. Induction of AP-1 by TPA or UV irradiation occurs within minutes and does not require new protein synthesis (12–14). UV irradiation causes changes in c-Jun phosphorylation which require activation of Raf-1 and protein-tyrosine kinases (38, 39). Interestingly, the UV response can even be inhibited by the antioxidants *N*-acetylcysteine and glutathione, suggesting a contribution of redox-sensitive kinases in this process (38). In contrast to UV and TPA treatment, which induce AP-1 activation initially by posttranslational mechanisms, antioxidant-increased AP-1 activity apparently required *de novo* synthesis. An increase in AP-1 activation by PDTC and thioredoxin was not observed in the presence of the translational inhibitor cycloheximide (data not shown). Moreover, thioredoxin and PDTC caused a rapid and transient activation of c-*jun* and c-*fos* gene transcription. In the case of c-*jun*, the time course of transcription paralleled the induction by TPA; in the case of c-*fos*, however, mRNA expression was clearly more persistent after antioxidant treatment. It may be that a compositional change of AP-1 could affect its transactivating activity. It is known that heterodimeric (Jun/Fos) complexes are stronger transcriptional activators than homodimeric (Jun/Jun) complexes. Induction of c-Jun *de novo* synthesis is mediated by a positive autoregulatory mechanism of its proximal AP-1 element, whereas induction of c-Fos is conferred by the serum response element (40).

In conclusion, our results reveal that the activation of NF-κB and AP-1 is regulated by thioredoxin and other antioxidants in a strikingly opposite way. Whereas NF-κB can be regarded as an oxidative stress-responsive factor that is activated by posttranslational processes, AP-1 has a far more complex pattern of regulation and can be activated by prooxidant *and* antioxidant conditions. Thioredoxin can be assumed to exert an important role in the redox regulation of these transcription factors. In agreement with our results, Meyer *et al.* have reported that PDTC increases AP-1 activity by a mechanism requiring *de novo* synthesis of the AP-1 subunits (41). They further demonstrated that transcriptional activation of c-*fos* was conferred by the serum response element, which may suggest the serum response factor as the primary target of the antioxidant effect.

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