Thioredoxin Nuclear Translocation and Interaction with Redox Factor-1 Activates the Activator Protein-1 Transcription Factor in Response to Ionizing Radiation

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ABSTRACT

Thioredoxin (TRX) is a cytoplasmic, redox-sensitive signaling factor believed to participate in the regulation of nuclear transcription factors mediating cellular responses to environmental stress. Activation of the activator protein (AP)-1 transcription factor is thought to be mediated in part by redox-sensitive interactions between the nuclear signaling protein redox factor-1 (Ref-1) and TRX. In this study, the role of TRX and Ref-1 in the activation of the AP-1 complex was examined in HeLa and Jurkat cell lines exposed to ionizing radiation (IR). After exposure to IR, nuclear levels of immunoreactive TRX increased, accompanied by an increase in AP-1 DNA binding activity. It was shown that a physical interaction between Ref-1 and TRX occurs within the nucleus and is enhanced after exposure to IR. Furthermore, TRX immunoprecipitated from irradiated cells was capable of activating AP-1 DNA binding activity in nonirradiated nuclear extracts. In addition, immunodepletion of Ref-1 from nuclear extracts demonstrated that the increase in AP-1 DNA binding activity after IR was also dependent upon the presence of Ref-1 from irradiated cells. Finally, the ability of both TRX and Ref-1 from irradiated cells to stimulate AP-1 DNA binding in nonirradiated nuclear extracts was abolished by chemical oxidation and restored by chemical reduction. These results indicate that, in response to IR, TRX and Ref-1 undergo changes in redox state that contribute to the activation of AP-1 DNA binding activity. These experiments suggest that a redox-sensitive signaling pathway leading from TRX to Ref-1 to the AP-1 complex participates in the up-regulation of DNA binding activity in response to ionizing radiation.

INTRODUCTION

Eukaryotic cells have evolved adaptive responses to multiple forms of environmental stress by initiating genetically preprogrammed signaling pathways (1, 2). These adaptive responses include the activation of cellular machinery involved in DNA repair, cell cycle arrest, apoptosis, gene induction, and lethality (3, 4). In response to a wide variety of environmental stresses including ionizing radiation, a class of proto-oncogenes (including c-Fos and c-Jun) referred to as immediate-early response genes are activated (5–7). These genes encode nuclear transcription factors (e.g., AP-1) involved in the transmission of inter- and intracellular information through multiple cellular signaling pathways (2, 8, 9). Thus, these gene products may function in short-term changes in cellular phenotype by modulating the expression of specific target genes involved in cellular defenses against exogenous cytotoxic agents, such as IR (6, 10).

A long-standing hypothesis in radiation biology states that the IR-induced activation of cellular signaling pathways is initiated at the cytoplasmic membrane (4, 11, 12). In recent years, several studies have demonstrated that genes encoding growth factors and cytokines involved in cytoplasmic membrane signaling are targets of IR (4, 13, 14). Because c-Fos and c-Jun are nuclear proteins that interact to form the activated AP-1 transcription factor, they are not likely to bridge the physical barrier between the cytoplasm and the nucleus in the IR-induced signaling pathway (6, 15). Instead, upstream signaling factors probably exist to pass the cytoplasmic signal into the nucleus.

One potential candidate for such a role is TRX, a small, ubiquitous, multifunctional protein containing a redox-active disulfide/dithiol within the conserved active site -Cys-Gly-Pro-Cys- (16, 17). TRX, also known as adult T cell leukemia-derived factor, has both intracellular and extracellular functions and is a key regulator of cellular signaling in response to various cellular stresses (18–20). Similar to AP-1, TRX is an inducible factor that shows cytoprotective activity against oxidative stress-induced apoptosis (21, 22) and exhibits autocrine growth-promoting effects (23–25). Agents such as phorbol 12-myristate 13-acetate and IR are known to generate intracellular free radicals such as \( \cdot OH \) and \( \cdot O_2^- \) (26–28) that are believed to mediate many of their biological effects. In tissue culture cells treated with phorbol 12-myristate 13-acetate, TRX is imported into the nucleus and forms a physical interaction with the Ref-1 gene product, Ref-1 (29). Ref-1 (also designated APE, HAP-1, and APEX) functions as both a nuclear DNA repair enzyme and as a reversible regulator of the DNA binding activity of several nuclear transcription factors, including AP-1, by altering the oxidation/reduction (redox) state of specific cysteine residues located in the basic DNA binding region of these transcription factors (15, 30–32). One possible role for the transient induction of AP-1 may be the initiation of protective or reparative cellular responses to the damaging effects of cellular stressing agents, including IR (6, 10). Therefore, the passage of redox signals through a series of sulfhydryl switches from TRX to Ref-1, finally resulting in the activation of the AP-1 transcription factor, might represent an important pathway for the transmission of radiation-induced signaling from the cytoplasm to the nucleus.

**In vitro** Ref-1 activity is induced by chemical reducing agents such as DTT; however, in the absence of such reducing agents, other cytoplasmic cellular signaling factors, such as TRX, TRX reductase, and NAP(P)H are hypothesized to activate the Ref-1 protein (15, 31, 33). These findings implicate Ref-1 as a central target protein in posttranslational, redox-sensitive signaling cascades that use thioldContaining proteins to activate specific sets of redox-sensitive proteins that could serve to induce alterations in gene expression in response to oxidative stress. Because IR generates intracellular free radicals and irradiated cells have been shown to respond to this insult by increasing the production of NADPH via the pentose cycle (34), it seems logical.

6688
to investigate the relationship between IR and redox-sensitive signaling proteins in the cellular responses to IR. The current study investigates the role of TRX and Ref-1 in the transduction of IR-induced signals from the cytoplasm to the nucleus, resulting in increased AP-1 DNA binding activity in HeLa and Jurkat cells. IR-induced subcellular relocalization of TRX from the cytoplasm to the nucleus in response to IR was demonstrated by Western blot analysis of nuclear extracts and confirmed by indirect immunofluorescence cell staining. A physical interaction between TRX and Ref-1 after exposure to IR was demonstrated using transient transfection two-hybrid assays. The addition of Ref-1 and TRX from irradiated cells to nonirradiated nuclear extracts was shown to activate the DNA binding activity of AP-1. Furthermore, alterations in the redox state of TRX and Ref-1 immunoprecipitated from irradiated cells were shown to be involved in the ability of these proteins to activate AP-1 DNA binding activity. Immunodepletion of Ref-1 from nuclear extracts showed inhibition of inducible AP-1 DNA binding activity in response to IR. Overall, these results suggest that the molecular cascade leading to AP-1 activation in response to IR involves the passage of redox signals through TRX from the cytoplasm to the nucleus, followed by interaction with Ref-1, possibly through a physical interaction between Ref-1 and TRX.

MATERIALS AND METHODS

Cell Culture and IR Conditions. HeLa (human cervical carcinoma) cells were grown in modified Eagle’s medium α supplemented with 10% HI calf serum, Jurkat cells (human leukemia cell line) were grown in RPMI 1640 supplemented with 5% HI fetal bovine serum, and Cos-1 (African green monkey kidney) cells were grown in DMEM supplemented with 10% HI FCS with penicillin and streptomycin (100 units/ml). Cells were grown in a humidified 5% CO2 atmosphere at 37°C. Prior to irradiation, cells were serum starvation with penicillin and streptomycin (100 units/ml). Cells were grown in a humidified atmosphere containing an enclosed incubator. Control, nonirradiated cells were placed into a similar incubator.

Nuclear and Cytoplasm Subcellular Fractionation. Cells at various time points after exposure to IR were washed twice with PBS, scraped with 750 µl of PBS-2.5 mM EDTA, and centrifuged at 14,000 rpm for 2 min at 4°C. The cell pellet was suspended in 400 µl of buffer A. After incubating for 15 min on ice, 25 µl of 10% NP40 were added. The cellular suspension was vortexed for 10 quick mixings and centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant (cytoplasmic fraction) was transferred to a new microcentrifuge tube, and the supernatant and cell pellets were stored at −80°C overnight. The nuclear pellet was thawed on ice for 15 min and suspended in 60 µl of extraction buffer [10 mM HEPES (pH 7.2), 422 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 5.3% glycerol], incubated at 4°C for 30 min, and spun for 10 min at 14,000 rpm. The supernatant (nuclear fraction) was transferred to a new microcentrifuge tube and stored at −80°C. Verification of the subcellular fractions was determined by immunoblotting both the nuclear and cytoplasmic extracts with Ref-1, a nuclear protein, and 1-xb, a cytoplasmic protein (data not shown). Protein concentrations were determined using the Bradford method (per the manufacturer’s specification; Bio-Rad Laboratories, Hercules, CA).

SDS-PAGE and Western Blot Analysis. Equal amounts of protein (20 µg) from nuclear or cytoplasmic cellular extracts were mixed with 5× Laemmli lysis buffer (3) and boiled for 5 min. Samples were separated on denaturing SDS-polyacrylamide gels and transferred to nitrocellulose filter paper using a semidyary apparatus (Owl Scientific Plastics, Inc., Portsmouth, NH). The nitrocellulose filter was prepared and developed as described previously (27), and Western blot analysis was performed using an anti-TRX antibody (American Diagnostica, Inc., Greenwich, CT).

EMSA. EMSAs were performed as described previously using a 32p-radiolabeled oligonucleotide corresponding to the consensus AP-1 DNA binding site (27). Nuclear extracts (10 µg) were incubated with poly(doxoxygenosin–doxoyctydidylic acid) for 10 min on ice, followed by the addition of radiolabeled oligonucleotide (200,000 cpm of radiolabeled probe/reaction) and incubation at 25°C for 20 min. No reducing agents were added to the EMSAs or the buffers used to make nuclear extracts. Samples were run on a 4.5% non-denaturing PAGE gel, dried, and exposed to a phosphorimager screen using a STORM 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). For immunodepletion experiments, cells were lysed by the addition of 1 ml of E1A lysis buffer (ELB: 50 mM HEPES (Sigma), pH 7.2, 250 mM NaCl (Fisher Scientific), 2 mM EDTA (Sigma), and 0.1% NP40 (BDH Chemicals Ltd.)). Cellular lysates were precleared with 25 µl of protein A (Santa Cruz Biotechnology, Inc.). Immunodepletion of Ref-1 and TRX was performed immediately after the extracts were prepared by adding 25 µl of protein A (Santa Cruz Biotechnology, Inc.) and 5 µl of anti-Ref-1 antibody (polyclonal; Santa Cruz Biotechnology, Inc.) or 5 µl of anti-TRX antibody (polyclonal; American Diagnostic, Inc.). These reactions were shaken slowly at 4°C for 2 h, and the IP was spun at 12,000 rpm for 1 min and washed in 750 ml of E1A buffer at 4°C three times. For the immunoprecipitation experiments where immunoprecipitated TRX (Fig. 3B) or Ref-1 (Fig. 5B) was added to various cellular extracts, the immunoprecipitated complex was added to 20 µg of EMSA cellular extract for 45 min at 4°C with gentle shaking, and EMSA was performed (27, 35). Chemical modification of immunoprecipitated Ref-1 and TRX was performed by adding either diamide (80 µM; Sigma D-3648, 98% pure), NEM (50 µM; Sigma E-3876, 98% pure), or DTT (8 mM; Sigma D-9163, 99% pure) to immunoprecipitation/protein A pellet for 30 min at 4°C. All chemicals were filter sterilized and used without further purification. To remove the free residual chemicals from the immunoprecipitation reaction, the pellets were washed eight times in E1A buffer at 4°C. Briefly, for each wash 750 ml of E1A buffer were added to the immunoprecipitation/protein A pellet, followed by gentle shaking by tapping the Eppendorf tube against the countertop for 30 s, followed by spinning at 12,000 rpm for 1 min. The immunoprecipitation/protein A pellet was added to 20 µg of EMSA cellular extract for 45 min at 4°C with gentle shaking, and EMSAs were performed (27, 35).

Indirect Immunofluorescence Cell Staining. Cells were grown on coverslips in 60-mm culture plates and fixed with 3.7% paraformaldehyde in PBS containing 10% FCS for 20 min at room temperature, followed by permeabilization for 10 min using 0.2% (wt/vol) Triton X-100 in PBS. After incubation (1 h) with primary antibodies for TRX (American Diagnostica) slides were incubated (1 h) with either dye-labeled antimonuex or antirabbit IgG. The slides with stained, and cells were mounted in 90% glycerol with 1 mg/ml p-phenylenediamine. Cells were examined using a confocal microscope, MRC 600 (Bio-Rad Laboratories, Hercules, CA).

Transient Cotransfections and Mammalian Two-Hybrid Assay. A cDNA of TRX fused in-frame with the transcription factor VP-16 downstream of the CMV promoter (pCMV-TRX-VP-16) and a cDNA of Ref-1 fused in-frame with the DNA binding domain of Gal-4 (pCMV-Ref-1-Gal-4) were used (29). Cos-1 cells were plated at 2 × 105 cells per 100-mm plate, serum starved (0.5% FCS) for 8 h, and transfected via calcium phosphate precipitation. In the cotransfection, 2 µg of pCMV-TRX-VP-16 and/or pCMV-Ref-1-Gal-4 were transfected with the reporter construct, ptk-3x-Gal-4-LUC containing three copies of the Gal-4 binding site upstream of the LUC gene in ptk-LUC (29). As an internal control, 1 µg of the β-gal expression plasmid (pCMV-β-gal) was used. Transfected cells were exposed to 10 Gy of IR 36 h after transfection and harvested after 10 h. LUC activity was determined using a luminometer (Zylyx Corp., Maryville, TN). β-gal activity was determined (Promega Corp., Madison, WI), and the relative-fold induction of LUC activity was calculated by normalizing to the β-gal activity.

RESULTS

Induction of AP-1 DNA Binding Activity after Irradiation. c-Fos and c-Jun are members of a multigene family consisting of an array of heterodimeric protein complexes that bind the specific AP-1 cis-acting DNA regulatory elements. Given that AP-1 may be initiated by a variety of heterodimeric interactions, the effect of IR on AP-1 DNA binding activity was examined. To investigate this, AP-1 DNA binding activity was measured by performing EMSAs with extracts from HeLa and Jurkat cells. Serum-starved cells were irradiated with 10 Gy of IR and then allowed to recover for 1, 2, 3, 4,
REDOX REGULATION OF THIOREDOXIN AND Ref-1 ACTIVATION OF AP-1

and 6 h at 37°C before the cells were harvested using a subcellular fractionation technique. An increase in DNA binding activity in HeLa cells, as compared with nonirradiated cell extracts, was first noted at 1 h (Fig. 1A, Lane 1 versus Lane 2), reached a peak induction at 2 h (Lane 3), and returned to nearly baseline at 6 h (Lane 6). Supershift experiments with anti-Fos and anti-Jun antibodies and competition experiments with a cold AP-1 oligonucleotide confirmed the specificity of the AP-1 complex in this system (27). These experiments were repeated in Jurkat cells with a similar induction of AP-1 binding at 2–4 h after IR (Fig. 1B, Lane 1 versus Lanes 2–6). Transient assays with 2x-AP-1-LUC were also done and confirmed a similar in induction in AP-1-dependent gene expression (data not shown) as has been shown by others (10). Thus, an increase in AP-1 DNA binding activity is noted in both cell lines in response to IR.

**Radiation Induces Translocation of TRX from the Cytoplasm to the Nucleus.** Nuclear localization of TRX has been shown previously in cells treated with phorbol esters, which are known to induce oxidative stress (29). IR also produces free radical intermediates, such as superoxide (O₂⁻), hydroxyl radicals (•OH), and carbon centered radicals (•R) that are involved in the production of oxidative stress (26, 28, 36). In addition, TRX has been shown to increase AP-1 DNA binding activity of recombinant Fos and Jun proteins, as determined in a cell-free assay system (15, 31, 32). These results suggest that translocation of TRX into the nucleus may be one mechanism by which IR results in the induction of AP-1 DNA binding activity.

HeLa and Jurkat cells were irradiated, and the nuclear and cytoplasmic cellular extracts were prepared by a subcellular fractionation. Increases in nuclear TRX protein levels in response to IR were observed in both HeLa and Jurkat cells (Fig. 2A). These increases were first seen at 1 h after radiation (Fig. 2A, Lane 2), reached a maximum at roughly 3 h (Lane 4), and returned to nearly baseline at 6 h (Lane 7). In addition, HeLa cells with or without exposure to IR were examined by an indirect immunofluorescence method using antibodies raised against TRX. TRX was located predominantly in the cytoplasm of nonirradiated cells and appeared to accumulate in the nucleus 1 h after exposure to IR (Fig. 2B). Nuclear TRX was no longer seen after 6 h (data not shown). There appears to be a close temporal correlation between the nuclear localization of TRX and the induction of AP-1 DNA binding, as shown in Fig. 2A. These results indicate that IR may activate a cellular signaling pathway(s) that results in the translocation of TRX into the nucleus.

**TRX Induces AP-1 DNA Binding in HeLa Cells.** Although the results described above demonstrate TRX nuclear translocation in response to IR, they do not establish that TRX participates in the induction of AP-1 DNA binding activity after IR. The role of TRX in IR-induced activation of AP-1 DNA binding activity was initially examined by adding increasing amounts of cytoplasmic extract (containing TRX) from irradiated HeLa cells to a fixed amount of nuclear extract from nonirradiated HeLa cells. The presence of c-Fos, c-Jun, and Ref-1 protein in the nuclear fraction and the presence of TRX in the cytoplasmic fraction were confirmed using immunoblotting (data not shown). As shown in Fig. 3A, increasing the amount of the cytoplasmic fraction from irradiated cells appears to increase AP-1 DNA binding activity in a dose-dependent manner up to 4 μg of extract. The increase in DNA binding activity appears to be saturated above this amount. These results suggest that a cytoplasmic factor from irradiated cells increases AP-1 DNA binding activity in extracts from unirradiated cells. Because TRX is predominantly a cytoplasmic protein that is translocated to the nucleus upon exposure to IR (Fig. 2A), TRX may participate in the induction of AP-1 DNA binding after IR.

To further investigate the role of TRX in the activation of AP-1 DNA binding activity, TRX was immunoprecipitated from the cyto-

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**Fig. 1.** Analysis of AP-1 DNA binding activity in response to IR by EMSA. HeLa (A) and Jurkat (B) cells exposed to 10 Gy of IR and incubated at 37°C until harvest are shown. Ten μg of nuclear extracts were isolated and analyzed at 1, 2, 3, 4, and 6 h via EMSA using an AP-1-specific, [32P]labeled oligonucleotide. Arrows: AP-1 complex, free AP-1 oligonucleotide, and nonspecific (NS) binding. All results are presented as relative-fold increase above control, nonirradiated cell extracts (Lane 1).

**Fig. 2.** The effect of IR on nuclear TRX protein levels. A. Western blot analysis of nuclear TRX protein levels in response to IR using an anti-TRX antibody. HeLa (A, upper panel) and Jurkat (A, lower panel) cells were exposed to 10 Gy of IR, incubated at 37°C, and harvested via a subcellular fractionation method at 1, 2, 3, 4, and 6 h. Equal protein loading was determined using a Bradford protein assay. All results are presented as relative-fold increase above baseline control (C), nonirradiated cells. B. Indirect immunofluorescence cell staining with anti-TRX antibody. Left image: nonirradiated HeLa cell with TRX localized to the cytoplasm. Right image: HeLa cells 1 h after they were exposed to 10 Gy of IR, where staining for TRX is localized to the nucleus.
controls (EMSAs for AP-1 were performed as described). Lanes 7 and 8 EMSA was performed as above. EMSA with cytoplasmic extract alone are shown as controls. Lane 3 Control, IP protein A (Fig. 3B, nonirradiated HeLa cells, and EMSA was performed 40 min later. Lane 4 nonirradiated cells (Lane 3) and IP TRX/protein A alone (Lane 2) were combined with the results showing translocation of TRX to the nucleus (Fig. 2) is consistent with the concept that cytoplasmic TRX activates AP-1 DNA binding by passing an IR-induced signal from the cytoplasm to the nucleus. 

Redox Alterations in the TRX Protein That Regulates AP-1 DNA Binding Activity. To determine whether the oxidation/reduction status of TRX protein regulates AP-1 DNA binding activity, TRX was immunoprecipitated from irradiated cells and split into five equal fractions that were subsequently treated with various chemicals that alter protein thiol oxidation/reduction (redox) status. After three washing steps with 750 ml of E1A buffer and repelleting, the IP TRX was treated with diamide (a sulfhydryl oxidizing agent), NEM (a sulfhydryl alkylating agent), or DTT (a sulfhydryl reducing agent) for 30 min on ice and washed eight times to remove any residual chemical. The effects of adding TRX IP from HeLa cells to the control nuclear extract from nonirradiated cells that were not treated with chemicals are shown as a control (Fig. 4A, Lane 1). The addition of diamide or NEM to IP TRX decreased AP-1 DNA binding activity (Fig. 4A, Lanes 2 and 3). In contrast to treatment with diamide and NEM, treatment of the IP TRX with DTT increased AP-1 DNA binding compared with the control sample (Fig. 4A, Lane 1 versus Lane 4). Finally, a fraction of IP TRX was treated with diamide, washed eight times to remove any free chemical, and subsequently treated with DTT. This treatment appeared to restore the ability of the IP TRX to induce AP-1 DNA binding (Fig. 4A, Lane 1 versus Lane 3). These results suggest that alterations in the redox status of TRX play a central role in the regulation of AP-1 DNA binding after IR.

TRX Physically Interacts with Ref-1 and This Interaction Is Enhanced by IR. To determine whether TRX interacts with Ref-1 after IR, a transient transfection two-hybrid system using Cos-1 cells

A. 
Cytoplasmic extract - 1 2 4 6 8 2 6  
Nuclear extract + + + + + + + 

B. 
IP Thioredoxin (IR) - - + + 
Nuclear extract (Con) - + + + 

AP-1 complex → 
Free probe → 

Fig. 3. A, a cytoplasmic factor induces AP-1 DNA binding activity. Increasing amounts of cytoplasmic extract from irradiated HeLa cells were added to the nuclear extract from nonirradiated cells. Nuclear extract alone is shown as a control (Lane 1). Increasing amounts of cytoplasmic (1–8 μg) were added to 10 μg of nuclear extract (Lanes 2–6), and EMSA was performed as above. EMSA with cytoplasmic extract alone are shown as controls (Lanes 7 and 8). EMSA for AP-1 was performed as described. Arrows in B, AP-1 complex and free unbound AP-1 oligonucleotide. B, AP-1 DNA binding activity after addition of TRX to nuclear extracts from HeLa cells. IP TRX/protein A pellets from the cytoplasmic fraction of irradiated HeLa cells were added to nuclear extracts from control, nonirradiated cells (Lane 4). Nuclear extract alone (Lane 2) and IP TRX/protein A alone (Lane 3) are shown as controls.

plasmic extracts obtained from irradiated HeLa cells. The IP TRX/protein A pellet was combined with 10 μg of nuclear extract from nonirradiated HeLa cells, and EMSA was performed 40 min later. Control, IP protein A (Fig. 3B, Lane 1) and IP TRX (Lane 3), both without any nuclear extract, are shown as controls. Nonirradiated nuclear extract was also run as a control to establish the baseline AP-1 DNA binding activity (Lane 2) and is similar to the result shown in Fig. 3A, Lane 1. When cytoplasmic TRX IP from irradiated cells was added to nonirradiated nuclear cell extract, a marked increase in AP-1 DNA binding activity was seen (Fig. 3B, Lane 4). Western blot analysis of the IP TRX using anti-Ref-1 antibody demonstrated an absence of Ref-1 protein in the IP TRX (data not shown). This result combined with the results showing translocation of TRX to the nucleus (Fig. 2) is consistent with the concept that cytoplasmic TRX activates AP-1 DNA binding by passing an IR-induced signal from the cytoplasm to the nucleus.

Redox Alterations in the TRX Protein That Regulates AP-1 DNA Binding Activity. To determine whether the oxidation/reduction status of TRX protein regulates AP-1 DNA binding activity, TRX was immunoprecipitated from irradiated cells and split into five equal fractions that were subsequently treated with various chemicals that alter protein thiol oxidation/reduction (redox) status. After three washing steps with 750 ml of E1A buffer and repelleting, the IP TRX was

A. 
Diamide - + - - + + + + 
NEM - - + + + + + + 
DTT - - - + + + + + + 

B. 
AP-1 complex → 
Free probe → 

Fig. 4. A, the ability of IP TRX to regulate AP-1 DNA binding activity is altered by chemical agents that alter the oxidation or reduction status of TRX. Either diamide (Lane 2), NEM (Lane 3), or DTT (Lane 4) were added to the TRX/protein A complex IP from the cytoplasm of irradiated HeLa cells (2 h after IR) after pelleting. A fraction of IP TRX was treated with diamide, washed eight times, and then treated with DTT (Lane 5). IP TRX that did not receive any treatment with oxidizing or reducing agents is shown as control (Lane 1). EMSA for AP-1 was performed as described. Arrows, AP-1 complex and free unbound AP-1 oligonucleotide. B, TRX forms a physical association with Ref-1 that is enhanced by IR. Cells were transfected with pCMV-TRX-VP-16, pCMV-Gal-4-Ref-1, or both with ptk-3x-Gal-4-LUC as a reporter plasmid. pUC was used to standardize each transfection to a total of 30 μg of plasmid DNA. The relative-fold induction of LUC activity was normalized by β-gal activity via cotransfection of pSV-40-β-gal. The results are presented as relative-fold induction in LUC activity over the baseline (Lane 1). All results are the means of three separate experiments, all done in duplicate. The results of individual transfections varied by <25%.

6691
was performed. These transfections were performed using Ref-1/Gal-4 and TRX/VP-16 fusion proteins expressed by a CMV expression vector and a LUC reporter cassette containing three Gal-4 DNA binding sites (p3x-Gal-4tk-LUC; Ref. 29). Cotransfection of p3x-Gal-4tk-LUC with either CMV-Ref-1/Gal-4 or CMV-TRX/VP-16 showed no increase in LUC activity when compared with transfection with p3x-Gal-4tk-LUC alone (Fig. 4B, Lanes 3–4 and 5–6 versus Lanes 1–2). Cotransfection of p3x-Gal-4tk-LUC with both CMV-Ref-1/Gal-4 and CMV-TRX/VP-16 in nonirradiated cells resulted in a 6-fold increase in LUC activity (Fig. 4B, Lane 7). Ten h after exposure to 10 Gy of IR, an additional increase in LUC activity to 11-fold above baseline was observed (Fig. 4B, Lane 8). These results suggest that a physical interaction between TRX and Ref-1 occurs and that this interaction is enhanced in response to IR.

**Immunodepletion of Ref-1 Impairs Inducible, but not Basal, AP-1 DNA Binding Activity.** Ref-1 has been shown to regulate AP-1 activity through a redox-sensitive mechanism (27, 31, 32, 35); therefore, the role of Ref-1 in the regulation of IR-induced increases in AP-1 DNA binding activity was examined. Irradiated cellular extracts were depleted of Ref-1 by immunodepletion with anti-Ref-1 antibody to determine the role of Ref-1 in the induction of AP-1 DNA binding activity after IR. HeLa cell extracts from the experiments shown above (Fig. 1) were treated with the addition of protein A and anti-Ref-1 antibody, followed by gentle shaking at 4°C for 2 h and spinning at 12,000 rpm for 1 min to pellet out the Ref-1 antibody complex. Western blot analysis confirmed the removal of Ref-1 protein from the extracts as well as the presence of Ref-1 in the IP complex (data not shown). The continued presence of c-Fos/c-Jun proteins in the Ref-1-depleted extracts as well as the lack of c-Fos/c-Jun protein in the IP complex was established by Western analysis (data not shown).

Immunodepletion of Ref-1 from nonirradiated cells did not alter the levels of AP-1 DNA binding activity detected by EMSA (Fig. 5A, Lane 1 versus Lane 3). These results suggest that the presence of Ref-1 in the extracts was not required for AP-1 DNA binding activity in nonirradiated cells. Nuclear extracts from cells harvested 1 h after exposure to IR and immunoprecipitated with protein A only (Fig. 5A, Lane 2) were used as a positive control, and similar to Fig. 1 (Lanes 2–4), a significant increase in AP-1 DNA binding activity was observed. In contrast, no increase in AP-1 DNA binding activity was observed in nuclear extracts from irradiated cells immunodepleted of Ref-1 and isolated at 1 and 2 h after irradiation (Fig. 5A, Lanes 4 and 5). These results indicate that the presence of Ref-1 is required for the IR-induced increase in AP-1 DNA binding activity.

**Restoration of IR-induced AP-1 DNA Binding Activity after Addition of Ref-1 IP to Immunodepleted Extracts.** To confirm that Ref-1 from irradiated cells must be present in the HeLa cell extracts for IR-induced increases in AP-1 DNA binding activity to occur, IP containing Ref-1 from irradiated or nonirradiated cells was reintroduced to the Ref-1-immunodepleted extracts from irradiated cells harvested 2 h after IR (Fig. 5B). When Ref-1 IP from irradiated cells was added back to Ref-1 without extracts from irradiated cells (Fig. 5B, Lane 3), the IR-induced increase in AP-1 DNA binding was restored (Fig. 5B, compare Lane 3 with Lane 2). When Ref-1 IP from nonirradiated cells was added to Ref-1 without irradiated cell extracts (Fig. 5B, Lane 5), a small, but reproducible increase in AP-1 DNA binding was observed (Fig. 5B, compare Lane 5 with Lane 2). Interestingly, when Ref-1 IP from irradiated cells was added to Ref-1 without extracts from nonirradiated cells (Fig. 5B, Lane 4), AP-1 DNA binding increased to levels seen in cells exposed to IR (Fig. 5B, compare Lane 4 with Lane 1). Equal amounts of IP Ref-1 from control and irradiated cells were confirmed by Western analysis (data not shown). This ruled out the possibility that the induction of AP-1 DNA binding was attributable to increased levels of IP Ref-1 protein. In addition, Western blot analysis confirmed the continued presence of c-Fos and c-Jun proteins in the Ref-1-depleted extracts as well as the absence of c-Fos and c-Jun protein in the IP complex (data not shown). Finally, we confirmed that recombinant bacterially expressed His-tagged Ref-1 protein also activates AP-1 DNA binding activity (data not shown), as shown by others (31, 32). These results indicate that the presence of Ref-1 from IR-treated cells is required to activate inducible, but not basal, AP-1 DNA binding. These experiments are
consistent with previous studies investigating Ref-1 activation of AP-1 in response to oxidative stress, including 12-O-tetradecanoylphorbol-13-acetate and H$_2$O$_2$ (4, 10, 14).

**Redox Alterations in the Ref-1 Protein from Irradiated Cells Regulates AP-1 DNA Binding Activity.** In Fig. 4A, it was demonstrated that TRX regulation of AP-1 DNA binding is altered by chemical oxidation and reduction. It was also shown that TRX physically interacts with Ref-1. Thus, the role of redox status in Ref-1 regulation of AP-1 DNA binding activity was examined. Ref-1 from irradiated cells was immunoprecipitated and treated in the presence of either diamide (a sulfhydryl oxidizing agent), NEM (a sulfhydryl specific alkylating agent), or DTT (a sulfhydryl reducing agent) and washed eight times to remove free chemical. The IP Ref-1 was then added to nonirradiated Ref-1-immunodepleted cell extracts, and AP-1 DNA binding activity was examined. Ref-1 IP from irradiated cells without chemicals is shown as a control (Fig. 5C, Lane 1). The addition of diamide or NEM to IP Ref-1 from irradiated cells (Fig. 5C, Lanes 2 and 3) decreased AP-1 DNA binding activity. Ref-1 IP from irradiated cells and treated with DTT significantly increased AP-1 DNA binding compared with Ref-1 IP from irradiated cells that were not treated with chemicals (Fig. 5C, Lane 1 versus Lane 4). These results suggest that alterations in redox status of the Ref-1 protein from irradiated cells play a central role in the enhancement of AP-1 DNA binding activity after exposure to IR.

**DISCUSSION**

Stress-activated signaling cascades are tightly regulated cellular processes that allow eukaryotic cells to respond to environmental stress, including therapeutic modalities such as chemotherapy and radiotherapy (37–39). These signaling pathways and factors appear to sense stress-induced changes in the intracellular oxidation/reduction (redox) status, providing the cell the ability to mount a response to stressing agents through a series of thiol-containing molecules, such as TRX and Ref-1 (21, 29). These signaling pathways and transcription factors appear to play a major role in maintaining the steady-state intracellular balance between prooxidant production, antioxidant capacity, and the repair of oxidative damage.

Key contributors in altering the intracellular redox potential are ROIs. ROIs can be formed by a variety of extracellular stressing agents including multiple exogenous genotoxic agents, such as inflammatory cytokines, chemical carcinogens, chemotherapeutic agents, and irradiation (9, 26, 28). Alterations in steady-state levels of ROIs and the subsequent alteration of the intracellular redox potential are considered to be a primary mechanism regulating cellular signaling factors that link external stimuli with signal transduction in cellular response to stress (40). It has been shown that IR results in the generation of multiple ROIs including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH), and organic hydroperoxides (ROOH; Refs. 26 and 28). Because IR generates ROIs and activates redox-sensitive signaling factors, such as AP-1, the role of TRX and/or Ref-1 in bridging the physical barrier between the cytoplasm and the nucleus and regulating the induction of AP-1 in response to IR was examined.

The current work confirms that TRX induces AP-1 DNA binding activity. In addition, subcellular fractionation demonstrated that in response to IR, TRX is transported into the nucleus, and this result was confirmed via indirect immunofluorescence cell staining. Furthermore, TRX IP from irradiated cells added to nonirradiated nuclear extracts resulted in activation of AP-1 DNA binding activity. It was also shown that the ability of TRX to stimulate AP-1 DNA binding activity was abolished by chemical oxidation and increased by chemical reduction. The results of these experiments strongly suggest that TRX becomes reduced and is transported from the cytoplasm to the nucleus in response to IR, resulting in the induction of AP-1 DNA binding activity. For many years, it has been known that exposure to IR and other oxidants results in an immediate increase in NADPH pools caused by stimulation of glucose metabolism through the pentose cycle (34). Because NADPH pools coupled with TRX reductase provide the reducing equivalents for TRX, these results are consistent with the hypothesis that IR-induced stimulation of NADPH production could be linked to radiation-induced signal transduction through redox changes in TRX (Fig. 6).

EMSA extracts immunodepleted of Ref-1 protein demonstrated that the increase in AP-1 DNA binding activity after IR is dependent upon the presence of Ref-1 and that Ref-1 regulates inducible, but not basal, AP-1 DNA binding activity. This result was confirmed by the restoration of IR-inducible AP-1 DNA binding upon addition of Ref-1 IP from irradiated cells to immunodepleted extracts. Similar to TRX, the ability of Ref-1 to stimulate AP-1 DNA binding was abolished by chemical oxidation and increased by chemical reduction. These results suggest that changes in the oxidation/reduction status of both TRX and Ref-1 play a role in the induction of AP-1 DNA binding activity in response to IR but does not show a linear pathway from TRX to Ref-1 regulating the DNA binding of AP-1. To support this, transient transfection two-hybrid assays were performed that demonstrated physical interaction between TRX and Ref-1 in the nucleus and that this interaction is increased after IR. The increase in LUC activity observed in the cotransfection with p3x-Gal-4/tk-LUC, CMV-Ref-1/ Gal-4, and CMV-TRX/VP-16 in nonirradiated cells may result from leakage of the TRX/VP-16 fusion protein from the cytoplasm into the nucleus attributable to the VP-16 nuclear localization sequence. Overall, these results suggest that in response to IR a change in the redox...
state of TRX may result in the regulation of the DNA binding activity of the AP-1 transcription factor via the interaction of Ref-1 and TRX. Although the activation of transcription factors, such as AP-1, ultimately occurs within the nucleus, a long-standing hypothesis in radiation biology states that the initiation of cellular signaling pathways resulting from irradiation exposure occurs at the cytoplasmic membrane (4, 9, 14). This raises several interesting questions regarding the cytoplasmic factors and pathways activated by IR as well as the specific factors that pass the signal from the cytoplasm to the nucleus. One mechanism of transcriptional regulation involves the movement of specific signaling factors from one subcellular location to another. Two examples of this are the extracellular signal-regulated kinases, also known as mitogen-activated protein kinases, and nuclear factor-xB, both of which are rapidly transported to the nucleus in response to exposure to IR (3, 41). The results presented in this report identify TRX as another signaling factor that is transported to the nucleus in response to IR, resulting in the activation of nuclear transcription factors.

One of the intracellular functions of TRX is to inhibit protein-nucleic acid interactions of nuclear transcription factors (29). TRX has two redox-active cysteine residues, -Cys-Gly-Pro-Cys-, in an active center and participates in redox reactions through the reversible oxidation/reduction of these thiol residues (16, 42, 43). In this regard, TRX serves as a source of reducing equivalents to alter protein function and subsequently regulate nuclear transcription factor activity. This suggests that alterations in intracellular oxidation/reduction reactions may induce alterations in the redox state of TRX initiating a signaling cascade (19, 44) in response to a variety of environmental stresses.

Once TRX has reached the nucleus, at least one mechanism regulating AP-1 DNA binding activity involves the nuclear redox-sensitive signaling protein Ref-1 (3, 35). This signal transduction appears to occur through a critical cysteine residue (motif), located in the basic DNA binding domain of c-Fos/c-Jun, that is flanked by the basic amino acids lysine and arginine (KCR). The redox status of this critical cysteine plays a central role in the regulation of c-Fos/c-Jun (AP-1) DNA binding activity (3, 15, 31, 32, 45). Interestingly, genetic amino acids lysine and arginine (KCR). The redox status of this DNA binding domain of c-Fos/c-Jun, that is flanked by the basic cysteine residues that are required for functional AP-1 DNA binding activity involves the nuclear redox-sensitive sites (1, 15).

In this model, the redox status of these critical cysteine residue(s) is strongly influenced by its local protein microenvironment (47). Subtle changes in cellular redox potentially alter the ionization state of the cysteine sulfur molecule (-CH₂-SH to -CH₂-S⁻), resulting in profound changes in protein activity (23). The critical cysteine(s) would act as a redox-sensitive “sulfhydryl switch” that reversibly modulates protein activity (31, 32). The redox potential then could be passed along specific signal transduction cascades using proteins with redox-sensitive sites (1, 15).

The results presented in this report are consistent with the model that IR results in environmental stress and the formation of ROSs that initiates a cellular response pathway at the level of the cytoplasmic membrane. IR has also been shown to stimulate NAPDH production through the pentose pathway and as such represents a possible mechanism linking IR-induced changes in metabolism to activation of transcription factors via TRX and Ref-1 (Fig. 6; Ref. 28). In this model, IR induction of NAPDH levels may activate a pathway involving TRX reductase that is upstream of TRX (Fig. 6). Because reduced, but not oxidized, TRX activates AP-1 DNA binding activity (Fig. 4A, Lanes 2 and 3 versus Lane 4), it would seem logical to suggest that an upstream factor such as TRX reductase reduces TRX prior to nuclear translocation. Reduced TRX interacts with Ref-1 (Fig. 4B) and passes a redox signal to Ref-1, which must also be in a reduced state to activate AP-1 DNA binding (Fig. 5C). Finally, Ref-1 appears to pass a redox signal to the AP-1 complex to induce DNA binding activity (Fig. 6). The results of these experiments suggest a stress-activated, redox-sensitive signaling pathway involving proteins containing critical cysteine residues that are required for functional activity (Fig. 6). The potential linkage between stress-activated metabolic pathways for increasing NADPH synthesis and TRX/Ref-1 also provides a plausible explanation for why IR and other oxidants can activate transcription factors (i.e., AP-1) that require reduction to become activated in vitro.

The discovery that TRX is a redox-sensitive cytoplasmic signaling factor activating AP-1 in response to IR identifies TRX as a potential target for radio-modifying agents. Furthermore, the subsequent determination of signaling factors upstream of TRX may also identify cellular targets for altering the inherent radiosensitivity of tumor cells. Finally, TRX nuclear translocation and interaction with Ref-1 firmly establishes a link between cytoplasmic events after radiation, resulting in the activation of nuclear transcription factors that potentially govern cellular responses to IR. Taken together with previous investigations (21), these results support the concept that a common central pathway(s) mediating cellular responses to IR or other types of environmental or metabolic oxidative stress may involve redox-sensitive signaling pathways leading to AP-1 activation via Ref-1 and redox-sensitive cytoplasmic signaling factors such as TRX and TRX reductase.

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Thioredoxin Nuclear Translocation and Interaction with Redox Factor-1 Activates the Activator Protein-1 Transcription Factor in Response to Ionizing Radiation

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