

Prx1 Suppresses Radiation-Induced c-Jun NH₂-Terminal Kinase Signaling in Lung Cancer Cells through Interaction with the Glutathione S-Transferase Pi/c-Jun NH₂-Terminal Kinase Complex

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Abstract

Radiotherapy is one of the major treatment modalities for lung cancer. Cell killing by ionizing radiation is mediated primarily through the reactive oxygen species (ROS) and ROS-driven oxidative stress. Prx1, a peroxiredoxin family member, was shown to be frequently elevated in lung cancer cells and tissues. Although the antioxidant function of Prx1 is expected to affect the radiotherapy response of lung cancer, the physiologic significance of its peroxidase activity in irradiated cells is unclear because the catalytic Cys⁵² is easily inactivated by ROS due to its overoxidation to sulfinic or sulfonic acid. In this study, we investigated the role of Prx1 in radiation sensitivity of human lung cancer cells, with special emphasis on the redox status of the catalytic Cys⁵². We found that overexpression of Prx1 enhances the clonogenic survival of irradiated cells and suppresses ionizing radiation-induced c-Jun NH₂-terminal kinase (JNK) activation and apoptosis. The peroxidase activity of Prx1, however, is not essential for inhibiting JNK activation. The latter effect is mediated through its association with the glutathione S-transferase pi (GSTpi)-JNK complex, thereby preventing JNK release from the complex. Reduced JNK activation is observed when the peroxidase activity of Prx1 is compromised by Cys⁵² overoxidation or in the presence of the Cys⁵² to Ser⁵² mutant (Prx1C52S) lacking peroxidase activity. We show that both Prx1 and Prx1C52S interact with the GSTpi-JNK complex and suppress the release of JNK from the complex. Our study provides new insight into the antiapoptotic function of Prx1 in modulating radiosensitivity and provides the impetus to monitor the influence of Prx1 levels in the management of lung cancer. (Cancer Res 2006; 66(14): 7136-42)

Introduction

Lung cancer is a lethal disease with a mortality rate of at least 120,000 death per year in the United States (1). Despite advances in the understanding of the molecular/genetic basis of lung cancer and improvement in therapy, overall survival has not increased during the past 20 years. Radiotherapy is one of the major treatment modalities of non-small cell lung cancer, which represents >80% of total pulmonary malignancies. Because radiation-induced cytotoxicity is mediated primarily by the generation of reactive oxygen species (ROS) and ROS-driven

oxidative stress, the role of ROS-scavenging proteins has come under intensive investigation because their modification might severe as an approach to enhance the efficacy of radiotherapy.

Peroxiredoxins are thiol-specific antioxidant proteins. They are found in mammals, yeast, and bacteria and are classified largely based on having either one (1-Cys) or two (2-Cys) conserved cysteine residues (2). Prx1 is a major 2-Cys peroxiredoxin family member. The catalytic mechanism of the 2-Cys peroxiredoxins is unique among the peroxide-detoxifying enzymes. For example, glutathione peroxidase reduces peroxides while oxidizing a cosubstrate (in this case, glutathione). Prx1 contains a cysteine at the catalytic site (Cys⁵²) and detoxifies peroxides at the expense of Cys⁵² oxidation through intermolecular disulfide formation with the other conserved Cys¹⁷³ residue. The disulfide bond is reduced back to the active thiol form (Cys-SH) by various mechanisms (2–4). We and others previously reported that Prx1 is frequently elevated in human lung cancer cells and tissues (5–9). Based on the observations in cultured cells and animals, a survival enhancing function of Prx1 has been proposed.

Although the antioxidant activity of Prx1 may increase radioresistance, the physiologic significance of the peroxidase function of Prx1 is unclear because it is highly susceptible to inactivation by oxidative stress. Peroxiredoxins have been shown to be converted to the oxidized form as a result of the Cys-SH overoxidation to Cys-sulfinic (Cys-SO₂H) or Cys-sulfonic acid (Cys-SO₃H) during peroxide detoxification (10–13). When the catalytic Cys⁵² of Prx1 is overoxidized, the peroxidase activity is lost. Recent studies suggest that the active site Cys overoxidation in itself may be physiologically significant because it allows a mechanism of structural and functional switching of 2-Cys peroxiredoxin from a peroxidase enzyme to a molecular chaperone under stress conditions (14, 15). The above hypothesis is consistent with the behavior of Prx1 interacting physically with various cellular proteins to modulate their activities. This newly identified role of Prx1 is independent of its peroxide detoxifying function. Using a yeast two-hybrid system, the interaction of Prx1 with the Src homology-3 domain of c-Abl (16), the Myc Box II domain of c-Myc (17), and the macrophage inhibiting factor, MIF (18) has been shown. The interaction of Prx1 with growth regulatory proteins may in part be responsible for the wide range of effects attributed to Prx1 (19–23).

This study was undertaken to address the physiologic relevance of the active site Cys redox status and/or the antioxidant function of Prx1 in regulating radiation sensitivity of human lung cancer cells. We report our work showing that Prx1 suppression of c-Jun NH₂-terminal kinase (JNK) signaling and apoptosis in irradiated human lung cancer cells is mediated through interaction of Prx1 with the glutathione S-transferase pi (GSTpi)-JNK complex. Furthermore, the decreased JNK activation by Prx1 is independent of the antioxidant activity of Prx1.

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Materials and Methods

Cell culture and radiation treatment. Human lung cancer 1170i cells (24) were maintained in RPMI 1640 supplemented with 10% (v/v) bovine calf serum and antibiotics and were grown at 37°C in an atmosphere of 5% CO₂ and 95% air. Cells in logarithmic growth were exposed to ionizing radiation using a ¹³⁷Cs Gammacell 40 Irradiator (MDS Nordion, Ontario, Canada) at a dose of 2, 5, or 10 Gy. After irradiation, cells were returned to the incubator and cultured for various lengths of time as indicated below.

Construction of adenoviral vectors. The entire coding region of the human Prx1 gene from Prx1/pET-17b (25) was subcloned into the pCR3.1 expression vector (Invitrogen, San Diego, CA). Site-directed mutagenesis of Cys⁵² to Ser⁵² was done to generate Prx1C52S using the Quikchange mutagenesis kit (Stratagene, La Jolla, CA). The substitution was confirmed by DNA sequencing. The *Xba*I/*Hind*III fragment released from Prx1/pCR3.1 or Prx1C52S/pCR3.1 was subcloned into the *Xba*I/*Hind*III site of the adenoviral shuttle vector pAdlox. Recombinant adenoviruses were constructed essentially by employing the *Cre-lox* recombination system (26). In brief, by selecting against ψ 5, we generated a recombinant adenovirus carrying either Prx1 or Prx1C52S in place of the *E1* gene by cotransfecting a shuttle vector with a *loxP* site (pAdlox) and ψ 5 DNA into CRE8 cells. Transfections were done by using the LipofectAMINE plus reagent (Invitrogen). Cells (5×10^5) were split into a six-well plate 1 day before transfection. For the production of recombinant adenovirus, 2 μ g each of *Sfi*I-digested Adlox/Prx1 fragment or Adlox/Prx1C52S was cotransfected with 2 μ g of ψ 5 viral genomic DNA into CRE8 cells according to the manufacturer's instructions. The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and ψ 5 viral DNA. Plaques were harvested and purified, and multiplicity of infection (MOI) was determined.

Western blot analysis. Cells were rinsed thrice with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-Cl (pH 7.4), 1% NP40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL each of aprotinin and leupeptin, and 1 mmol/L Na₃VO₄]. After centrifugation at 12,000 \times g for 30 minutes, the supernatant was collected, and protein concentration was determined using the Lowry method (27). Equal amounts of proteins [10 μ g for Prx1, GSTpi, poly(ADP-ribose) polymerase (PARP), and β -actin or 30 μ g for phosphorylated JNK (pJNK) and JNK] were separated on 12% SDS-PAGE gel and blotted onto nitrocellulose membranes. The blots were incubated with anti-Prx1 (Lab Frontier, Seoul, Korea), GSTpi (Calbiochem, La Jolla, CA), PARP (Cell Signaling, Beverly, MA), pJNK (Santa Cruz Biotechnology, Santa Cruz, CA), JNK (Santa Cruz Biotechnology), and β -actin (Sigma, St. Louis, MO) antibodies. Detection of immunoreactive bands was done by using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ). The experiments were repeated at least thrice.

Immunoprecipitation. Immunoprecipitation was carried out with 500 μ g of cell lysates and 1 μ g of anti-GSTpi, anti-JNK, or anti-Prx1 overnight at 4°C. After the addition of 25 μ L of Protein G-agarose (Santa Cruz Biotechnology), the lysates were incubated for an additional 4 hours. To validate specific protein interactions, rabbit IgG (Santa Cruz Biotechnology) or mouse IgG (Santa Cruz Biotechnology) was used as negative control. The beads were washed thrice with the lysis buffer, separated by SDS-PAGE, and immunoblotted with GSTpi, JNK, or Prx1 antibodies. The proteins were detected with the ECL system (Amersham Biosciences).

Clonogenic survival assay. To determine the effect of radiation on clonogenic survival, cells were irradiated at different doses of ionizing radiation. Clonogenic survival was determined as previously described (28). Colonies were stained with crystal violet after 12 days, and colonies containing ≥ 50 cells were counted. Statistical analysis was done by Student's *t* test.

Apoptosis assay. Apoptosis was monitored by flow cytometry analysis with the Yo-Pro-1/propidium iodide Vybrant staining kit (Molecular Probes, Eugene, OR) and by microscopic observation with the Annexin V-Cy3.18 kit (Sigma). Statistical analysis was done by Student's *t* test.

Two-dimensional electrophoresis and two-dimensional Western blot. Two-dimensional electrophoresis was done as previously described

with minor modifications (29). In brief, nonlinear pH 3 to 10 gradient strips (Amersham Biosciences) were used for isoelectric focusing. The samples were applied onto the strips by overnight in-gel rehydration with a thiourea-urea mixture as a denaturing agent. The gels were then migrated in an IPGphor II apparatus (Amersham Biosciences) up to 20,000 V hour. The immobilized pH gradient strips were then equilibrated in SDS buffer and placed on top of a 12% SDS-PAGE. After migration, the gels were transferred to nitrocellulose membrane and probed with Prx1 (Lab Frontier) or Prx-SO_{2/3}H (Lab Frontier) antibody to determine Prx1 oxidation status.

Expression and purification of recombinant proteins. Recombinant human Prx1 and Prx1C52S proteins were purified by sequential ion exchange chromatography and size exclusion chromatography following the previously described procedure (30). The cell extract was loaded onto DEAE-Sepharose (GE Healthcare, Piscataway, NJ) and equilibrated with 20 mmol/L Tris-Cl (pH 7.5). The unbound fractions were dialyzed with 50 mmol/L sodium phosphate buffer (pH 6.5). The dialyzed proteins were loaded onto SP-Sepharose (GE Healthcare) and equilibrated with 50 mmol/L sodium phosphate buffer (pH 6.5). The bound proteins were eluted with a linear gradient of sodium chloride. The fractions containing Prx1 or Prx1C52S were pooled, loaded onto Superdex 200 (16/60, GE Healthcare), and equilibrated with 50 mmol/L sodium phosphate buffer (pH 7) containing 0.1 mol/L NaCl. The fractions containing Prx1 or Prx1C52S were pooled and stored at -80°C. Yeast thioredoxin and thioredoxin reductase proteins were prepared following the previously described procedure (31).

Preparation of oxidized Prx1. To generate oxidized Prx1 *in vitro*, we followed a published method (32) with minor modifications. A quantity of 500 μ g of Prx1 was incubated in 50 mmol/L Tris-Cl (pH 7.5) containing 100 μ g of thioredoxin, 1 mmol/L EDTA, and 10 mmol/L DTT. Oxidation was started by the addition of 20 μ L of 200 mmol/L H₂O₂ to the 1 mL reaction mixture. After 30 minutes at room temperature, another 20 μ L aliquot of H₂O₂ was added, and this procedure was repeated four times. To remove thioredoxin, the oxidized protein was mixed with Q-Sepharose (GE Healthcare), equilibrated with 50 mmol/L Tris-Cl (pH 7.5), and centrifuged. The supernatant was collected and dialyzed with 50 mmol/L sodium phosphate buffer (pH 7) containing 0.1 mol/L NaCl. The oxidation of Cys⁵² at the catalytic site was confirmed by Western blot with the Prx-SO_{2/3}H antibody (Lab Frontier).

Peroxidase assay. The peroxidase activity of the purified recombinant proteins was measured by using an established procedure (31). Briefly, 1 μ g of Prx1 was incubated in 50 mmol/L HEPES (pH 7) containing 200 μ mol/L NADPH, 3 μ mol/L thioredoxin, and 1.5 μ mol/L thioredoxin reductase. The mixture was incubated at 30°C for 5 minutes, and H₂O₂ was added. NADPH oxidation was monitored at 30°C by following absorbance reduction at 340 nm.

***In vitro* Prx1 and GSTpi pull-down assay.** *In vitro* pull-down assay was done as described previously with minor modifications (17). To examine the interaction between Prx1 and GSTpi, 0.5 μ g each of purified Prx1, Prx1C52C, or oxidized Prx1 was incubated with an equal amount of GSTpi (Oxford Biomedical Research, Oxford, MI) in 20 mmol/L Tris-Cl (pH 7.4) at 4°C overnight. After 1 day, Prx1 or rabbit IgG antibody was added to the Prx1-GSTpi reaction mixture. Prx1 or rabbit IgG antibody pull-down assays were carried out. The pull-down proteins were probed for GSTpi in the presence or absence of DTT.

Statistical analysis. Statistical significance was examined using Student's *t* tests. The two-sample *t* test was used for two-group comparisons. Values were reported as mean \pm SD. *P* values < 0.05 were considered significant and indicated by asterisks in the figures.

Results

Prx1 increases clonogenic survival and reduces apoptotic cell death. To investigate the role of Prx1 elevation in radioresistance of human lung cancer cells, we generated an adenoviral Prx1 expression vector Ad.Prx1. Empty vector without Prx1 insertion (Ad.con) was also generated to serve as a control. Figure 1A shows the Western blot data of the adenoviral vector-driven Prx1

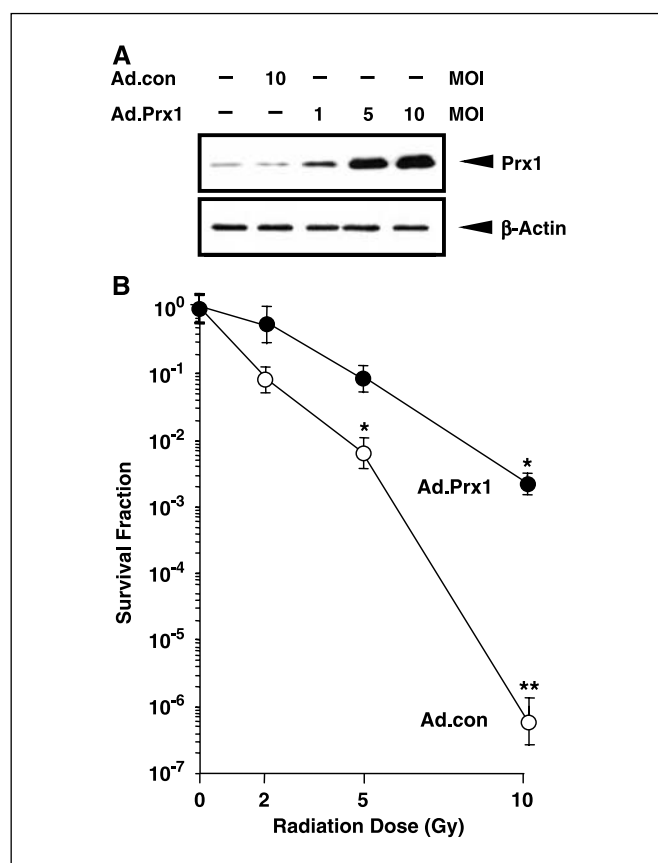


Figure 1. Prx1 overexpression increases clonogenic survival of irradiated cells. **A**, human lung cancer 1170i cells were infected with Ad.con or Ad.Prx1 at various MOI. At 24 hours after infection, cells were harvested, and equal amounts of proteins (10 μ g) were subjected to Western blot analysis using Prx1 antibody as a probe. Analysis of β -actin expression was done as a loading control. **B**, cells infected with Ad.con or Ad.Prx1 were exposed to increasing doses of ionizing radiation. Cell survival was determined by clonogenic assay. Points, mean of three independent experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$, compared with the respective unirradiated controls.

expression in 1170i cells as a function of MOI. Because cytopathic effects were noted when the MOI was >10 for both Ad.con and Ad.Prx1 vectors, all subsequent experiments were done using viral vectors at MOI of 5. As shown in Fig. 1B, when Ad.con- or Ad.Prx1-infected cells were exposed to increasing doses of ionizing radiation, increased clonogenic survival was observed in Prx1 overexpressing cells. The results suggest that Prx1 plays a role in enhancing cell survival following radiation treatment.

To test the effect of Prx1 on ionizing radiation-induced apoptosis, the Ad.con- or Ad.Prx1-infected cells were irradiated at 5 Gy. At 24 hours after irradiation, flow cytometry was done after staining the cells with Yo-Pro1 and propidium iodide. Typical dot plots of the analysis are shown in Fig. 2A. Staining cells simultaneously with Yo-Pro-1 and propidium iodide allows the resolution of intact cells (double negative), early apoptotic cells (Yo-Pro-1 positive and propidium iodide negative), and late apoptotic/necrotic cells (double positive). A histogram showing the mean value of three independent determinations is presented in Fig. 2B. Overexpression of Prx1 reduced apoptotic cell death markedly upon exposure to ionizing radiation. Because the activation of JNK or p38 mitogen-activated protein kinase (MAPK) has been shown to mediate oxidative stress-induced apoptosis

(33–35), we also looked at the effect of Prx1 on the activation of these MAPKs in irradiated cells. Our results showed that ionizing radiation stimulated JNK phosphorylation, which peaked at 6 hours and remained elevated at 24 hours after irradiation (Fig. 2C). Consistent with the apoptosis analysis, Prx1 overexpression significantly suppressed JNK activation and PARP cleavage in irradiated cells (Fig. 2C). Ionizing radiation did not stimulate the activation of p38 MAPK (data not shown).

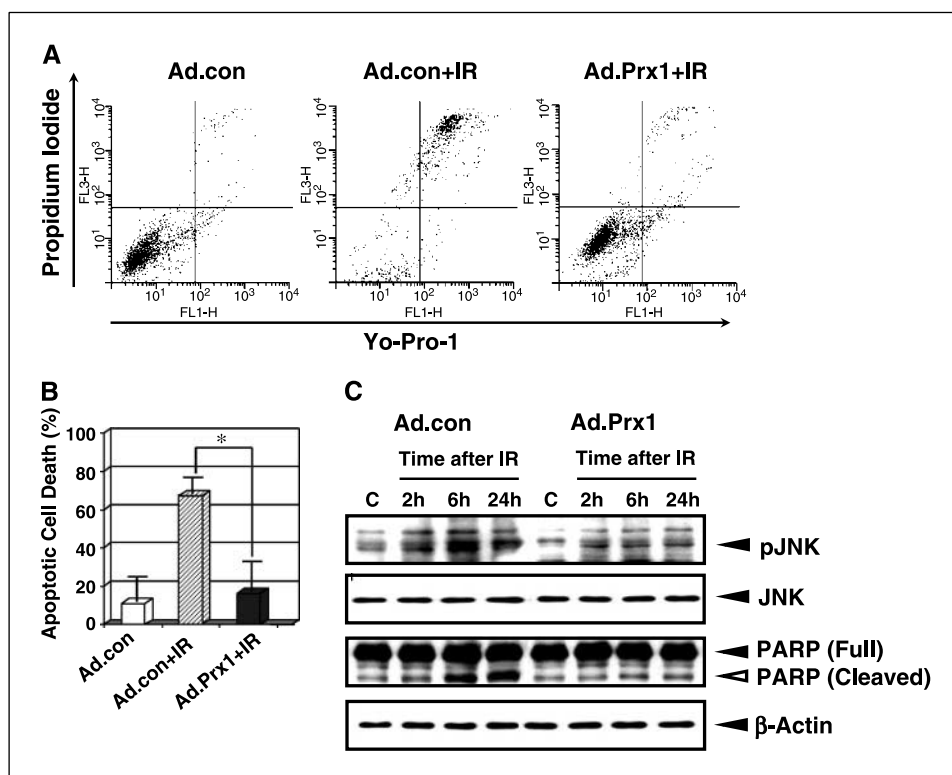
JNK inhibitory function of Prx1 is independent of its antioxidant activity. When we examined the oxidation status of Prx1, we found that the active site Cys⁵² was oxidized and inactivated in both Ad.con- and Ad.Prx1-infected cells after irradiation. Figure 3A shows the two-dimensional Western blots of Ad.Prx1-infected cells harvested at 6 hours after 5 Gy irradiation. Oxidation of Prx1 resulted in an acidic shift of the two-dimensional Western blot when probed with the Prx1 antibody (Fig. 3A, left). Oxidation of the active site Cys⁵²-SH to Cys⁵²-SO_{2/3}H was further confirmed by using the Prx SO_{2/3}H antibody, which specifically recognizes the oxidized Cys⁵² (Fig. 3A, right).

To confirm the lack of antioxidant activity of oxidized Prx1, we subcloned human Prx1 cDNA into a bacterial expression vector, purified the Prx1 protein, and oxidized it *in vitro*. As shown in Fig. 3B, Prx1 was susceptible to oxidation upon exposure to as low as 50 μ mol/L H₂O₂. The peroxidase activity of the wild-type and oxidized Prx1 was monitored by following the decrease in absorbance at 340 nm resulting from NADPH oxidation coupled to H₂O₂ reduction. When we tested the peroxidase activity of the oxidized Prx1, we were able to verify the complete loss of activity (Fig. 3C). Additionally, we generated mutant Prx1 by replacing the catalytic site Cys⁵² with Ser⁵² (Prx1C52S). As shown in Fig. 3C, Prx1C52S, which served as a negative control, also failed to exhibit any peroxidase activity.

We then constructed the adenoviral vector (Ad.C52S) to test directly if mutant Prx1 lacking antioxidant activity can modulate radiosensitivity. As shown in Fig. 3D, when Prx1C52S was overexpressed, decreased JNK phosphorylation and PARP cleavage were evident in irradiated cells, showing that the antioxidant activity is not essential for the JNK inhibitory function of Prx1. Importantly, the antiapoptotic effect of Prx1C52S was comparable with that seen in the wild-type Prx1 overexpressing cells. These results confirmed the antioxidant activity and the JNK inhibitory function of Prx1 are dissociated from each other. Because the JNK inhibitory effect of Prx1 was most pronounced at 6 hours after ionizing radiation, subsequent experiments were analyzed at this time point.

Ionizing radiation stimulates JNK release/activation from the GSTpi-JNK complex. GSTpi has been shown to play an important role in suppressing JNK activation by forming the GSTpi-JNK complex (36–38). When cells are exposed to UV or H₂O₂, GSTpi is oxidized, thereby triggering a dissociation of the GSTpi-JNK complex and releasing JNK for activation. First, we examined whether the GSTpi-JNK complex is detectable in human lung cancer cells, and whether ionizing radiation stimulates JNK release from GSTpi-JNK. We carried out GSTpi immunoprecipitation before and after exposure to ionizing radiation. As shown in Fig. 4A, when the GSTpi antibody was used for immunoprecipitation, JNK was coimmunoprecipitated with GSTpi. The interaction of GSTpi and JNK, however, was reduced significantly when cells were irradiated. Reciprocal immunoprecipitation using JNK antibody confirmed the presence of GSTpi-JNK in control cells and showed that ionizing radiation stimulated a dissociation of the

Figure 2. Prx1 overexpression reduces apoptosis and inhibits JNK activation. **A**, apoptotic cell death was analyzed at 24 hours after 5 Gy irradiation by flow cytometry of Yo-Pro1- and propidium iodide-stained cells. Intact cells (double negative, *bottom left quadrant*); early apoptotic cells (Yo-Pro1 positive and propidium iodide negative, *bottom right quadrant*); late apoptotic/necrotic cells (double positive, *top right quadrant*). Ad.con-infected cells served as the control. **B**, comparison of percent apoptotic cell death (early and late) at 24 hours after ionizing radiation (IR). *, $P < 0.05$, compared with Ad.con-infected irradiated cells. **C**, Western blots of pJNK, total JNK, and PARP cleavage at different times after 5 Gy irradiation. Analysis of β -actin expression was done as a loading control.



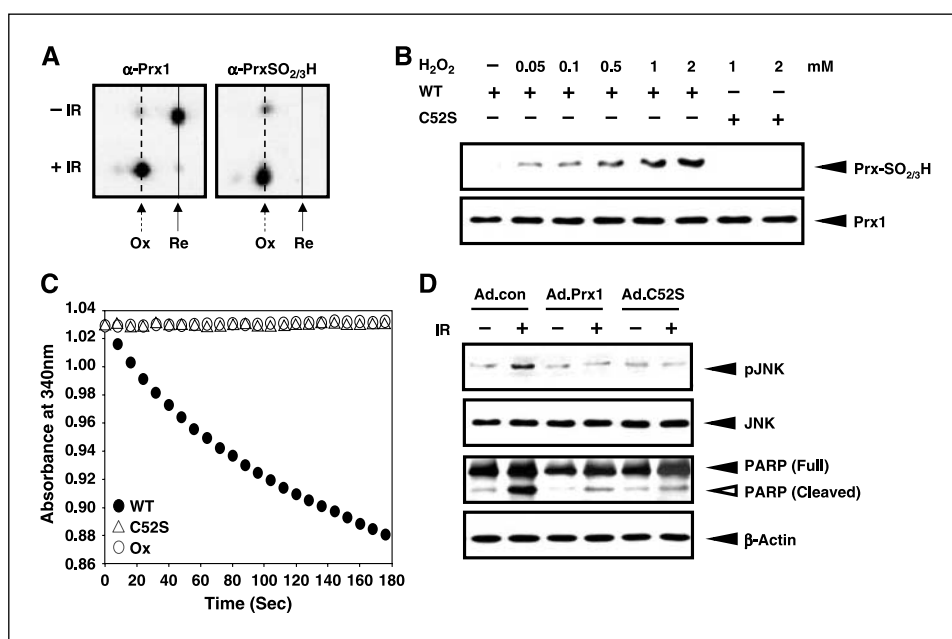
GSTpi-JNK complex, resulting in JNK release (Fig. 4B). Interestingly, our results also indicated a possible association of endogenous Prx1 with the GSTpi-JNK complex. This was supported by the coimmunoprecipitation of Prx1 with the GSTpi or JNK in control, unirradiated cells. When cells were irradiated, the interaction of endogenous Prx1 with GSTpi was still detectable, whereas the interaction with JNK completely disappeared.

Prx1 overexpression suppresses JNK release/activation from the GSTpi-JNK complex.

Next, we tested if Prx1 overexpression

reduces JNK release from the GSTpi-JNK complex. We found that ionizing radiation-stimulated JNK release was significantly diminished in cells overexpressing Prx1 (Ad.Prx1). Figure 5 shows the results of immunoprecipitation with GSTpi or JNK antibody. Prx1 overexpression suppressed the dissociation of the GSTpi-JNK complex. However, Prx1 remained associated with GSTpi-JNK in irradiated cells when Prx1 was overexpressed. The results support the idea that the Prx1 elevation may strengthen/stabilize the GSTpi-JNK complex, thereby preventing JNK release. Overexpression of

Figure 3. Peroxidase and antiapoptotic activities of wild-type Prx1, oxidized Prx1, and Prx1C52S. **A**, Ad.Prx1-infected cells were harvested before ($-IR$) or 6 hours after irradiation ($+IR$). Oxidation status of Prx1 was analyzed by two-dimensional gel electrophoresis followed by immunoblot using Prx1 antibody (*left*). The Prx1 antibody recognizes both oxidized (Ox) and reduced (Re) forms of Prx1. Note the acidic shift of oxidized Prx1. Oxidation status of the active site Cys⁵² was probed by using Prx-SO_{2/3}H antibody (*right*). **B**, reaction mixtures containing 0.1 μ g of recombinant Prx1 (WT) or Prx1C52S (C52S) proteins were exposed to H₂O₂ for 30 minutes. The oxidation status of the active site Cys⁵² was confirmed by using Prx-SO_{2/3}H antibody. **C**, reaction mixtures containing 0.1 μ g of recombinant Prx1 (WT), *in vitro* overoxidized Prx1 (Ox), or Prx1C52S (C52S) were assayed for peroxidase activity, which was monitored as a decrease in absorbance at 340 nm (A_{340}). The assay is described in Materials and Methods. **D**, cells infected with Ad.con, Ad.Prx1, or Ad.C52S were irradiated at 5 Gy. Cells were harvested at 6 hours after irradiation and processed for Western blot analysis to probe for JNK activation and PARP cleavage.



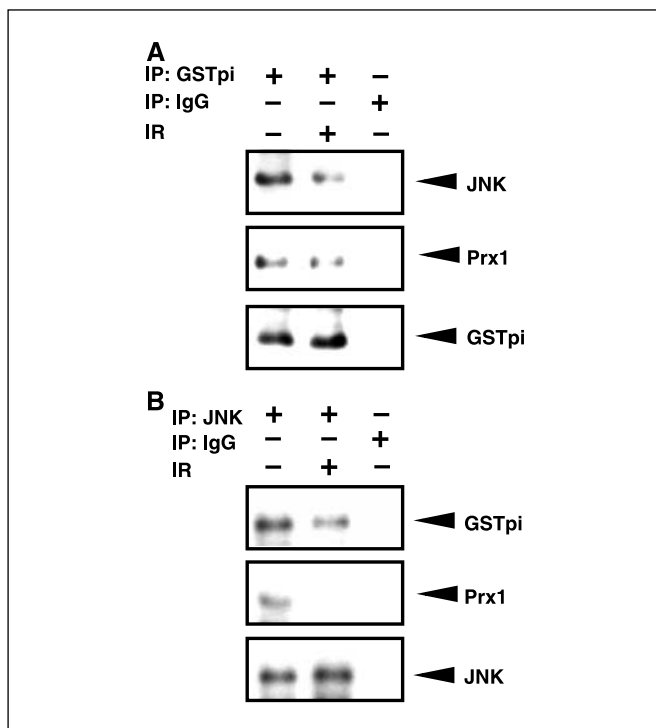


Figure 4. Effect of ionizing radiation (IR) on the GSTpi-JNK complex. *A*, cells were harvested at 6 hours after 5 Gy irradiation. Equal amounts of proteins (500 μ g) were subjected to immunoprecipitation (IP) with GSTpi antibody. Rabbit IgG immunoprecipitation was done as negative control. The immunoprecipitates were probed for the presence of JNK or Prx1 by Western blot. GSTpi Western blot was performed as a loading control. *B*, aliquots of proteins (500 μ g) were subjected to reciprocal immunoprecipitation with JNK antibody. Mouse IgG immunoprecipitation was done as negative control. The immunoprecipitates were probed for the presence of GSTpi or Prx1 by Western blot. JNK Western blot was done as a loading control.

Prx1C52S also suppressed the dissociation of JNK from the GSTpi-JNK complex as effectively as wild-type Prx1 in irradiated cells. These findings clearly showed that the antioxidant activity of Prx1 is not essential for the JNK inhibitory effect of Prx1.

Our results seem to corroborate the idea that the Prx1-GSTpi-JNK complex may strengthen/stabilize an association of GSTpi and JNK. Considering the interaction of both Prx1 and Prx1C52S with GSTpi, the Cys⁵²-SH-mediated heterodimerization between Prx1 and GSTpi would seem unlikely. To address a direct physical interaction of Prx1 with GSTpi, we carried out *in vitro* pull-down experiments with purified Prx1 and GSTpi proteins. As shown in Fig. 6, the interaction of Prx1 and GSTpi was not affected by the presence or absence of the reducing agent (DTT), confirming that the physical interaction between Prx1 and GSTpi was not dependent on the active site Cys⁵²-mediated mixed disulfide formation. The interaction of Prx1C52S or Cys⁵²-oxidized Prx1 with GSTpi further corroborated that the interaction of Prx1 with GSTpi is independent of the antioxidant activity of Prx1.

Discussion

During the past 20 years, much effort has been devoted to reduce the death rate of lung cancer patients. The improvement in overall survival, however, has been minimal, and <15% of the patients will survive for 5 years after diagnosis. Understanding the molecular make up of lung cancer cells responsible for treatment resistance is

highly desirable because new target-based strategies can be developed to improve the clinical outcome. In this study, we showed an antiapoptotic role of Prx1 in irradiated lung cancer cells and elucidated the mechanism of JNK inhibition by Prx1 at the molecular level. The JNK belongs to a family of stress-activated protein kinase enzymes and was originally identified by its ability to phosphorylate c-Jun in response to UV. It is now being recognized as a critical regulator of cell proliferation, cell survival, cell death, DNA repair, and metabolism (39). For the first time, we showed that Prx1 suppresses radiation-induced JNK activation and apoptosis in human lung cancer cells through interaction with the GSTpi-JNK complex. We also showed that the antioxidant activity of Prx1 is not essential for JNK inhibition by Prx1 because mutant Prx1 (Prx1C52S) lacking antioxidant activity is as efficient as the wild-type Prx1 in stabilizing the GSTpi-JNK complex and reducing JNK release/activation. The JNK inhibitory and antiapoptotic activities of Prx1 suggest that lung cancer with higher Prx1 levels is likely to be resistant not only to radiation but also to multiple anticancer agents targeting the JNK and apoptotic pathways.

Despite the initial biochemical characterization of Prx1 as a peroxidase enzyme, we found that Prx1 is overoxidized and inactivated in cells exposed to ionizing radiation. This is consistent with previous reports showing the inactivation of Prx1 during the H₂O₂ catalysis (10–13). These observations cast a shadow of doubt about the physiologic significance of peroxiredoxins as antioxidant

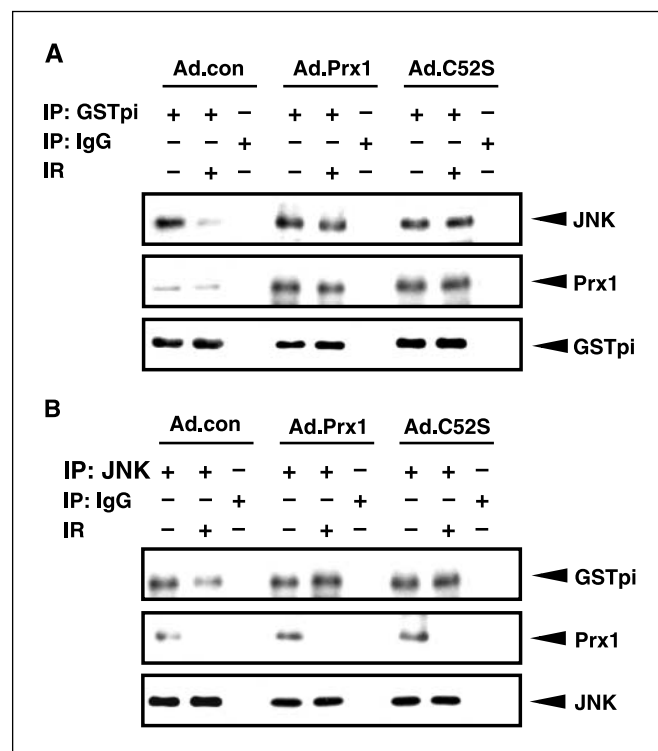


Figure 5. Effect of Prx1 or Prx1C52S on JNK release from the GSTpi-JNK complex. *A*, cells infected with Ad.con, Ad.Prx1, or Ad.C52S were irradiated (5 Gy) and harvested at 6 hours after ionizing radiation (IR). Equal amounts of proteins (500 μ g) were subjected to immunoprecipitation with GSTpi antibody. Rabbit IgG immunoprecipitation was done as negative control. The immunoprecipitates were probed for the presence of JNK or Prx1 by Western blot. GSTpi Western blot was done as a loading control. *B*, aliquots of proteins (500 μ g) were subjected to reciprocal immunoprecipitation with JNK antibody. Mouse IgG immunoprecipitation was done as negative control. The immunoprecipitates were probed for the presence of GSTpi or Prx1 by Western blot. JNK Western blot was done as a loading control.

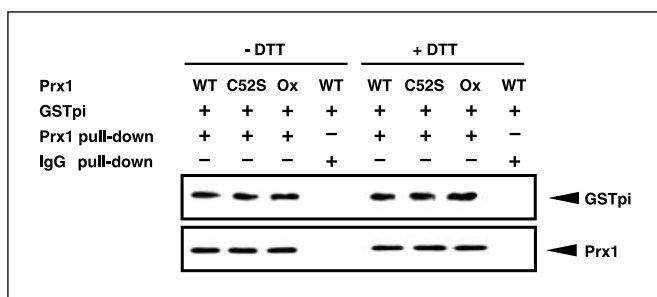


Figure 6. Prx1 and GSTpi pull-down assay. Equal amount (0.5 μ g) of purified wild-type Prx1 (WT), Prx1C52S (C52S), or *in vitro* oxidized Prx1 (Ox) each was incubated with 0.5 μ g of purified GSTpi protein. Physical interaction of Prx1 with GSTpi was tested by Prx1 antibody pull-down assay. Proteins were separated by SDS-PAGE under nonreducing (- DTT) or reducing (+ DTT) condition and probed for the presence of GSTpi by Western blot. Rabbit IgG pull-down assay was carried out as a negative control.

enzymes. Recent studies from several groups suggest that the active site Cys overoxidation may play an important role in the structural and functional switching of 2-Cys peroxiredoxin from a peroxidase enzyme to a molecular chaperone, thereby allowing it to interact with various cellular proteins (14, 15, 40). This hypothesis is in contrast to what was observed in the yeast system. In yeast, the reduced state Cys was proposed to be critical for peroxiredoxin to interact with other proteins. The Cys⁴⁸-SH of Tpx1, the yeast homologue of mammalian 2-Cys peroxiredoxin, was proposed to act as a center for Tpx1 to form a mixed disulfide with other Cys-containing proteins in regulating their activities (41, 42). As an example, Tpx1 suppresses JNK activity via mixed disulfide bond formation with Sty1, a yeast homologue of mammalian JNK, upon H₂O₂ stimulation (42). In irradiated human lung cancer cells, however, the reduced state Cys⁵² is not essential for the JNK suppressive effect of Prx1. The interaction of Prx1 with the GSTpi-JNK complex is evident when the active site Cys⁵² is overoxidized/inactivated or when the Cys⁵² is mutated to Ser⁵² (Prx1C52S). Although the molecular mechanisms identified in the H₂O₂-stimulated yeast differ from that found in the irradiated human lung cancer cells, the inhibition of JNK signaling seems to be one of the conserved functions of Prx1. In addition, the observations from the yeast systems (41, 42) showing the requirement of peroxidases for oxidative signaling may not be mutually exclusive with our findings because Prx1 may be needed for activation of factors other than JNK, or that JNK could be activated by peroxidases other than Prx1.

The ability of Prx1 to interact with various critical proliferation/apoptosis regulatory proteins has been shown previously. In yeast two-hybrid screening studies, Prx1 has been identified as an important regulating protein for c-Abl (16) or c-Myc (17). Prx1 was proposed as a tumor suppressor based on the role of Prx1 in inhibiting c-Abl tyrosine kinase activity via protein-protein

interaction in NIH3T3 fibroblast cells. The interaction of Prx1 with c-Myc is complex. Although Prx1 confers resistance to oxidative stress and thus increases cell survival, on the other hand, it inhibits the expression of selective c-Myc target genes, which are involved in promoting cell proliferation (17). These findings led to the suggestion that Prx1 can mimic or enhance some of the c-Myc properties while inhibiting others. No information was provided on the involvement of the active site Cys in Prx1 interaction with c-Abl or c-Myc. The role of the antioxidant function of Prx1 in regulating c-Abl was also not investigated.

Until recently, the catalytic site Cys-SH overoxidation to sulfinic (SO₂H) or sulfonic acid (SO₃H), which leads to inactivation of peroxidase function, has been thought to be an irreversible process. Several groups have suggested that oxidized Prx1, produced during the exposure of cells to H₂O₂, can be "retro-reduced" to the catalytically active reduced state (32, 40, 43). Although we did not observe a noticeable retro-reduction of oxidized Prx1 until 6 hours after irradiation, we cannot eliminate a possible retro-reduction of Cys⁵²-SH at later time points. The reappearance of reduced Prx1 at 24 hours after irradiation (data not shown) is likely to result in part from the retro-reduction of oxidized Prx1 combined with *de novo* synthesis of Prx1. Prx1 with the reduced active site may play a role in reducing oxidative stress propagated by ionizing radiation exposure and in forming mixed disulfide bond with other proteins containing the redox-sensitive cysteine residues.

Our study provides the rationale of the need to monitor Prx1 levels in lung cancer management. However, considering the dysregulated expression of many antioxidant proteins in various cancer cell types, caution should be exercised so that the data may not be overinterpreted. Our study suggest that, in addition to serving as a predictive marker for clinical outcome, Prx1 may also serve as a target to increase therapeutic efficacy of multiple anticancer agents. Although much effort is being made to understand the behavior of Prx1, virtually no information exists on the mechanisms responsible for the activation of the human *Prx1* gene. Identifying the key regulatory components and understanding the molecular basis of *Prx1* gene regulation will provide clues to develop ways to inhibit abnormal Prx1 up-regulation in lung cancer cells. It will be important to investigate whether augmented Prx1 expression in human lung cancer can be reversed, and whether reducing Prx1 levels can increase the therapeutic efficacy of lung cancer treatment in preclinical models.

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