Molecular Medicine 8(2): 95–102, 2002

Nonredundant Antioxidant Defense by Multiple Two-Cysteine Peroxiredoxins in Human Prostate Cancer Cells

Chuanlu Shen and Carl Nathan

Department of Microbiology & Immunology, Weill Medical College, and Immunology Program, Weill Graduate School of Medical Sciences of Cornell University, New York, New York, USA

Contributed by C. Nathan. Accepted February 28, 2002

Abstract

Background: Peroxiredoxins (Prxs) are antioxidant enzymes expressed by most free-living organisms, often in multiple isoforms. Because mammalian Prxs have not been experimentally deleted or inhibited, it is not known how much they contribute to antioxidant defense, nor whether the multiple isoforms afford redundant or additive protection.
Materials and Methods: Expression of the four members of the 2-Cys family of human Prxs was tested in human tumor cell lines. Monospecific antibodies were developed and used to monitor the extent and specificity of inhibition of expression of each isoform in prostate cancer cells stably transfected with antisense constructs.
Results: Seventeen tumor lines transcribed genes for all four human Prxs. Prostate cancer cells coexpressed each isoform at the protein level. Stable transfection with antisense allowed partial, selective suppression of Prx 1, 2, 3, or 4. Prostate cancer cells were rendered more sensitive to hydrogen peroxide or an organic hydroperoxide when Prx 1, 2, or 3 but not 4 was partially suppressed, bringing them into the range of sensitivity of mouse cells. The effect of partially suppressing a single Prx was comparable to that of depleting glutathione. In contrast, sensitization to adriamycin, an antitumor agent with a redox-active quinone, followed the partial suppression of Prxs 1, 2, or 4 but not 3. Individual suppression of Prxs 1–4 had no effect on sensitivity of the cells to reactive nitrogen intermediates, tumor necrosis factor (TNF), paclitaxel (Taxol), or etoposide.
Conclusions: The 2-Cys Prxs act in a mutually nonredundant and sometimes stress-specific fashion to protect human cells from oxidant injury. The substantial resistance of human cells to hydroperoxides may result in part from the additive action of multiple Prxs.

Introduction

In vitro, reactive oxygen intermediates (ROI) produced by activated granulocytes and macrophages can kill comparable numbers of mouse tumor cells (1–3). ROI-dependent killing becomes particularly effective when the tumor cells' antioxidant defenses are inhibited by interference with the synthesis, oxidation, or reduction of glutathione (4–6). ROI may be generated within tumors when X-irradiation ionizes water, apoptosis disrupts mitochondrial electron transport (7), certain chemotherapeutic agents undergo autoxidation (8–11), T cells (12) or immune complexes (2) activate macrophages, or complement-fixing antibodies and cytokines attract and trigger neutrophils (13). Even the systemic injection of an H_2O_2-producing oxidase coupled to polystyrene microbeads cured mice of a disseminating malignancy (14). However, efforts to exploit ROI to kill human cancer cells were set back when it was discovered that lysis of many human cells requires one to two orders of magnitude more H_2O_2 than does lysis of mouse cells (15). The basis for this species difference remains unexplained (15).

Address correspondence and reprint requests to: C. Nathan, Box 62, Weill Medical College, 1300 York Avenue, New York, NY 10021. Phone: (212) 746-6505; fax: (212) 746-8587; e-mail: cnathan@med.cornell.edu.

More than a century following the discovery of catalase, our understanding of mammalian cell-based antioxidant defenses is still growing. Among them, diverse defenses against hydroperoxides have been identified, including the glutathione and thioredoxin redox cycles, methionine sulfoxide reductase (16,17), α-keto acids (18,19), and peroxiredoxins (20–22). In human cells, we have little insight into the degree to which these systems are additive or mutually redundant. For example, simultaneous inhibition of glutathione synthesis and catalase in some human cancer cells did not sensitize them to H_2O_2(15), suggesting that those defenses were redundant with some other pathway(s).

Peroxiredoxins (Prxs) are an enzyme superfamily whose first-discovered members were the nonflavin (colorless or "C") chains of bacterial alkylhydroperoxide reductase, termed AhpC (23). Eukaryotic homologs called "thiol-specific antioxidants" and then "thioredoxin peroxidases" were discovered in yeast (24) and rat (25). As it became clear that not all these proteins use thioredoxin, they were renamed peroxiredoxins (21). Genome sequencing indicates that Prxs are the most widely represented antioxidant enzymes recognized (22). In human, isoforms 1–4, the 2-Cys subgroup, share two conserved motifs centered on Cys residues. Isoform 5 differs because

its C-terminal cysteine is not in the conserved position (26,27). Isoform 6 conserves only the Cys nearer the NH_2-terminus, which is the catalytic site. Recombinant mammalian Prxs can use thioredoxin, cyclophilin (28) and/or unidentified electron donors for the reduction of H_2O_2 and organic peroxides.

Forced overexpression of mammalian Prxs led to a gain of function (29,30). However, there have apparently been no studies in which mammalian Prxs have been deleted or inhibited. One study reported suppressing a single human Prx isoform by an antisense approach (31), but the possible impact on other isoforms was not investigated. Thus, the physiologic contributions of mammalian Prxs, acting individually or collectively, remain undefined. Bacterial peroxiredoxins confer resistance not just to ROI but also to reactive nitrogen intermediates (RNI) (32) by acting as components of a peroxynitrite reductase (33). It is unknown whether mammalian Prxs may act in a similar manner.

The present study addresses the following questions. Despite their high sequence conservation, can the individual Prxs of the 2-Cys family be monitored individually at the protein level? If so, are they broadly or selectively expressed by human cancer cells? Can their expression be suppressed selectively? Is the protection they afford redundant or additive among themselves and with glutathione? Does each Prx protect against the same stresses? Is protection evident only against exogenously supplied H_2O_2 and organic peroxides, or also against potentially related stresses, such as a chemotherapeutic agent that generates intracellular ROI, a cytokine whose induction of apoptosis involves endogenous ROI generation, and RNI?

Materials and Methods

Cell Culture

P815 mouse mastocytoma cells are described elsewhere (14). All other cells were human. Prostate cancer cell lines PC3, Du145, LNCaP, and TSU-Pr1 were gifts from Dr. A. Houghton (Memorial Sloan Kettering Cancer Center, New York, NY), who had obtained them from the American Type Culture Collection. They were cultured in complete medium (DMEM with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM of glutamine). Melanoma cell lines SK-MEL-28 and -64 and renal carcinoma lines SKRC 1, 7, 8, 9, 29, 39, 44, and 49 were also gifts of Dr. Houghton from lines established at the Sloan-Kettering Institute; they were cultured in complete medium formulated as above except based on MEM and enriched with nonessential amino acids. For the renal epithelial cell line 293 (ATCC), complete medium was based on RPMI 1640. For chronic myelogenous leukemia cell line K562 and breast cancer cell lines BT20 and MDA-MB-231 (ATCC), complete medium was based on IMDM. Cells were cultured at 37°C in 5% CO_2.

RT-PCR

Total RNA was purified from $\sim 5 \times 10^6$ cells by a single-step method using TRI Reagent (Molecular Research Center, Cincinnati, OH). One microgram of total RNA was reverse-transcribed to first strand cDNA for 15 min at 42°C with 50 U of MuLV reverse transcriptase and 2.5 μM random hexamer or oligo $d(T)_{16}$ primers (Perkin-Elmer, Norwalk, CT). Amplification of these cDNAs by PCR was performed using the following primers:

Prx 1 (forward), 5′-CTTGCCTGGTGTCGGTGGTTAGT-3′;
Prx 1 (reverse), 5′-CGGCTGAATCTGAAGTCTTGGTTTT-3′;
Prx 2 (forward), 5′-GCCCACGCAGCTTTCAGTCA-3′;
Prx 2 (reverse), 5′-AGCCAGCCTAATTGTGTTTG-3′;
Prx 3 (forward), 5′-AGATGGCGGCTGCTGTA-3′;
Prx 3 (reverse), 5′-AGTAAGGCTAAGAAAGAAGAGTGTT-3′;
Prx 4 (forward), 5′-GTTTCTGCGCTCGCGTGGTCAT3′; and
Prx 4 (reverse), 5′-GAGAACTTTCAAGCATCATAACT-3′.

Production of Specific Antisera

Full-length Prx 1, 2, and 3 cDNAs were cloned from 293 cells and Prx 4 cDNA from K562 cells by RT-PCR in the pT7-blue blunt vector (Novagen, Madison, WI) and sequenced. Subclones placed downstream of an IPTG-inducible promoter in the pQE vector were used to transform *Escherichia coli* M15 (pREP4). Hexahistidine fusion proteins were induced with IPTG and purified on Ni^+-NTA resin followed by SDS-PAGE. Synthetic peptides were conjugated to keyhole limpet hemocyanin. Electroeluted recombinant proteins or conjugated peptides were injected into rabbits. For Western blot, recombinant proteins and tumor cell lysates were separated by SDS-PAGE and electroblotted onto a 0.2-μm pore nitrocellulose membrane, which was blocked in 5% nonfat milk in TBST (25 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20), probed with antiserum, washed with TBST and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) for detection by enhanced chemiluminescence (NEN Life Science, Boston, MA or Pierce, Rockford, IL).

Stable Transfection with Antisense Constructs

Fragments of plasmids containing the entire coding regions of Prx 1, 3, or 4 or nt 1-224 of Prx 2 were subcloned in reverse orientation into pcDNA3 (Invitrogen, Carlsbad, CA) to generate antisense Prx isoform plasmids pcASPrx 1–4. PC3 cells were transfected with insert-free pcDNA3 vector or the above antisense Prx isoform expression plasmids using LipofectAMINE 2000 Reagent (Life Technologies, Inc., Grand Island, NY). Stable transfectants were selected in culture medium containing 400 μg/ml G418 and 5 mM pyruvate.

Cytotoxicity Assays

Cells were plated at 4×10^3 cells per well in 96-well plates. After 24 hr, the cells were exposed to complete medium containing the indicated cytotoxic agents for the specified time. Subsequently, 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO; 5 mg/ml in PBS) was added per well. After 4 hr, supernatant was replaced with 100 μl isopropanol/0.05 M HCl. Absorbance of solubilized formazan was measured at 570 nm. The percentage of viable cells was calculated relative to cells similarly cultured with diluent alone. To block glutathione synthesis, 1 mM buthionine sulfoximine (BSO) (Sigma) was added 24 hr prior to H_2O_2 and remained present during the 24 hr following addition of H_2O_2. Other test agents were paclitaxel (Sigma), etoposide (Sigma), tumor necrosis factor (TNF) (Genentech, So. San Francisco, CA), (Z)-1-[2-aminoethyl)-*N*-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate (DETA NONOate) (Alexis, San Diego, CA) and *S*-nitrosoglutathione (GSNO) (Alexis).

Results

Production of Isoform-Specific Antisera

Before attempting to suppress individual Prxs, we needed a means to monitor the expression of each isoform at the protein level. This was challenging because the 2-Cys Prxs are 55–75% identical (Fig. 1A).

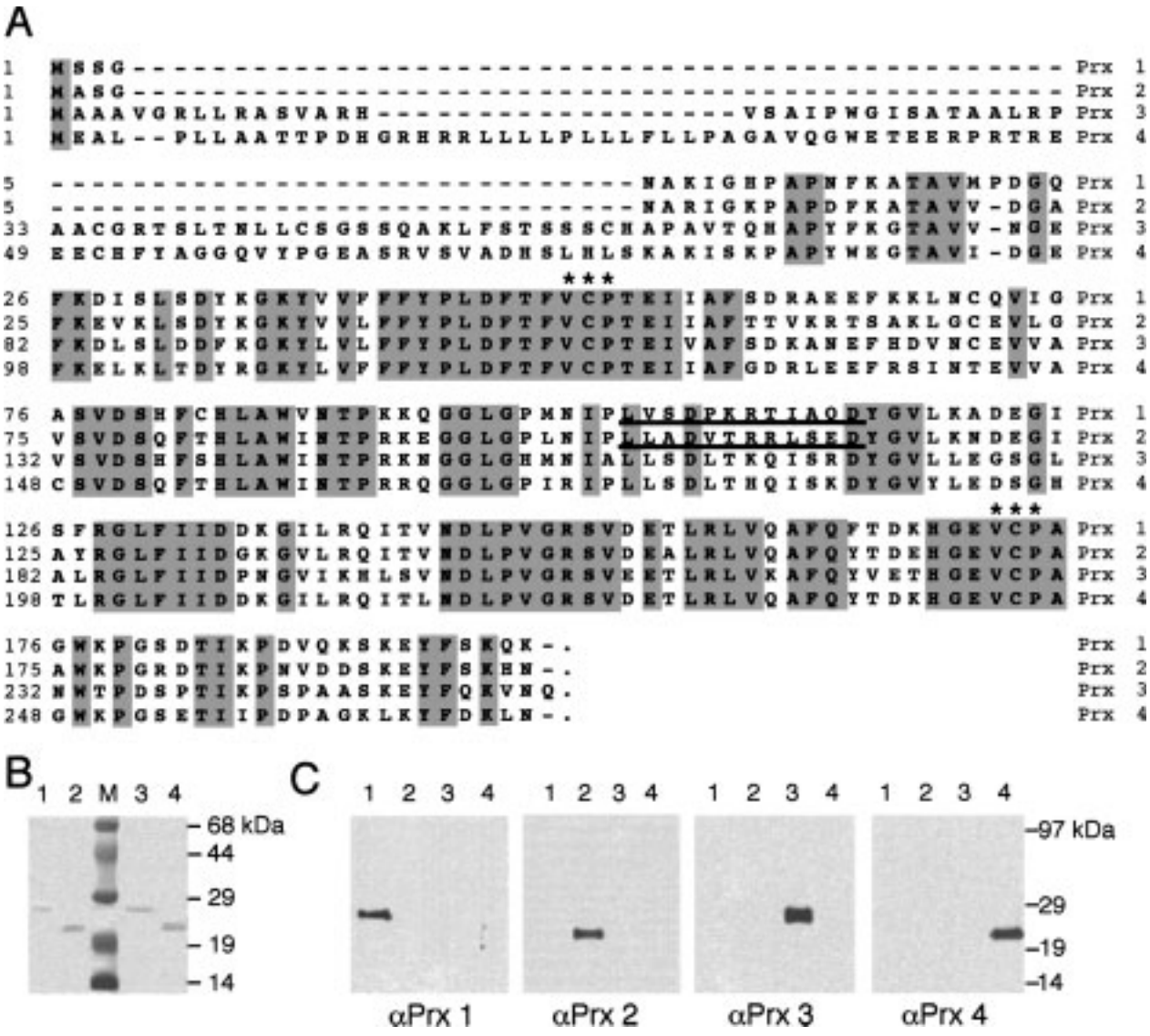

Fig. 1. Generation of monospecific antisera for four human 2-Cys Prxs. (A) Sequence alignment. Deduced amino acid sequences of Prx 1 (GenBank X67951), Prx 2 (Z22548), Prx 3 (D49396) and Prx 4 (U25182) were aligned using MegAlign. Identical residues are shaded. Asterisks mark VCP motifs. Sequences of synthetic peptide immunogens are underlined. **(B)** Purification of recombinant proteins. SDS/PAGE of Prxs 1–4 in lanes of the same number after Ni^{2+}-NTA chromatography and electroelution from gel slices. M, molecular weight markers. **(C)** Isoform selectivity of antisera. One microgram of purified recombinant human Prx 1, 2, 3, or 4 was loaded in each lane of the same number, subjected to SDS/15% PAGE and immunoblotted with antisera (α) against Prx 1, 2, 3, and 4.

We purified recombinant Prx 1, 2, 3, and 4 to homogeneity (Fig. 1B) both to generate and test antisera. Prx 3 and 4 elicited isoform-specific antisera (Fig. 1C), but antisera against Prx 1 and 2 cross-reacted with other Prxs (data not shown). We raised additional antisera against synthetic peptides LVSDPKRTIAQD (for Prx 1) and LLADVTRRLSED (for Prx 2) (underlined in Fig. 1A). The resulting high-titered (>1:10,000) antisera detected only the homologous Prxs by western blot (Fig. 1C).

Widespread Expression of Prxs 1–4 in Human Cancer Cell Lines

We then asked if some human cancer cell lines might express only a single isoform of 2-Cys Prxs. If so, targeting that isoform might produce a cell lacking any 2-Cys Prxs. However, all 17 cancer cell lines tested expressed mRNA for all four Prx isoforms, as assessed by RT-PCR. Results for prostate cancer and melanoma cell lines are illustrated in Figure 2A. Similar results were obtained for chronic myelogenous leukemia cell line K562, breast cancer lines BT20 and MDA-MB-231, and renal cell carcinoma lines SKRC 1, 7, 8, 9, 29, 39, 44, and 49 (data not shown). Prostate cancer cell lines LNCaP, TSU-Pr1, Du145, and PC3 were selected for further study. Each expressed Prx 1–4 mRNA as assessed by Northern blot (not shown) and protein as revealed by Western blot (Fig. 2B). Preimmune sera were nonreactive (Fig. 2B).

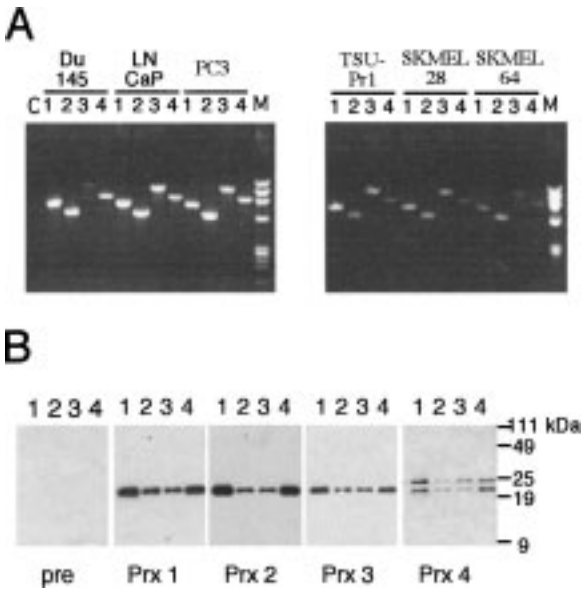

Fig. 2. Expression of 2-Cys Prx isoforms in human cancer cells. (A) Expression of mRNA. Total RNA from prostate cancer cells Du145, LNCaP, PC3, and TSU-Pr1 and from melanoma cells SK-MEL-28 and -64 was subjected to RT-PCR using primers specific for Prx 1 (lanes 1), 2 (lanes 2), 3 (lanes 3), or 4 (lanes 4). C, control with RT omitted. M, molecular markers. **(B)** Expression of protein. Extracts from LNCaP (lanes 1), TSU-Pr1 (lanes 2), Du145 (lanes 3), and PC3 (lanes 4) were separated by SDS/15% PAGE and immunoblotted with antisera specific for the Prx isoform designated beneath each panel. Each preimmune serum was nonreactive, as illustrated for Prx 1 (pre). Equal loading was confirmed with anti-tubulin antibody (not shown).

The Western blots demonstrated that each anti-Prx antiserum bound to only a single polypeptide species in each of the cell lines, except for anti-Prx 4, which bound to a doublet. The apparent Mr of the faster migrating member of the doublet corresponded to the Mr predicted for Prx 4, and this species was also detected in the conditioned medium (not shown), suggesting that it represents the mature protein. The apparent Mr of the slower migrating species in the doublet corresponded to the Mr predicted for pro-Prx 4, including its signal peptide. Immunocytochemistry revealed intense, diffuse cytoplasmic staining of prostate cancer cells by each antiserum, including anti-Prx 4 (not shown). Thus, Prx 4 partitioned between intracellular and extracellular compartments. Moreover, immunocytochemistry demonstrated that there were no subsets of cells expressing one or another Prx isoform. On the contrary, all cells examined expressed all four of the 2-Cys Prxs.

Antisense Inhibition of Expression of 2-Cys Prxs

Compared to parental PC3 cells and to PC3 cells stably transfected with a vector encoding only the selection marker, about one in four of the stably transfected clones expressed lower levels of the targeted Prx. Figure 3A illustrates results for two clones per isoform. Longer exposure of films showed that Prx expression was never completely extinguished (not shown). As estimated by densitometry, expression was suppressed by 40–90% in the clones chosen for functional studies. Specificity was stringent for suppression of Prx 3 and Prx 4, in that the clones expressing diminished levels of either of these Prxs expressed normal levels of each of the other three (Fig. 3A). However, one of the clones targeted for suppression of Prx 1 also had decreased expression of Prxs 2 and 4, and one of the clones targeted for suppression of Prx 2 also had decreased expression of Prx 4 (Fig. 3A).

Effect of Prx Suppression on Sensitivity of PC3 Cells to Hydroperoxides

On average, exposure to 326 μM H_2O_2 produced a 50% reduction in the number of viable, vector-transformed PC3 cells recovered 24 hr later (Table 1). As illustrated in Figure 3B and summarized in Table 1, partial reduction in the expression of Prxs 1, 2, or 3 shifted the concentration–response curve to the left, sensitizing PC3 cells to the cytotoxic effect of H_2O_2. This was seen for each of two clones targeted for each of the three Prxs. The two independent clones for each targeted Prx behaved the same as each other, even though targeting was not completely specific in the case of pcASPrx1-2 and pcASPrx2-12, as noted above. In contrast, suppression of Prx 4 did not sensitize PC3 cells to killing by H_2O_2 (Fig. 3B). Decreased expression of Prx 2 brought the human

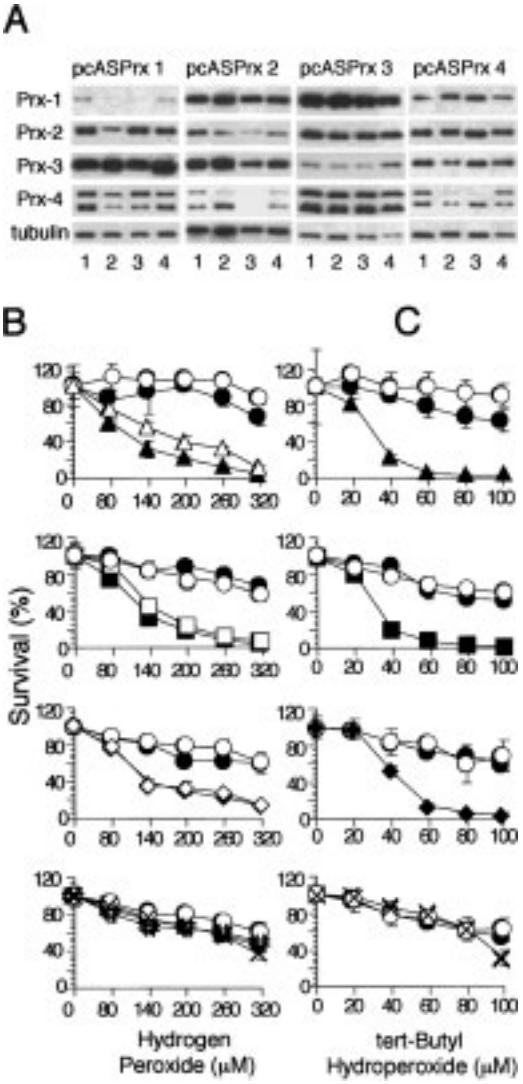

Fig. 3. Antisense-mediated suppression of Prxs in PC3 cells: sensitization to hydroperoxides. (A) Prx levels. Extracts from parental PC3 cells (lanes 1), PC3 cells stably transfected with insert-free pcDNA3 vector (lanes 4), and two independently selected clones transfected with antisense to Prx 1 (pcASPrx1-2 and -8; lanes 2 and 3), Prx 2 (pcASPrx2-5 and -12; lanes 2 and 3), Prx 3 (pcASPrx3-11 and -12; lanes 2 and 3), or Prx 4 (pcASPrx4-6 and -11; lanes 2 and 3) were resolved by SDS/15% PAGE and analyzed by immunoblot with antisera specific for Prx 1, 2, 3, and 4. Anti-tubulin blot tested equal loading. **(B and C)** Effect of suppressed Prx levels on sensitivity to hydrogen peroxide **(B)** or tBHP **(C)**. PC3 cells modified as detailed below were exposed to the indicated concentrations of hydroperoxides and their survival determined by the MTT assay 24 hr later. Cell numbers were normalized to those measured in the absence of the peroxide. Means ± SD of quadruplicates from one experiment representative of the number summarized in Table 1 (most error bars fall within the symbols). Parental PC3 cells (filled circles) and PC3 cells stably transfected with insert-free pcDNA3 vector (open circles) are included in each panel, along with one or two of the following clones transfected with antisense constructs: pcASPrx1-2 (filled triangles) and pcASPrx1-8 (open triangles); pcASPrx2-5 (open squares) and pcASPrx2-12 (filled squares); pcASPrx3-11 (open diamonds) and pcASPrx3-12 (filled diamonds); and pcASPrx4-6 (crosses) and pcASPrx4-11 (ampersands). Most crosses and ampersands are overlying.

Table 1. Sensitivity of human PC3 prostate cancer cells and mouse P815 mastocytoma cells to hydrogen peroxide

Cell	Experiments (No.)	LD_{50} (10^{-6} M)*	$p^{\dagger}$
P815	2	44 ± 1.4	0.0285
PC3 parental	12	244 ± 102	n/a
PC3 vector only	22	326 ± 152	0.1041
PC3 vector + BSO	9	102 ± 60	0.0016
pcASPrx 1	10	102 ± 21	0.0024
pcASPrx 2	8	59 ± 8	0.0002
pcASPrx 3	8	84 ± 26	0.0016
pcASPrx 4	9	232 ± 81	0.7891

*Values are means ± SD for the interpolated doses that were lethal to 50% of the cells, where each datum consists of an LD_{50} derived from an independent experiment.

†Comparison to results for PC3 cells (ANOVA and Kruskal-Wallis analysis).

prostate cancer cells into the same range of H_2O_2 sensitivity as P815, a mouse tumor tested under the same assay conditions (Table 1 and refs. 1 and 19).

To compare the degree of protection afforded by physiologic levels of individual Prxs with that afforded by the glutathione redox cycle, we tested the effect of BSO, a specific inhibitor of γ-glutamylcysteine synthetase (34). Cellular levels of glutathione are markedly suppressed within 24 hr of addition of BSO (4,5,34). BSO sensitized PC3 cells to H_2O_2 to about the same degree as partial inhibition of expression of Prxs 1, 2, or 3 (Table 1).

Tert-butyl hydroperoxide (tBHP) is a prototypical organic oxidant that induces cell death upon oxidation of lipids, protein thiols, and glutathione, release of Ca^{2+} from the endoplasmic reticulum, and permeability transition in mitochondria (35). We next examined the effect of antisense against 2-Cys Prx isoforms on sensitivity of PC3 to tBHP. The LD_{50} was about 30 μM, 30 μM, and 40 μM for PC3 cells expressing antisense against Prx 1, 2, and 3, respectively, whereas the LD_{50} for parental PC3 cells and cells expressing control vector was not reached at the highest concentration tested (100 μM). In contrast, suppression of Prx 4 had little effect (Fig. 3C).

Effect of Prx Suppression on Sensitivity to Other Stresses

Adriamycin, an effective antitumor agent, contains an aminosugar in a glycosidic bond with adriamycinone, a naphthacenequinone. A one-electron reduction of ring B of adriamycinone leads to the formation of a semiquinone radical. Under aerobic conditions, the unpaired electron is donated to oxygen, forming superoxide (8–11). In this manner adriamycin can use intracellular reductants to catalyze the formation of

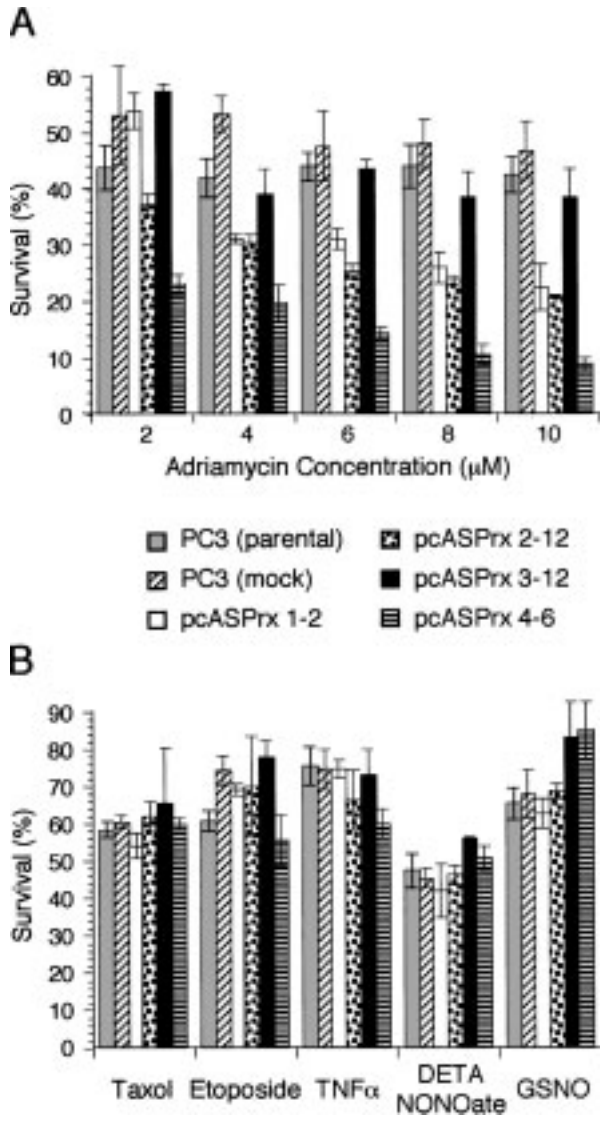

Fig. 4. Effect of antisense suppression of Prx isoforms on sensitivity of PC3 cells to agents other than hydroperoxides. Survival of the parental cells PC3 and transfectants after incubation in the indicated concentrations of **(A)** adriamycin or **(B)** paclitaxel (0.02 mM) for 24 hr, etoposide (0.01 mM) for 72 hr, or TNF (25 ng/ml), DETA NONOate (0.5 mM), or *S*-nitrosoglutathione (GSNO) (1.25 mM) for 48 hr. Means ± SD from quadruplicates in one experiment representative of at least two performed.

ROI. Inhibition of the expression of Prxs 1, 2, or 4 sensitized PC3 cells to adriamycin. In contrast, suppression of Prx 3 had no effect (Fig. 4A).

The cytotoxic action of TNF is related to its ability to induce apoptosis in a process dependent in part on the generation of ROI (36). However, individual suppression of each of the 2-Cys Prxs had no effect on sensitivity of PC3 cells to TNF (Fig. 4B). Likewise, no nonredundant role was defined for individual 2-Cys Prxs in resistance to RNI in the form of an NO-donating compound, DETA NONOate (0.1–2.0 mM), and an *S*-nitrosothiol, GSNO (0.3–5.0 mM), whose cytotoxicity for human cells was previously demonstrated (32).

Finally, we tested two additional chemotherapeutic agents, paclitaxel (5–40 μM) and etoposide (0.1–40.0 μM), for which no direct oxidative action has been described. Individual suppression of the 2-Cys Prxs had no impact on sensitivity of PC3 cells to these compounds (Fig. 4B).

Discussion

Prxs are highly conserved and widely expressed among prokaryotes and eukaryotes, often with multiple isoforms per species. All 17 human tumor cell populations we studied expressed all four of the 2-Cys Prxs, and the prostate cancer lines expressed all four Prxs in every cell. Most human cells also express catalase and enzymes that cycle reduced and oxidized glutathione and thioredoxin. Thus, the question arises: why are there so many distinct systems to catabolize hydroperoxides in a single cell, and in particular, so many isoforms of Prx? Are these defenses redundant, or does each make a unique contribution? Redundancy of defenses might suggest that cells are subject to stresses that exert evolutionary pressure through generation of hydroperoxides, and that different stresses can differentially inactivate defense pathways. This situation would select for expression of diverse defenses with distinct vulnerabilities. Alternatively, additive defenses might be optimal if they each protected distinct subcellular compartments or could only protect the same compartment incompletely.

If one seeks to enhance radiotherapy, immunotherapy, or chemotherapy by inhibiting antioxidant defenses in tumor cells, it becomes important to understand the biological significance of the multiplicity of these defenses. For example, most human breast cancer cells markedly overexpressed Prxs 1, 2, and 3 compared to the normal cells in the same surgical specimens (37). If Prx 1, 2, and 3 are functionally redundant, it might be necessary to inhibit all of them to sensitize the tumor cells to an oxidant stress. If these Prxs are functionally additive, inhibition of just one might sensitize the cells, and inhibition of more Prxs might sensitize them further.

The main inference from the present experiments is that some of the protective effects of 2-Cys Prxs in human prostate cancer cell lines are nonredundant both with the glutathione system and with each other. In the one previous report of antisense suppression of a Prx (31), Prx 2 was found to be elevated in clinically radioresistant tumors of the head and neck compared to radiosensitive tumors. Suppression of Prx 2 in cells cultured from the radioresistant tumors was associated with enhancement of their sensitivity to radiation in vitro. The study by Park et al. (31) is consistent with our finding that some actions of human 2-Cys Prx isoforms are nonredundant, and supports the implication that

inhibition of even one Prx may have therapeutic potential.

When tested against a single form of oxidant stress, such as H_2O_2, tBHP, or adriamycin, three Prxs each substantially protected the cells against cytotoxicity in a mutually nonredundant manner. This was evident even though the suppression of each Prx was only partial. Because the sensitizing effect of partially suppressing just one Prx was similar to the effect of depleting glutathione, it seems possible that the combined action of multiple 2-Cys Prxs may account for a substantial proportion of the antihydroperoxide defense of human prostate cancer cells. It remains to be explored whether differences in Prx expression or Prx-reducing systems may help explain why human cells are often more resistant to H_2O_2 than mouse cells (15).

The specificity of antisense effects is a critical issue (38). Specificity was evaluated here by two types of internal controls. First, in six of eight clones studied, suppressing any one isoform had little or no effect on expression of three others. This demonstrated predominant but not perfect specificity at the biochemical level. Second, suppressing Prx 3 sensitized the cells to two stresses but not a third; suppressing Prx 4 had the opposite pattern of effects; and suppressing any of the four isoforms had no effect on cellular sensitivity to five additional stresses. Thus, antisense did not render the cells nonspecifically sensitive to injury, demonstrating specificity at the functional level. Nonetheless, spreading of suppression beyond the targeted isoform was observed in two of eight clones. This underscores the importance of testing multiple clones and evaluating multiple control proteins within them.

We first tried to suppress Prx expression by transfection with antisense phosphorothioate oligonucleotides. This resulted in marginally diminished expression of Prx 1 and 2 but not 3 and 4 (not shown). We turned to stable transfection with antisense expression plasmids in a tissue culture medium that lacked pyruvate. Only six to eight positive clones were obtained from $\sim 10^6$ cells per transfection, and in none of them was the targeted Prx suppressed. In contrast, when pyruvate was included in the selection medium, ~100 positive clones were recovered per transfection, in about one-fourth of which the targeted Prx was suppressed. The long-known ability of pyruvate to enhance cloning efficiency has been attributed to the rapid, stoichiometric, nonenzymatic, oxidative decarboxylation that α-ketoacids undergo upon encounter with H_2O_2 (19). In fact, extracellular pyruvate constitutes a physiologic antioxidant defense (19). Given that tissue culture is an oxidizing environment, the usual requirement for pyruvate for efficient cell cloning may have been exacerbated by suppression of Prx. That is, clones in which a Prx was suppressed may have faced a selection disadvantage until pyruvate was replenished.

Finally, these experiments have established that individual Prxs may protect against different stresses. Prx 1, 2, and 3 protected cells against H_2O_2 and tBHP, whereas Prxs 1, 2, and 4 protected cells against adriamycin. Prx 3 did not appear to protect against adriamycin and Prx 4 did not appear to protect against H_2O_2 and tBHP. None of the Prxs tested appeared to provide nonredundant protection against RNI, TNF, paclitaxel, or etoposide. However, negative results in knock-down experiments do not exclude the possibility that Prxs may protect against these or other stresses. Prx isoforms may offer redundant protection in some settings, so that knocking down a single isoform is inconsequential. Alternatively, it may be necessary to drive down the level of a given Prx more extensively than we were able to do with antisense constructs. Prx 5 or 6 may be critical in certain settings. To probe Prx biology more deeply, it will be necessary to develop means to suppress or inhibit all the isoforms in any combination in the same cells.

Acknowledgments

We thank Martha Cathcart for generously teaching us how to design DNA antisense oligonucleotides, although the results of those experiments were not included here. We thank Dr. A. Houghton for cell lines, Drs. A. Ding and N. Brot for reviewing the manuscript, and the Cancer Research Institute for supporting this work. The Department of Microbiology and Immunology is supported by the William Randolph Hearst Foundation.

References

1. Nathan CF, Silverstein SC, Brukner LH, Cohn ZA. (1979) Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. *J. Exp. Med.* **149:** 100–113.
2. Nathan CF, Cohn ZA. (1980) Role of oxygen-dependent mechanisms in antibody-induced lysis of tumor cells by activated macrophages. *J. Exp. Med.* **152:** 198–208.
3. Nathan CF, Klebanoff SJ. (1982) Augmentation of spontaneous macrophage-mediated cytolysis by eosinophil peroxidase. *J. Exp. Med.* **155:** 1291–1308.
4. Nathan CF, Arrick BA, Murray HW, DeSantis NM, Cohn ZA. (1981) Tumor cell antioxidant defenses: Inhibition of the glutathione redox cycle enhances macrophage-mediated cytolysis. *J. Exp. Med.* **153:** 766–782.
5. Arrick BA, Nathan CF, Griffith OW, Cohn ZA. (1982) Glutathione depletion sensitizes tumor cells to oxidative cytolysis. *J. Biol. Chem.* **257:** 1231–1237.
6. Arrick BA, Nathan CF, Cohn ZA. (1983) Inhibition of glutathione synthesis augments lysis of murine tumor cells by sulfhydryl-reactive antineoplastics. *J. Clin. Invest.* **71:** 258–267.
7. Vrablic AS, Albright CD, Craciunescu CN, Salganik RI, Zeisel SH. (2001) Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-O-octadecyl-2-methyl-rac-glycero-3-phosphocholine in p53-defective hepatocytes. *FASEB J.* **15:** 1739–1744.
8. Doroshow JH. (1983) Effect of anthracycline antibiotics on oxygen radical formation in rat heart. *Cancer Res.* **43:** 460–472.
9. Doroshow JH. (1983) Anthracycline antibiotic-stimulated superoxide, hydrogen peroxide and hydroxyl radical production by NADH dehydrogenase. *Cancer Res.* **43:** 4543–4551.
10. Bachur NR, Gee MV, Friedman RD. (1982) Nuclear catalyzed antibiotic free radical formation. *Cancer Res.* **42:** 1078–1081.

11. Svingen BA, Powis G. (1981) Pulse radiolysis studies of antitumor quinones: Radical lifetimes, reactivity with oxygen, and one-electron reduction potentials. *Arch. Biochem. Biophys.* **209:** 119–126.
12. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H. (1998) The central role of CD4(+) T cells in the antitumor immune response. *J. Exp. Med.* **188:** 2357–2368.
13. Minasian LM, Szatrowski TP, Rosenblum M, et al. (1994) Hemorrhagic tumor necrosis during a pilot trial of tumor necrosis factor-α and anti-GD3 ganglioside monoclonal antibody in patients with metastatic melanoma. *Blood* **83:** 56–64.
14. Nathan CF, Cohn Z. (1981) A. Antitumor effects of hydrogen peroxide in vivo. *J. Exp. Med.* **154:** 1539–1553.
15. O'Donnell-Tormey J, DeBoer C, Nathan CF. (1985) Resistance of human tumor cells in vitro to oxidative cytolysis. *J. Clin. Invest.* **76:** 80–86.
16. Brot N, Weissbach L, Werth J, Weissbach H. (1981) Enzymatic reduction of protein-bound methionine sulfoxide. *Proc. Natl. Acad. Sci. U.S.A.* **8:** 2155–2158.
17. Moskovitz J, Berlett BS, Poston JM, Stadtman ER. (1997) The yeast peptide-methionine sulfoxide reductase functions as an antioxidant in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 9585–9589.
18. Andrae U, Singh J, Ziegler-Skylakakis K. (1985) Pyruvate and related alpha-ketoacids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity. *Toxicol. Lett.* **28:** 93–98.
19. O'Donnell-Tormey J, Nathan CF, Lanks K, DeBoer C, de la Harpe J. (1987) Secretion of pyruvate: an antioxidant defense of mammalian cells. *J. Exp. Med.* **165:** 500–514.
20. Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: Alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 7017–7021.
21. Chae HZ, Chung SJ, Rhee SG. (1994) Thioredoxin-dependent peroxide reductase from yeast. *J. Biol. Chem.* **269:** 27670–27678.
22. Jin DY, Jeang KT. (2000). Peroxiredoxins in cell signaling and HIV infection. In Sen CK, Sies H, Baeuerle PA (eds). *Antioxidation and Redox Regulation of Genes*. San Diego: Academic Press; 381–407.
23. Storz G, Jacobson FS, Tartaglia LA, Morgan RW, Silveira LA, Ames BN. (1989) An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: Genetic characterization and cloning of ahp. *J. Bacteriol.* **171:** 2049–2055.
24. Chae HZ, Kim IH, Kim K, Rhee SG. (1993) Cloning, sequencing, and mutation of thiol-specific antioxidant gene of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268:** 16815–16821.
25. Chae HZ, Rhee SG. (1994) A thiol-specific antioxidant and sequence homology to various proteins of unknown function. *Biofactors* **4:** 177–180.
26. Knoops B, Clippe A, Bogard C, et al. Cloning and characterization of AOEB166, a novel mammalian antioxidant enzyme of the peroxiredoxin family. *J. Biol. Chem.* **274:** 30451–30458.
27. Yamashita H, Avraham S, Jiang S, et al. (1999) Characterization of human and murine PMP20 peroxisomal proteins that exhibit antioxidant activity in vitro. *J. Biol. Chem.* **274:** 29897–29904.
28. Lee SP, Hwang YS, Kim YJ, et al. (2001) Cyclophilin A binds to peroxiredoxins and activates their peroxidase activity. *J. Biol. Chem.* **276:** 29826–29832.
29. Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. (1998) Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J. Biol. Chem.* **273:** 6297–6302.
30. Kim H, Lee TH, Park ES, et al. (2000) Role of peroxiredoxins in regulating intracellular hydrogen peroxide and hydrogen peroxide-induced apoptosis in thyroid cells. *J. Biol. Chem.* **275:** 18266–18270.
31. Park SH, Chung YM, Lee YS, et al. (2000) Antisense of human peroxiredoxin II enhances radiation-induced cell death. *Clin. Cancer Res.* **6:** 4915–4920.
32. Chen L, Xie QW, Nathan C. (1998) Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Molecular Cell* **1:** 795–805.
33. Bryk R, Griffin P, Nathan C. (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* **407:** 211–215.
34. Griffith OW, Meister A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-*n*-butyl homocysteine sulfoximine). *J. Biol. Chem.* **254:** 7558–7560.
35. Henschke PN, Elliott SJ. (1995) Oxidized glutathione decreases luminal Ca2+ content of the endothelial cell ins(1,4,5)P3-sensitive Ca2+ store. *Biochem. J.* **312:** 485–489.
36. Goossens V, De Vos K, Vercammen D, et al. (1999) Redox regulation of TNF signaling. *Biofactors* **10:** 145–156.
37. Noh DY, Ahn SJ, Lee RA, Kim SW, Park IA, Chae HZ. (2001) Overexpression of peroxiredoxin in human breast cancer. *Anticancer Res.* **21:** 2085–2090.
38. Stein CA. (2001) The experimental use of antisense oligonucleotides: A guide for the perplexed. *J. Clin. Invest.* **108:** 641–644.