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Peroxiredoxin III, a Mitochondrion-specific Peroxidase, Regulates Apoptotic Signaling by Mitochondria*

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Various proapoptotic stimuli increase the production of superoxide and H_2O_2 by mitochondria. Whereas superoxide impairs mitochondrial function and is removed by Mn^{2+}-dependent superoxide dismutase, the role and metabolism of mitochondrial H_2O_2 during apoptosis have remained unclear. The effects on apoptotic signaling of depletion of peroxiredoxin (Prx) III, a mitochondrion-specific H_2O_2-scavenging enzyme, have now been investigated by RNA interference in HeLa cells. Depletion of Prx III resulted in increased intracellular levels of H_2O_2 and sensitized cells to induction of apoptosis by staurosporine or TNF-α. The rates of mitochondrial membrane potential collapse, cytochrome *c* release, and caspase activation were increased in Prx III-depleted cells, and these effects were reversed by ectopic expression of Prx III or mitochondrion-targeted catalase. Depletion of Prx III also exacerbated damage to mitochondrial macromolecules induced by the proapoptotic stimuli. Our results suggest that Prx III is a critical regulator of the abundance of mitochondrial H_2O_2, which itself promotes apoptosis in cooperation with other mediators of apoptotic signaling.

Normal cellular processes that involve oxygen result in the production of reactive oxygen species (ROS)[1] such as superoxide ($O_2^{\cdot-}$), H_2O_2, and the hydroxyl radical ($OH^{\cdot}$). Each of these species has the potential to oxidize macromolecules and thereby to induce mutation of DNA, impairment of protein function, and lipid peroxidation. Most ROS in unstimulated mammalian cells are generated as a result of the univalent reduction of molecular oxygen to $O_2^{\cdot-}$ by electrons that leak from the mitochondrial electron transport chain, mainly from complexes I and III (1–3). Given its charged nature, $O_2^{\cdot-}$ does not readily cross membranes; therefore, if not destroyed, it inhibits mitochondrial function by inactivating the Fe-S centers in the electron transport chain (complexes I and III) and the tricarboxylic acid cycle (aconitase) (4). The burden of $O_2^{\cdot-}$ production is largely countered by Mn^{2+}-dependent superoxide dismutase (MnSOD), an enzyme specifically localized in the mitochondrial matrix (5). The SOD reaction only partially relieves oxidative stress in mitochondria, however, given that its product, H_2O_2, is itself a mild oxidant and is readily converted to the more powerful oxidant $OH^{\cdot}$ via the Fenton reaction. Intracellular H_2O_2 is removed mostly by catalase, glutathione peroxidase (GPx), and peroxiredoxin (Prx).

GPx catalyzes the reduction of H_2O_2 and of various hydroperoxides with glutathione as the electron donor. There are at least four GPx isoforms in mammalian cells. GPx1 is the major isoform and is expressed in all tissues; it is localized predominantly in the cytosol, but a small proportion (~10%) of GPx1 molecules is also present in the matrix of mitochondria (6–9). With the exception of those in rat myocytes, mitochondria lack catalase (10). Mitochondria apparently also must import glutathione because they also lack the enzymes required for glutathione synthesis.

The newly identified Prx family of peroxidases includes at least six isoforms in mammalian cells (11–13). Among them, Prx III is synthesized with a mitochondrial targeting sequence, as is MnSOD, and is then transferred to mitochondria, where its targeting residues are cleaved during maturation (12–14). On reaction with H_2O_2, the redox-sensitive Cys residue of each subunit of the Prx homodimer is oxidized to Cys-SOH, which then reacts with a neighboring Cys-SH of the other subunit to form an intermolecular disulfide (15). This disulfide is reduced specifically by thioredoxin, not by glutathione or glutaredoxin (15). The reduced form of thioredoxin is then regenerated by thioredoxin reductase at the expense of NADPH (11–13). Mammalian mitochondria contain thioredoxin 2 and thioredoxin reductase 2, both of which are synthesized in the cytosol with mitochondrial targeting sequences and become localized specifically in mitochondria (16, 17).

Mitochondria play a central role in apoptosis by releasing cytochrome *c* and other proapoptotic proteins (reviewed in Refs. 18–21). The release of cytochrome *c* into the cytosol results in the activation of caspases by triggering formation of the apoptosome (22, 23). The mitochondrial production of ROS is also thought to be associated with the activation and propagation of apoptosis (4, 20, 21, 24–28). Indeed, generation of ROS by mitochondria appears to be an early event in apoptotic signaling initiated by TNF-α, ceramide, or glutamate (29–34), whereas other studies suggest that ROS production occurs relatively late in cells that have already committed to die (35,

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[1] The abbreviations used are: ROS, reactive oxygen species; CHX, cycloheximide; CM-H_2DCFDA, 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; $\Delta\Psi_m$, mitochondrial membrane potential; DEVD-AMC, Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin); DHR123, dihydrorhodamine 123; GPx, glutathione peroxidase; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanin iodide; mtDNA, mitochondrial DNA; NAO, 10-*N*-nonyl-acridine orange; PARP, poly(ADP-ribose) polymerase; Prx, peroxiredoxin; PTP, permeability transition pore; RNAi, RNA interference; siRNA, small interfering RNA; SOD, superoxide dismutase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; RT, reverse transcriptase.

36). Most of these various observations, however, did not make a distinction between O_2^- and H_2O_2, the former of which preferentially oxidizes certain metal ions whereas the latter oxidizes cysteine and methionine residues.

An important role for O_2^- in apoptotic signaling was previously suggested by the observations that overexpression of MnSOD conferred increased resistance to TNF-α-induced cytotoxicity, that expression of MnSOD antisense RNA increased the sensitivity of cells to TNF-α, and that induction of MnSOD expression in cells was protective under various proapoptotic conditions (37–40). Homozygous knockout of the MnSOD gene is lethal in mice, whereas mice that lack cytosolic SOD (Cu/ZnSOD) appear normal (41, 42). Experiments with heterozygous MnSOD knockout mice or cell lines derived from these animals have shown that the partial deficiency of MnSOD promotes cellular apoptotic events, such as cytochrome *c* release and the permeability transition of mitochondria, under various conditions (43–45). Deficiency of MnSOD activity was also associated with increased damage to mitochondrial proteins such as aconitase and NADH dehydrogenase (45).

Unlike O_2^-, whose effects on mitochondrial function are well established, the effects of H_2O_2 production in mitochondria remain unclear. Given the absence of catalase from mitochondria in most cell types, GPx1 has been thought to play the major role in the protection of these organelles against oxidative damage by H_2O_2 (3, 44, 46–50). However, homozygous GPx1 knockout mice appear healthy and do not manifest an increased sensitivity to hyperoxia or an increased content of protein carbonyl groups or lipid peroxides (9). Furthermore, hepatocytes isolated from these animals did not show an enhanced sensitivity to apoptosis initiated by TNF-α receptors or Fas (51). A protective role for GPx1 became apparent, however, when the GPx1 knockout and control mice, or cell lines derived from these animals, were subjected to extreme oxidative stress such as that associated with ischemia-reperfusion injury or treatment with paraquat or a bolus of H_2O_2 (48, 52). It remains unclear whether the effect of GPx1 knockout under these conditions was attributable to the absence of the enzyme from the cytosol or from mitochondria, or from both.

The specific localization of Prx III in mitochondria (12–14) together with the identification of its mitochondria-specific electron suppliers, namely thioredoxin 2 and thioredoxin reductase 2 (16, 17), suggest that these three proteins might provide a primary line of defense against H_2O_2 produced by the mitochondrial respiratory chain (53, 54), as MnSOD does against O_2^-. The role of Prx III in mitochondria has not been defined, however. Therefore, with the use of RNA interference (RNAi), we have now investigated the effects of depletion of endogenous Prx III on oxidative damage to mitochondrial components and on apoptotic events. Our results indicate that Prx III is much more abundant in mitochondria than is GPx1 and is a critical regulator of the mitochondrial H_2O_2 concentration, in contrast to the widely held view that GPx1 is the only important H_2O_2-metabolizing enzyme in mitochondria (3, 44, 48–50). We further demonstrate a regulatory function for Prx III during apoptosis induced by staurosporine or TNF-α.

EXPERIMENTAL PROCEDURES

Depletion of Prx III by RNAi—A small interfering RNA (siRNA) duplex targeting the 5′-AAGCCAAGUCCAGCUGCUUCC-3′ sequence in the open reading frame of human Prx III mRNA as well as si*CONTROL*® non-targeting siRNA were obtained from Dharmacon Research (Lafayette, CO). The RNAs were introduced into HeLa cells by transfection with the use of a Nucleofector instrument (Amaxa Biosystems, Cologne, Germany).

Preparation of Recombinant Proteins—The DNA sequence for human GPx1 was amplified by PCR from HeLa cell cDNA and cloned into the NdeI and EcoRI sites of pET17b (Novagen, Madison, WI). The resulting plasmid, pET17b-GPx1, was subjected to site-directed mutagenesis with the primers 5′-TGGCGTCCCTCTGCGGCACCACGGT-3′ and 5′-ACCGTGGTGCCGCAGAGGGACGCCA-3′ (mutated residue in bold) in order to replace selenocysteine at position 47 with cysteine. *Escherichia coli* BL21(DE3) cells harboring the mutated plasmid were cultured in Luria-Bertani broth and expression of the recombinant protein was induced by incubation of the cells for 3 h with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4 °C, resuspended in 10 vol of ice-cold extraction buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.5 mM aminoethylbenzene sulfonyl fluoride, 5 mM dithiothreitol), and then disrupted by pressure. After centrifugation of the cell lysate at 12,000 × *g* for 30 min at 4 °C, the supernatant was treated immediately on ice with streptomycin sulfate (final concentration, 1%) for 30 min and then centrifuged again at 12,000 × *g* for 30 min at 4 °C to remove the precipitated nucleic acid. Human GPx1 was purified from the resulting supernatant by a series of chromatographic steps including HPLC on TSK DEAE-5PW and TSK phenyl-5PW columns (Tosoh Bioscience, Montgomeryville, PA) as well as gel filtration on Superose-6 (Amersham Biosciences, Piscataway, NJ). Recombinant human Prx III was expressed in *E. coli* and purified as described previously (12).

Induction of Apoptosis—HeLa cells were exposed for the indicated times to 200 nM staurosporine (Calbiochem, San Diego, CA) or to the combination of TNF-α (15 ng/ml) (Invitrogen, La Jolla, CA) and cycloheximide (CHX) (10 μg/ml).

Subcellular Fractionation—Cytosolic and mitochondria-enriched fractions were prepared from HeLa cells with the use of a Subcellular Proteome Extraction kit (Calbiochem).

Immunoblot Analysis—HeLa cell lysates were prepared and immunoblot analysis was performed as described previously (55). Monoclonal antibodies to poly(ADP-ribose) polymerase (PARP) and to cytochrome *c* were obtained from BD PharMingen (San Diego, CA), those to β-actin were from Abcam (Cambridge, UK), polyclonal antibodies to caspase-3 and to caspase-9 were from Cell Signaling Technology (Beverly, MA), those to GPx1 were from LabFrontier (Seoul, Korea), and rabbit antiserum to Prx III, Prx I, and thioredoxin reductase 2 were described previously (12, 17). Carbonylated proteins in the mitochondrial fraction were labeled by derivatization of the carbonyl groups with 2,4-dinitrophenylhydrazine and then detected with antibodies specific for the latter moiety (Zymed Laboratories Inc., San Francisco, CA) (56).

Assay of Caspase-3 Activity—Caspase-3 activity was assayed by incubating cell lysate (10 μg of protein) with 200 μl of reaction buffer (100 mM Hepes-KOH (pH 7.5), 10% (w/v) sucrose, 0.1% CHAPS, 10 mM dithiothreitol) containing 25 μM Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin) (DEVD-AMC) (Biomol, Plymouth Meeting, PA). The fluorescence generated by cleavage of the artificial substrate was measured with a CytoFluor 4000 instrument (PerSeptive Biosystems, Framingham, MA) at excitation and emission wavelengths of 380 and 460 nm, respectively.

Confocal Microscopy—Cells on coverslips were fixed with 4% paraformaldehyde and permeabilized for 5 min with 0.2% Triton X-100. Endogenous Prx III was detected with specific antiserum (1:50 dilution) and Alexa-488-conjugated goat antibodies to rabbit IgG (Molecular Probes, Eugene, OR). Mitochondria were stained with 0.1 μM MitoTracker Red CMXRos and nuclei were stained with Hoechst 33342 (10 μg/ml), both from Molecular Probes. Confocal fluorescence images were obtained with an LSM510 microscope (Carl Zeiss, Tornwood, NJ). Apoptotic cells were quantified as a percentage of total cells on the basis of their condensed or fragmented nuclei as revealed by the Hoechst dye; at least 300 cells from five random fields were scored for each sample.

Flow Cytometry—A FACSCalibur flow cytometer (BD Biosciences) was used for all analyses, with a minimum of 2×10^4 cells per sample for each measurement. The excitation wavelength was 488 nm, and the observation wavelength was 530 nm for green fluorescence and 585 nm for red fluorescence. Relative change in fluorescence was analyzed with WinMDI software. For analysis of apoptosis, cells were stained with propidium iodide (25 μg/ml) as described previously (55) and the percentage of hypodiploid (apoptotic) cells was determined. For evaluation of cardiolipin peroxidation, cells were labeled with 5 μM 10-*N*-nonylacridine orange (NAO) (Molecular Probes) for 30 min and washed twice before measurement of the fluorescence emitted by cardiolipin-bound NAO. For evaluation of changes in the mitochondrial membrane potential ($\Delta\Psi_m$), cells (4×10^5) were incubated with 10 μg/ml of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanin iodide (JC-1) (Molecular Probes) for 20 min at 37 °C and the shifts in both red and green fluorescence emissions of JC-1 were measured.

Measurement of ROS—For measurement of intracellular ROS, detached cells were loaded with 5 μM 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Molecular Probes) at 37 °C

for 20 min, washed, and then analyzed immediately by flow cytometry. Direct visualization of mitochondrial ROS generation was achieved with the use of dihydrorhodamine 123 (DHR123) (Molecular Probes). To verify that mitochondria were indeed the site of ROS formation, we incubated cells with both 1 μM DHR123 and 1 μM MitoTracker Red for 20 min at 37 °C. Cells loaded with the fluorescent probes were imaged with an LSM510 confocal microscope fitted with an objective with a magnification of ×40 and an NA of 1.3. Excitation was performed at 458 nm for R123 and at 543 nm for Mitotracker Red. R123 fluorescence was passed through a 505/530-nm band-pass filter. Mitotracker Red fluorescence was passed through a 560-nm long-pass filter. For overlaid images, exposures were collected for equal times at the same plane of focus for both excitation wavelengths; the images were overlaid with a computer and R123 fluorescence was analyzed with MetaMorph software (Universal Imaging, Westchester, PA).

Quantitative RT-PCR—Total RNA was extracted from cells with the use of an RNeasy kit (Qiagen) and portions (2 μg) were subjected to reverse transcription in a final volume of 20 μl also with the use of a kit (Invitrogen). The resulting first-strand cDNA was diluted and used as a template for real-time PCR analysis with an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). A fluorogenic probe (5′-6-FAM-CTGTGGAGCAAACC-TAMRA-3′, where 6-FAM is 6-carboxyfluorescein and TAMRA is 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine and primer pair (5′-GGTATACTACGGTCAATGCTCTGAAA-3′, 5′-ACGATGGGCATGAAACTG-3′) for human cytochrome *c* oxidase were designed with the use of ABI Primer Express software (Applied Biosystems). Reactions for each sample were performed in triplicate with equal amounts of template cDNA. The amount of cytochrome *c* oxidase mRNA was normalized by that of human 18 S ribosomal RNA.

Adenovirus-mediated Gene Expression—The DNA sequence for Prx III was amplified from a mouse liver cDNA library (Clontech, Palo Alto, CA) and subcloned into pAd5MCSloxP (kindly provided by G. Nabel, NIH, Bethesda, MD), and a recombinant adenoviral genome was generated by *in vitro* Cre- and loxP-mediated recombination, as described (57). Viruses were propagated in HEK293 cells under endotoxin-free conditions and purified. Recombinant adenoviruses encoding human catalase with an MnSOD mitochondrial leader sequence (mitoCatalase; kindly provided by A. Cederbaum) (58), human MnSOD (obtained from the University of Iowa Gene transfer vector core) (59), or *E. coli* β-galactosidase (LacZ) were also generated. Adenoviral stocks were titered with the use of standard viral plaque assays, and HeLa cells were infected at a multiplicity of infection of 100 plaque-forming units per cell.

Data Analysis—All experiments were repeated at least three times, and quantitative data are presented as means ± S.D. of triplicate determinations from representative experiments.

RESULTS

To compare the amounts of Prx III and GPx1 in mitochondria, we prepared a mitochondria-enriched fraction from HeLa cells and subjected it to immunoblot analysis with antibodies specific for each of these two proteins (Fig. 1*a*). By comparing the resulting band intensities with those of protein standards, we estimated the amounts of Prx III and GPx1 in HeLa cell mitochondria to be ~1.90 and ~0.06 μg per milligram of soluble protein of the mitochondria-enriched organelles, respectively, indicating that Prx III is ~30 times more abundant than is GPx1. To selectively deplete Prx III from HeLa cells, we subjected the cells to transfection by electroporation with a siRNA targeted to Prx III mRNA; the transfection efficiency determined with rhodamine-labeled siRNA was ~95% (data not shown). As a control, cells were transfected with an RNA duplex of random sequence. Immunoblot (Fig. 1*b*) and immunofluorescence (Fig. 1*c*) analyses revealed that the amount of Prx III was greatly reduced by ~90% in cells transfected with the Prx III siRNA but was unaffected by the control RNA. Cells transfected with the control RNA or the Prx III siRNA are hereafter designated Prx III-replete and Prx III-depleted cells, respectively.

To determine whether Prx III inhibits the accumulation of cellular H_2O_2 under conditions that promote apoptosis, we first compared the abundance of ROS between Prx III-replete and

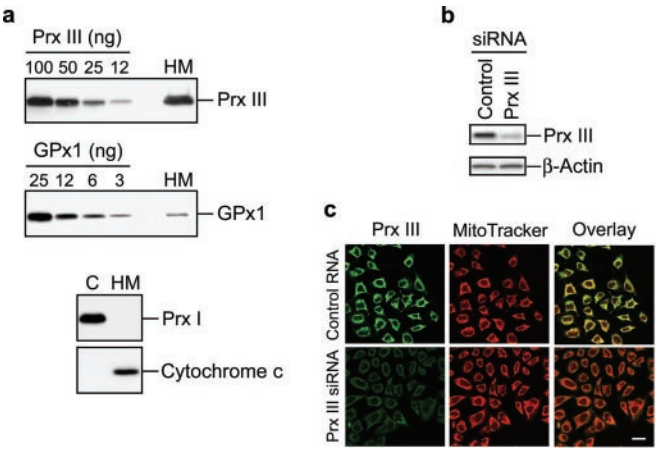

FIG. 1. **Depletion of Prx III by RNAi in HeLa cells.** *a*, HeLa cells were subjected to subcellular fractionation to yield mitochondria-enriched (heavy membrane or *HM*) and cytosolic (*C*) fractions. The HM fraction (45 μg of protein) and the indicated amounts of recombinant human Prx III or GPx1 were subjected to immunoblot analysis with the corresponding antibodies (*upper* and *middle panels*). Equal amounts (20 μg) of the C and HM fractions were also analyzed with antibodies specific for Prx I or for cytochrome *c* as cytosolic and mitochondrial markers, respectively (*bottom panel*). *b* and *c*, HeLa cells were transfected with a Prx III-specific siRNA or a control RNA. After 48 h, cell lysates were prepared and subjected to immunoblot analysis with antibodies to Prx III or to β-actin (*b*). Alternatively, the transfected cells were stained with antibodies to Prx III (*green*) and with MitoTracker Red and then examined by confocal microscopy (*c*). *Scale bar*, 20 μm.

Prx III-depleted cells with the use of the indicator CM-H_2DCFDA. This membrane-permeable dye passively diffuses into cells, where its acetate groups are cleaved and subsequent oxidation by various oxidants including H_2O_2 yields a fluorescent adduct that is trapped inside the cell (60, 61). Flow cytometric analysis revealed that the intracellular level of ROS was increased, even in the absence of proapoptotic stimuli, in Prx III-depleted cells compared with that in Prx III-replete cells (Fig. 2*a*). The increase in ROS abundance during the induction of apoptosis is attributable predominantly to ROS generation by mitochondria (4, 20, 21). Apoptosis is induced in HeLa cells by TNF-α, in the presence of cycloheximide, via a death receptor-mediated pathway, or by staurosporine via a mitochondria-dependent pathway that does not involve a death receptor. Both TNF-α and staurosporine induce the generation of ROS by mitochondria (29, 30, 32). The extent of the ROS accumulation induced by these two stimuli was also greater in Prx III-depleted cells than in Prx III-replete cells (Fig. 2*a*).

Oxidation of the nonfluorescent DHR123 to the highly fluorescent rhodamine 123 (R123) has been used to monitor the amount of ROS in mitochondria (62–64). The level of R123 fluorescence was markedly (about 2-fold) greater in Prx III-depleted cells stimulated with staurosporine or with TNF-α and CHX than in similarly treated Prx III-replete cells (Fig. 2*b*). The localization of mitochondria was verified by exposure of cells to MitoTracker Red, a mitochondria-specific fluorescent probe. Interpretation of results obtained with DHR123 requires caution, however, because oxidation of the dye can occur in the cytosol and the resulting cationic R123 can then be actively imported into mitochondria in a manner dependent on the $\Delta\Psi_m$. Our data nevertheless suggest that depletion of Prx III results in an initial increase in the level of H_2O_2 in mitochondria, and that the accumulated H_2O_2 then diffuses into the cytosol.

We next examined the effect of Prx III depletion on apoptosis in HeLa cells. Condensation and fragmentation of the nucleus were monitored with the use of the fluorochrome Hoechst 33342 (Fig. 3). In the absence of proapoptotic stimuli, cells with

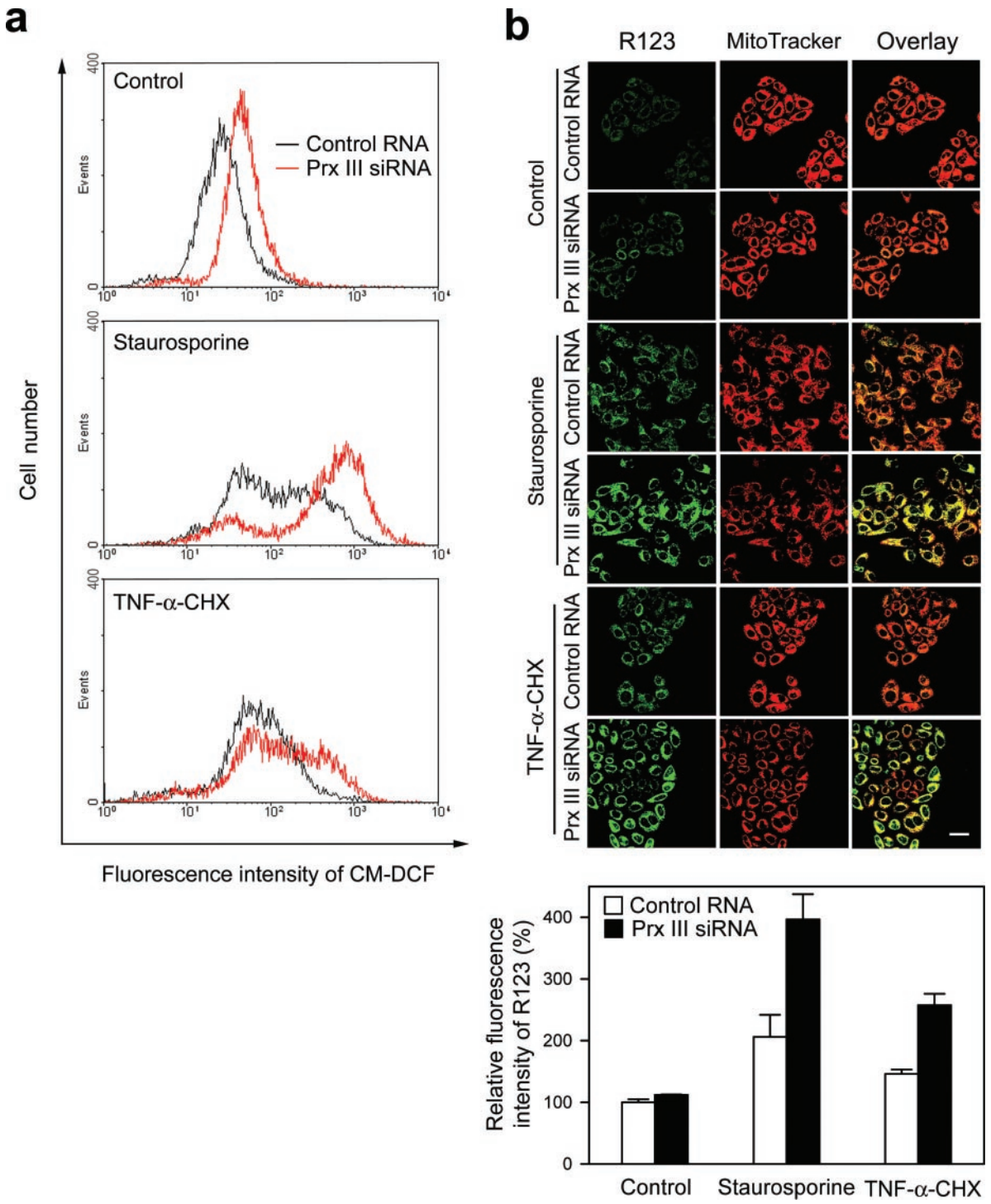

FIG. 2. **Effects of Prx III depletion on ROS accumulation.** *a*, 48 h after transfection with Prx III siRNA or control RNA, HeLa cells were cultured for 4 h in the absence (*control*) or presence of staurosporine or of TNF-α and CHX. ROS were detected with the use of CM-H_2DCFDA and flow cytometry. *b*, cells transfected and stimulated as in *a* were incubated with DHR123 (*green*) and MitoTracker Red and then examined by confocal microscopy. Representative microscopic fields are shown in the *upper panels* (*scale bar*, 20 μm), and quantitation of R123 fluorescence intensity relative to that of nonstimulated cells transfected with control RNA is shown in the *lower panel*; data are means ± S.D. of triplicates.

condensed or fragmented nuclei were virtually undetectable among both Prx III-replete and Prx III-depleted populations. After exposure to staurosporine or to TNF-α and CHX for 8 h, however, the nuclei of ~7 and ~10%, respectively, of Prx III-replete cells exhibited apoptotic characteristics, and these percentages were increased about 2-fold for Prx III-depleted cells.

Progress of mitochondria-mediated apoptosis is associated with a reduction in $\Delta\Psi_m$, the release of cytochrome *c*, and the activation of caspases. We therefore examined the effects of Prx III depletion on these events. The change in $\Delta\Psi_m$ was measured with the use of JC-1, which exists in the cytosol in a monomeric form that emits green fluorescence and forms aggregates in mitochondria that emit red fluorescence (65). The uptake of JC-1 into mitochondria is dependent on $\Delta\Psi_m$, so that collapse of the latter is associated with an increase in the fluorescence intensity of monomeric JC-1. Flow cytometric analysis of such green fluorescence indicated that treatment of HeLa cells with staurosporine or with TNF-α and CHX induced a time-dependent decrease

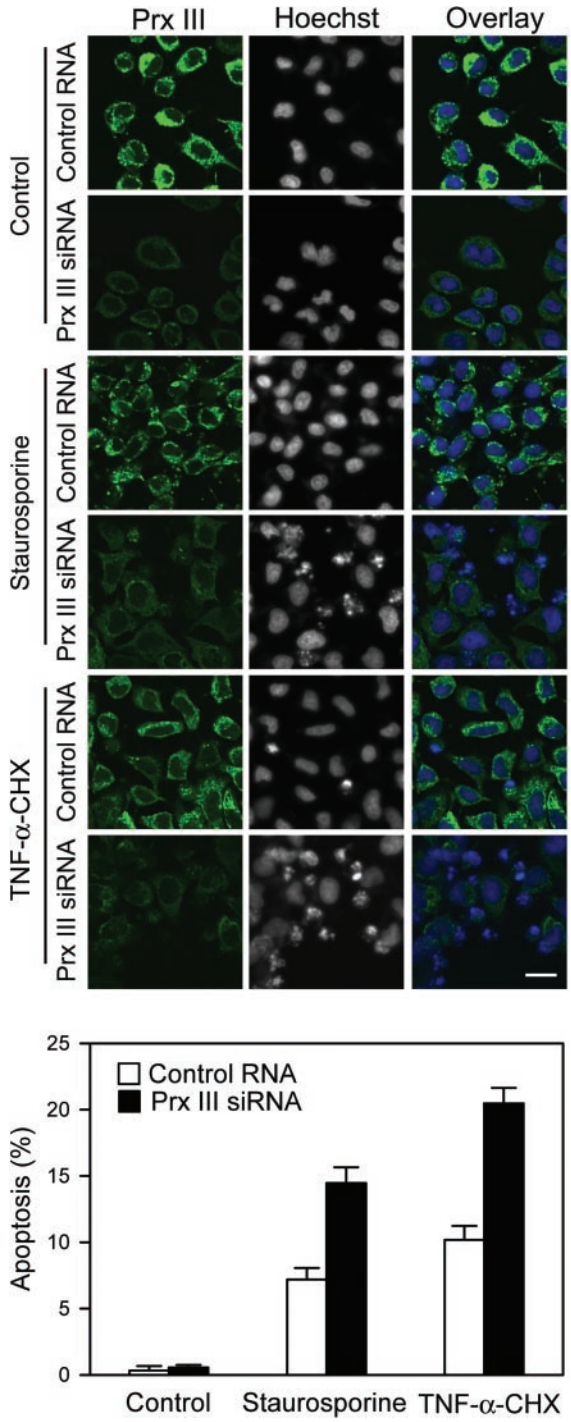

FIG. 3. **Effects of Prx III depletion on cell death.** Cells at 48 h post-transfection with Prx III siRNA or control RNA were cultured for 8 h in the absence (*Control*) or presence of staurosporine or of TNF-α and CHX. They were then stained with Hoechst 33342 (*blue*) and antibodies to Prx III (*green*) and were examined by confocal microscopy. Representative microscopic fields are shown in the *upper panels*; *scale bar*, 10 μm. The number of apoptotic cells (those with condensed or fragmented nuclei) was determined as a percentage of the total cell number (*lower panel*); data are means ± S.D. of triplicates.

in $\Delta\Psi_m$ and that this effect was enhanced by depletion of Prx III (Fig. 4*a*). Consistent with this latter observation, the stimulus-induced release of cytochrome *c* into the cytosol was faster in Prx III-depleted cells than in Prx III-replete cells (Fig. 4*b*). Furthermore, immunoblot analysis revealed that depletion of Prx III increased the rate of cleavage of procaspases-3 and -9 as well as that of the caspase substrate PARP (Fig. 4*c*). Measurement of caspase-3 activity with a specific fluorogenic substrate, DEVD-AMC, confirmed that the activation of caspase 3 by staurosporine or by TNF-α and CHX was enhanced in Prx III-depleted cells (Fig. 4*d*). These results thus suggested that Prx III functions as a suppressor of mitochondria-mediated apoptosis by eliminating H_2O_2 in mitochondria.

Hydrogen peroxide can damage cellular macromolecules such as proteins, lipids, and nucleic acids, especially after its conversion to $OH^{\cdot}$ (66, 67). Given that damage to mitochondrial DNA (mtDNA) impairs the production of mtRNA, a decrease in the amount of mtRNA reflects damage at both the DNA and RNA levels. We therefore measured the abundance of cytochrome *c* oxidase mRNA, a product of mtDNA, with the use of quantitative RT-PCR. Treatment for 4 h with staurosporine or with TNF-α and CHX resulted in a decrease of 25 or 14%, respectively, in the amount of cytochrome *c* oxidase mRNA in Prx III-replete cells and of corresponding decreases of 53 and 36%, respectively, in Prx III-depleted cells (Fig. 5*a*), suggesting that Prx III protects mitochondrial polynucleotides from oxidative damage.

The oxidative modification of proteins is accompanied by an increase in protein carbonyl content (68), which can be assessed by derivatization with 2,4-dinitrophenylhydrazine and subsequent immunoblot analysis with antibodies specific for the latter moiety. An overall increase in the amount of such immunoreactive proteins of all sizes was apparent in the mitochondria of cells treated with the proapoptotic stimuli, and this increase was greater in Prx III-depleted cells than in Prx III-replete cells (Fig. 5*b*). Lipid peroxidation in mitochondria can be detected with the fluorescent probe NAO, which specifically binds to a monolayer of cardiolipin but not to other types of phospholipid or to oxidized cardiolipin (61, 69). Cardiolipin in mitochondria contains a substantial proportion of highly unsaturated fatty acids and is therefore sensitive to oxidation. A decrease in the cellular fluorescence of NAO is thus thought to reflect peroxidation or other modification of cardiolipin. Treatment of cells with staurosporine or with TNF-α and CHX resulted in a decrease in NAO fluorescence, and this effect was more pronounced in Prx III-depleted cells than in Prx III-replete cells (Fig. 5*c*).

The RNAi approach has sometimes been found to silence nontargeted genes (70). To alleviate this concern in the present study, we examined whether expression of mouse Prx III in Prx III-depleted HeLa cells reversed the phenotypes conferred by transfection with the Prx III siRNA. In addition, to verify that the observed phenotypes were caused by the accumulation of H_2O_2 and not to that of other ROS, we expressed in the Prx III-depleted cells a form of human catalase that is targeted to mitochondria (mitoCatalase) (58). Expression of mouse Prx III or mitoCatalase was achieved by infection of cells with recombinant adenoviruses (Fig. 6*a*). The effects of mouse Prx III and mitoCatalase on the level of ROS were measured with the use of CM-H_2DCFDA and flow cytometry. As demonstrated in Fig. 2*a*, the level of ROS after staurosporine treatment was markedly greater in Prx III-depleted cells than in Prx III-replete cells (Fig. 6*b*). Expression of either mouse Prx III or mitoCatalase in the Prx III-depleted cells, however, reduced the staurosporine-induced accumulation of ROS to a level similar to that apparent in Prx III-replete cells that had been infected with a control adenovirus encoding β-galactosidase (LacZ). Similar results were obtained for cells stimulated with TNF-α and CHX.

The enhanced effects of staurosporine or of TNF-α and CHX on $\Delta\Psi_m$ (Fig. 6*c*) and on the extent of apoptosis (Fig. 6*d*) observed in Prx III-depleted cells, compared with those in Prx III-replete cells, were also no longer apparent after infection

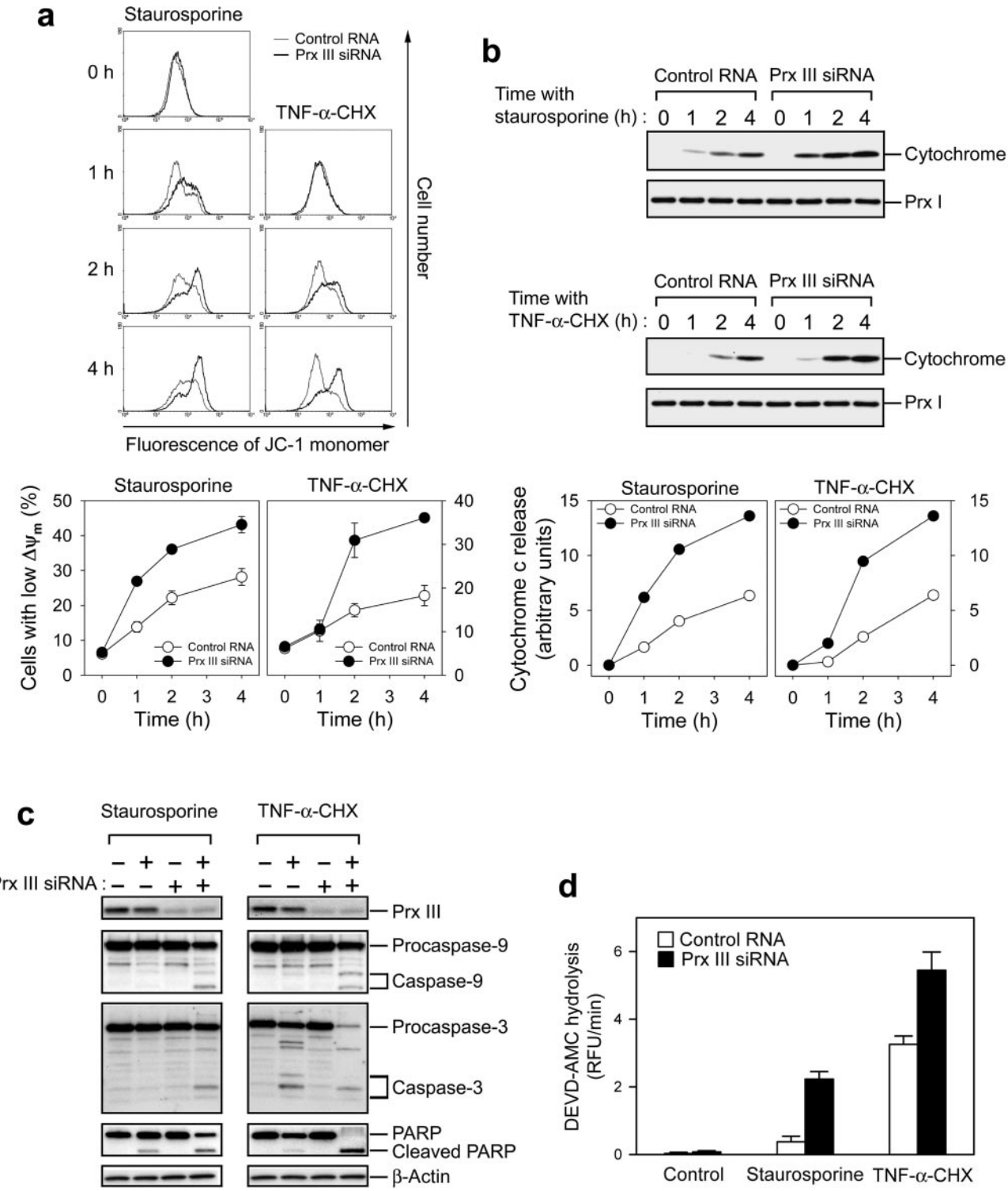

FIG. 4. **Effects of Prx III depletion on mitochondria-mediated apoptotic signaling.** *a*, 48 h after transfection with Prx III siRNA or control RNA, HeLa cells were cultured in the presence of staurosporine or of TNF-α and CHX for the indicated times and changes in $\Delta\Psi_m$ were then evaluated by staining with JC-1 and measurement of fluorescence of the JC-1 monomer by flow cytometry (*upper panels*). The *lower panels* show the percentage of cells with a low $\Delta\Psi_m$ (increased fluorescence of the JC-1 monomer) and are means ± S.D. of triplicates. *b*, cells transfected and stimulated as in *a* were subjected to subcellular fractionation, and the cytosolic fraction was subjected to immunoblot analysis with antibodies to cytochrome *c* and to Prx I (*upper panels*). The intensities of the cytochrome *c* bands were normalized by those of the Prx I bands and plotted against time (*lower panels*). *c* and *d*, cells transfected and stimulated for 6 h as in *a* were subjected to immunoblot analysis with antibodies to Prx III, to caspase-9, to caspase-3, to PARP, or to β-actin (*c*). Caspase-3 activity in cell lysates was also determined with the synthetic substrate DEVD-AMC (*d*); data are expressed as relative fluorescence units (*RFU*) per minute and are means ± S.D. of triplicates.

with adenoviruses encoding mouse Prx III or mitoCatalase. Overexpression of MnSOD did not mimic these effects of the latter two proteins (data not shown). Given that the only catalytic activity of catalase relates to the conversion of H_2O_2 to H_2O and O_2, these results suggest that the amplified responses observed in Prx III-depleted cells are attributable

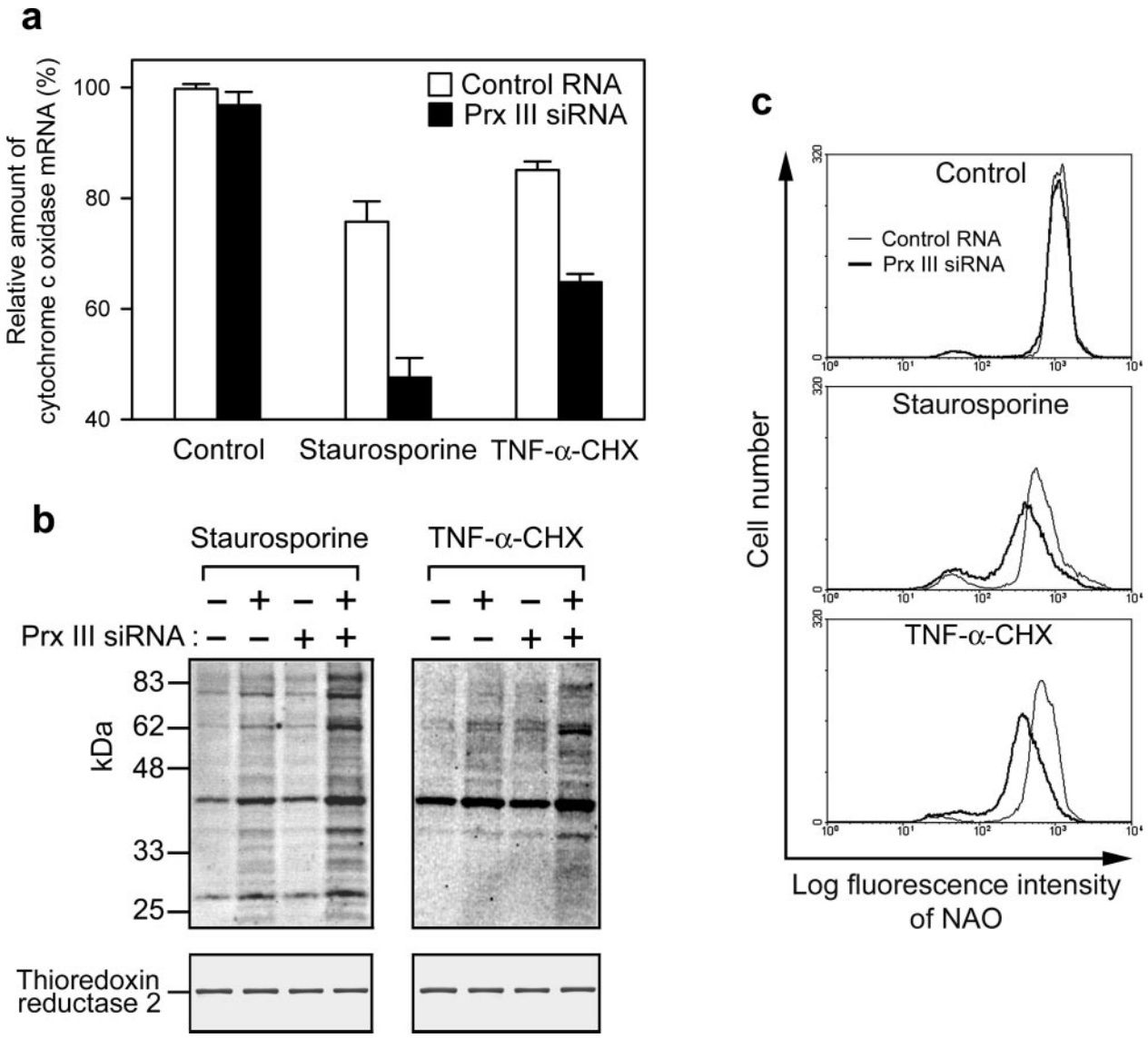

FIG. 5. **Effects of Prx III depletion on mitochondrial damage during induction of apoptosis.** *a*, HeLa cells at 48 h post-transfection with Prx III siRNA or control RNA were cultured for 4 h in the absence (*Control*) or presence of staurosporine or of TNF-α and CHX. Total RNA was then isolated and subjected to quantitative RT-PCR analysis of cytochrome *c* oxidase mRNA. Data are expressed as a percentage of the value for nonstimulated cells transfected with control RNA and are means ± S.D. of triplicates. *b*, cells transfected and stimulated as in *a* were subjected to subcellular fractionation, and the mitochondria-enriched fraction was subjected to derivatization with 2,4-dinitrophenylhydrazine and subsequent immunoblot analysis with antibodies to the latter molecule (*upper panels*) or to thioredoxin reductase 2 (*lower panels*) as loading control. The positions of molecular size standards are indicated in kilodaltons. *c*, cells transfected and stimulated as in *a* were incubated with NAO and analyzed by flow cytometry.

to the cellular accumulation of H_2O_2 that results from the loss of Prx III activity.

DISCUSSION

Most of the O_2^- generated by mitochondria is vectorially released into the mitochondrial matrix, where it is converted to H_2O_2 by a specific intramitochondrial MnSOD. Although O_2^- is not an effective oxidant, it impairs mitochondrial function by oxidizing the Fe-S centers of various enzymes. In addition, O_2^- might be converted to peroxynitrite, an extremely powerful oxidant, as a result of its reaction with nitric oxide produced by mitochondrial nitric-oxide synthase (50, 71). The participation of O_2^- in various stages of receptor-dependent or -independent apoptosis and the inhibition of such participation by MnSOD have been demonstrated (37, 39–45). However, although MnSOD relieves mitochondrial oxidative stress caused by O_2^-, it generates H_2O_2 and therefore a different type of oxidative stress. Hydrogen peroxide is sufficiently stable to diffuse out of mitochondria and to exert extramitochondrial effects, although its concentration within mitochondria remains much higher than that in the cytosol.

Many studies have addressed the mechanisms by which mitochondria deal with H_2O_2 and whether mitochondrial function is compromised by the mitochondrial production of this molecule. Most of these studies, however, considered GPx1 as the only mitochondrial enzyme that removes H_2O_2 (3, 44, 48–50) and their results were interpreted without regard to Prx III. In one previous study, overexpression of Prx III was shown to protect WEHI thymoma cells from apoptosis induced by hypoxia, a bolus of peroxide, or the anticancer drug imexon (72). In another study, Prx III overexpression was found to protect rat hippocampal neurons from excitotoxic injury (72). However, the effects of Prx III overexpression on mitochondrial function and apoptotic signaling were not investigated in these two studies. Furthermore, expression of recombinant Prx III on top

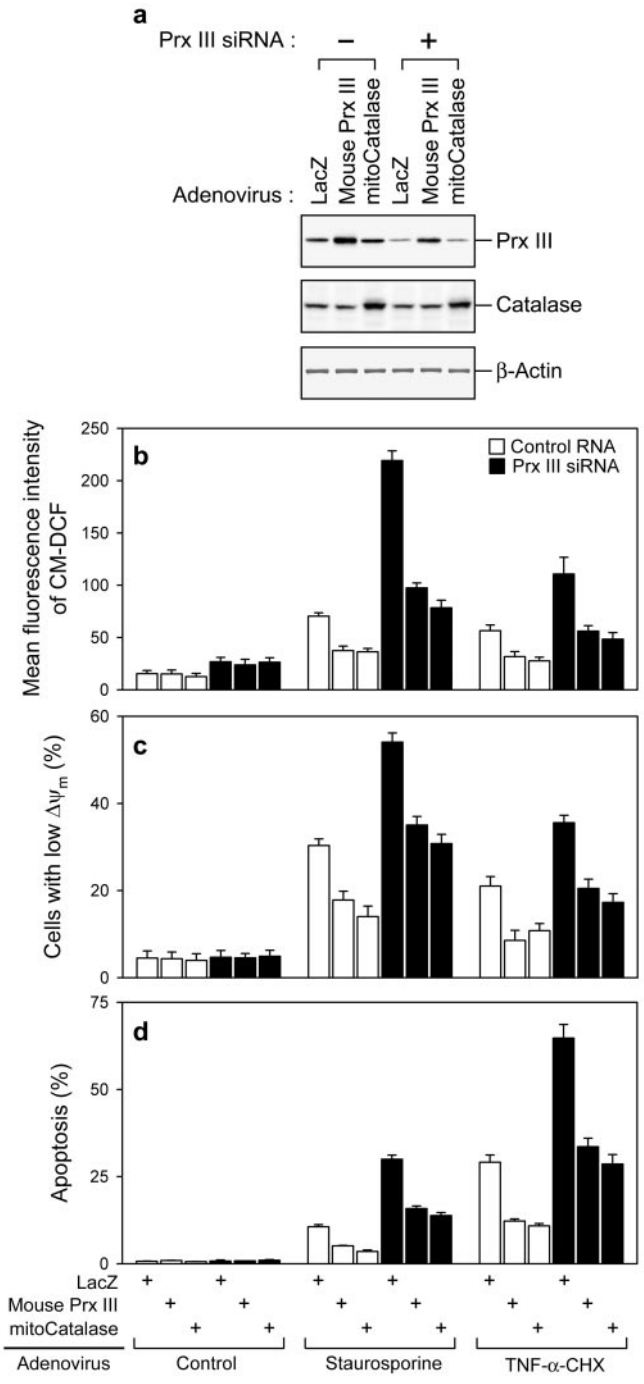

FIG. 6. **Effects of ectopic expression of mouse Prx III or mitochondrially targeted catalase on the apoptotic phenotypes conferred by Prx III depletion.** *a*, 24 h after transfection with Prx III siRNA or control RNA, HeLa cells were infected with adenoviral vectors for β-galactosidase (*LacZ*), mouse Prx III, or mitoCatalase and cultured for 36 h. Cell lysates were then subjected to immunoblot analysis with antibodies to Prx III, to catalase, or to β-actin. *b*, cells treated as in *a* were cultured for 4 h in the absence (*Control*) or presence of staurosporine or of TNF-α and CHX, after which intracellular ROS were detected with the use of CM-H_2DCFDA and flow cytometry. *c*, cells treated as in *b* were stained with JC-1 and subjected to flow cytometry for determination of the percentage of cells with a low $\Delta\Psi_m$. *d*, cells treated as in *b* were analyzed for DNA content by staining with propidium iodide and flow cytometry. Apoptotic cells were defined as those with a DNA content less than that typically present in G_1 phase of the cell cycle. Data in *b* through *d* are means ± S.D. of triplicates.

of the already high level of endogenous Prx III in mitochondria appears to saturate the mitochondrial import machinery and results in substantial accumulation of Prx III in the cytosol.

Our results now indicate that Prx III is ~30-fold more abundant in the mitochondria of HeLa cells than is GPx1. This observation is consistent with the kinetic prediction that mitochondrial GPx1 accounts for removal of only 15% of the H_2O_2 produced in mitochondria (50.). Studies based on disruption of the GPx1 gene have suggested that GPx1 is dispensable for the survival of cells or mice exposed to proapoptotic conditions or mild oxidative stress, probably because Prx III is able to functionally substitute for it in mitochondria and several cytosolic Prx isoforms do so in the cytosol. The production and elimination of H_2O_2 appear to be delicately balanced in mitochondria, with our data now indicating that partial depletion of Prx III tilts the balance in favor of production in unstimulated cells and to a greater extent in cells exposed to inducers of apoptosis.

Although many proapoptotic stimuli induce the intracellular accumulation of H_2O_2, a causal relation between the mitochondrial generation of H_2O_2 and apoptosis has not been previously demonstrated. It has thus not been known whether the production of H_2O_2 is a passive event that occurs as the result of MnSOD action or whether, like $O_2^{\cdot-}$, H_2O_2 is an active participant in the apoptotic process. Our results now provide support for the latter scenario. The increased mitochondrial accumulation of H_2O_2 that resulted from depletion of Prx III thus led to an increase in the rate of apoptosis induced either by staurosporine or by TNF-α and CHX. Recruitment of the mitochondrial apoptotic pathway results in mitochondrial membrane permeabilization, which is mediated by the formation of protein-permeable channels by Bcl-2 family proteins in the outer membrane and by the opening of the permeability transition pore (PTP) in the inner membrane. The increase in membrane permeability is accompanied by the release of proapoptotic proteins such as cytochrome *c* and by initiation of the caspase cascade. Our results indicate that opening of the PTP, as reflected in a loss of $\Delta\Psi_m$, was enhanced in Prx III-depleted cells. One potential site of H_2O_2 action in this regard is the adenine nucleotide translocator, which is a component of the PTP. Although H_2O_2 is a mild oxidant, it is able to specifically oxidize certain cysteine residues with a low acid dissociation constant (73). Cysteine 56 of the adenine nucleotide translocator is sensitive to oxidation and its oxidation induces opening of the PTP (74–76).

Permeabilization of the mitochondrial membranes may not be sufficient to release cytochrome *c* molecules that are tightly bound to the inner mitochondrial membrane through interaction with cardiolipin, an anionic phospholipid that is restricted to mitochondria and is present predominantly in the inner membrane. The dissociation of cytochrome *c* is facilitated by peroxidation of cardiolipin (77, 78). Lipid peroxidation is readily achieved by $OH^{\cdot}$ but not by H_2O_2. The increased accumulation of H_2O_2 in Prx III-depleted cells might thus result in a greater abundance of $OH^{\cdot}$, which in turn might be responsible for the increased levels of cardiolipin peroxidation and cytochrome *c* release observed in these cells.

We also observed increased damage to mitochondrial proteins and polynucleotides in Prx III-depleted cells. Such damage is also likely the result of oxidation by $OH^{\cdot}$ and might be expected to lead to functional impairment of proteins that contribute to the respiratory chain and thereby to a further increase in the generation of $O_2^{\cdot-}$ and H_2O_2 in a feedback cascade. Similarly, loss of cytochrome *c* from mitochondria will also contribute to disruption of the respiratory chain, increased production of $O_2^{\cdot-}$ and H_2O_2, and faster dissipation of $\Delta\Psi_m$ (63, 79). Activated caspases also generate feedback to mitochondria both by cleaving antiapoptotic Bcl-2 family proteins and thereby promoting membrane permeabilization (80) and by disrupting the functions of complexes I and II of the electron

transport chain and thereby increasing the generation of ROS (28). It thus appears that H_2O_2 generated by mitochondria, like cytochrome *c* and caspases, functions in conjunction with multiple factors to amplify the death signal, and that Prx III modulates the extent of such amplification by controlling the concentration of available H_2O_2.

In addition to H_2O_2, Prx enzymes act on soluble alkylhydroperoxides (13), although it is not known if they are able to reduce peroxidized phospholipds in a membrane environment. Bacterial Prx enzymes catalyze the reduction of peroxynitrite (Bryk, 2000 no. 199), but a similar activity has not been demonstrated for mammalian Prx isoforms, which are more fragile than are their bacterial homologs. Despite these additional activities or potential activities, it is likely that the effects of Prx III depletion on apoptosis described in the present study were predominantly attributable to the accumulation of H_2O_2, given that they were abolished by overexpression of catalase.

Apoptosis is essential during development and for maintenance of healthy tissues (81). Dysregulation of apoptosis has thus been found to contribute to many pathologies, including neurodegenerative disorders (82, 83), as well as to tumor promotion (84, 85). In this regard, some human cancers express increased levels of Prx III, which might protect the tumor cells against apoptosis (86, 87). The abundance of Prx III was found to be reduced in the brains of humans with Alzheimer's disease or Down syndrome, possibly rendering the neuronal cells of these individuals more vulnerable to cell death (88).

In conclusion, we have shown that Prx III is an important, if not the most important, H_2O_2-eliminating enzyme in mitochondria and that the mitochondrial generation of H_2O_2 contributes to apoptotic signaling independently of the direct contribution of $O_2^{\cdot-}$. The intracellular accumulation of H_2O_2 caused by Prx III depletion resulted in acceleration of apoptosis, with increased rates of $\Delta\Psi_m$ collapse, cytochrome *c* release, and caspase activation. Our data thus indicate that the widely accepted view that GPx1 is the only important H_2O_2-metabolizing enzyme in mitochondria is not correct.

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Peroxiredoxin III, a Mitochondrion-specific Peroxidase, Regulates Apoptotic Signaling by Mitochondria

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