Downregulation of peroxiredoxin V stimulates formation of etoposide-induced double-strand DNA breaks

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Abstract Antioxidant protein Peroxiredoxin V (PrxV) is located in mitochondria and peroxisomes but is also present in the nucleus. Here, we show that nuclear PrxV associates with coilin-containing bodies suggesting possible interaction of this protein with transcription complexes. We also studied etoposide-induced phosphorylation of histone H2AX (γ-H2AX) in human cells in which PrxV activity was downregulated (knockdown, KD-clones) or compromised by overexpression of redox-negative (RD) protein. In KD clones, but not in RD-clones, formation of etoposide-induced γ-H2AX was increased, indicating that PrxV inhibits conversion of topoisomerase II cleavage complexes into double-strand DNA breaks but this inhibition is not caused by its antioxidant activity.

Keywords: Peroxiredoxin V; Localization; Cajal body; siRNA interference; Etoposide; Histone γ-H2AX

1. Introduction

Peroxiredoxins belong to the recently identified protein family, which play an important role in antioxidant defense and in hydrogen peroxide-mediated signaling (rev. in [1–3]). These proteins contain one or two redox-active cysteine residues which can be oxidized to a sulphenic acid by a peroxide substrate and then recycled back to a thiol [1]. Peroxiredoxins I and II (PrxI and PrxII) are shown to be essential for antioxidant protection of red blood cells in mice [4,5] and for protection of mammalian cells against ionizing radiation [6,7].

Human PrxV gene has been originally cloned in 1997 as a gene, which is downregulated in adenovirus-infected cells (Genbank Accession Nos. U82615, U82616). In 1999 the full cDNA for this gene was isolated, sequenced and identified as gene, which product is able to repress RNA polymerase III-dependent transcription of Alu retroposons in vitro [8]. Later in 1999 identical genes have been independently cloned as genes encoding a peroxisomal antioxidant protein [9] and a major antioxidant protein of bronchoalveolar lavage fluid [10]. PrxV contains C-terminal peroxisome-targeting signal 1 allowing this protein to concentrate in peroxisomes [8,9]. Nevertheless, significant amount of PrxV is also present in mitochondria [10], cytosol and in the nuclei [8]. Mouse PrxV was shown to be able to suppress p53-dependent apoptosis of cultivated cells [11] and human PrxV was able to promote differentiation and reduce apoptosis in the muscle cells of mice [12]. PrxV is also demonstrated to have antioxidant functions in some mammalian tissues like lung, cartilage and brain [13–16]. However, it still remains unknown whether antioxidant defense is the only function of this protein. Since significant amount of PrxV is found in the nucleus [8,17] where it may have antioxidant function, this protein may also play a role in regulation of transcription because of its direct protein–protein interactions with transcription factors [8]. For example, PrxI (also named Pag protein) interacts with box II domain of the c-Myc protein which is important for transcriptional regulation [18].

In this study, we constructed and analyzed two types of stable clones of human cells with impaired PrxV activity: (1) clones with downregulation of overall amount of PrxV protein using siRNA technology (knockdown, KD-clones), (2) clones with overexpression of redox-negative PrxV variant (RN-clones) in which only antioxidant activity of this protein was compromised. Both types of clones show an increased amount of intracellular ROS. However, the comparison of the amount of etoposide-induced double-strand breaks in these clones revealed that they differentially respond to this drug, suggesting the involvement of a non-redox acivity of PrxV. We also found that a fraction of PrxV associates with distinct nuclear bodies containing p80 coilin consistent with possible interactions of PrxV with transcription complexes.

2. Materials and methods

2.1. Construction of siRNA plasmids

Vector mU6pro with U6 RNA gene promoter was obtained from Dr. D.L. Turner (map can be found http://sitemaker.umich.edu/dlturner.vectors/rna_interference_vectors). siRNA encoding synthetic oligonucleotides were subcloned between *Bbs*I and *Xba*I restriction sites. NEO gene was amplified using PCR from the Rc-CMV plasmid

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Abbreviations: PrxV, peroxiredoxin V; NES, nuclear export signal; RN, redox-negative; KD, knockdown; DTT, dithiothreitol; PBS, phosphate-buffered saline; γ-H2AX, Ser-139 phosphorylated histone H2AX

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and subcloned between *Apa*I and *Hin*dIII sites. Clones with insertions of siRNA oligonucleotides were identified using PCR and sequenced with vector primers GCTACATTTTACATGATAGGCTTGG (U6-forward) and CACAGGAAACAGCTATGACCAT (M13-rev). Sequences (5′ to 3′) of siRNA complementary oligonucleotides for control GFP (green fluorescence protein) gene and PrxV gene (hairpin is shown in italic) are given below:

GFP5-top: **TTT**GAAGAAGTCGTGCTGCTTC*ATG*GAAGCAGCACGACTTCTTC**TTTTT**

GFP5-bottom: **CTAGAAAAA**GAAGAAGTCGTGCTGCTTC*CAT*GAAGCAGCACGACTTCTT

PrxV-top: **TTT**GAGAACCTCTTGAGACGTC*GAT*GACGTCTCAAGAGGTTCTC**TTTTT**

PrxV-bottom: **CTAGAAAAA**GAGAACCTCTTGAGACGTC*AT*C*G*ACGTCTCAAGAGGTTCT

2.2. Construction of plasmid expressing RN PrxV and purification of the recombinant proteins

Human PrxV cDNA fragment containing the coding mitochondrial targeting sequence (Genbank sequence file AF231705) was first subcloned in proper orientation into unique *Bam*HI site of the bacterial expression vector pQE30 (Qiagen). To isolate the plasmid encoding RN PrxV, we used standard Oligonucleotide Site-directed Mutagenesis method with mutated PrxV oligonucleotide 5′ ccttcacccctgaTCTtccaagacacacc 3′ in which normal TGT codon encoding redox active Cysteine-100 was replaced by TCT codon encoding redox-inactive Serine-100. Replacement of this codon in the resulting plasmid pQE-PrxV-RN was confirmed by sequencing. Further, human PrxV and PrxV-RN were expressed in *Escherichia coli* strain TG1 with their mitochondrial presequence as 6 × His-tagged proteins and purified as described previously [8].

For the construction of pPrxV-RN-IRES-Neo, human PrxV-RN cDNA was amplified from pQE-PrxV-RN plasmid by PCR using forward primer 5′ TCA CGA ATT CAG TGG CCG TGG GGC GGG T 3′ (*Eco*RI site underlined) and reverse primer 5′ CCA AGG ATC CAT TTG CAG GGC CCC AG 3′ (*Bam*H1 site underlined) and cloned into *Eco*RI and *Bam*H1 sites of pIRES-Neo (Clontech).

2.3. Cell culture

HeLa and non-small lung carcinoma U1810 cells were cultivated in DMEM containing D-glucose (4.5 g/l) and supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (complete DMEM) in a 95% humidified atmosphere, in 5% CO_2 at 37 °C. Cells were passaged at a 1:10 ratio every 5 days with 0.05% trypsin–EDTA.

2.4. Isolation of stable clones of U1810 cells

U1810, a non-small cell lung carcinoma cells, grown on 24-well plate were transfected either with redox negative mutant PrxV-RN in pIRES-Neo or with mU6neo, which expressed siRNA against PrxV using Lipofectamine 2000 obtained from INVITROGEN. 0.8 μg of plasmid DNA or 1.6 μl of Lipofectamine was diluted in 50 μl of Opti-MEM, incubated for 5 min, then mixed and incubated for 20 min before addition to cells. After overnight incubation at 37 °C in CO_2 incubator DMEM plus 10% FCS were added, incubation continued for 10 h, then cells were re-plated at 1/10–1/40 dilution onto the 6-well plates and selection with G418 (1 mg/ml) started on the next day. Individual clones growing on G418 were analyzed using Western blotting with antibodies to PrxV. Three of U1810 clones with overexpression of PrxV-RN and three of these clones with decreased expression of PrxV as well as the mix of control clones obtained after transfection of U1810 cells with either empty pIRES-neo vector or GFP siRNA expressing mU6neo plasmid were used in preliminary experiments and showed similar results. More detailed investigation was performed using two clones of each variant and average data obtained from three independent experiments using all these clones are presented.

2.5. Immunofluorescence analysis

Cells grown on microscopic slides were washed with phosphate-buffered saline (PBS) and fixed 15 min with 4% formaldehyde before or after extraction with 0.1% solution of Triton X-100 in PBS for 1 min at room temperature. After permeabilization with 0.1% Triton X-100 in PBS for 10 min, cells were incubated overnight at 4 °C with the combination of two of the following primary antibodies in 5% solution of BSA in PBS: rabbit anti-human PrxV polyclonal antibodies (1/100) affinity purified as described in [8], and mouse anti-coilin monoclonal antibodies (1:25, BD Biosciences). After rinsing 4 × 5 min in PBS, the samples were incubated for 1 h with the following secondary antibodies in 5% BSA in PBS and with 1 μg/ml Hoechst 33342 (Sigma): TRITC-conjugated donkey anti-rabbit antibodies (1:175, Jackson) for PrxV and CY2-conjugated goat anti-mouse antibodies (1:400, Amersham Bioscience) for coilin. After final rinsing 4 × 5 min in PBS, the samples were mounted on glass slides using Pro-Long mountain medium (Molecular Probes) and analyzed on Olympus fluorescent microscope.

2.6. FACS analysis of sub-G1 cells

Propidium iodide (PI) staining of cells was used for apoptosis studies [19]. Control and transfected U1810 cells were seeded in 6-well plate for 36 h. Apoptosis was induced by treatment with 0.5 mM hydrogen peroxide or 8 Gy IR. After indicated times cells were collected by trypsinization, washed once with cold PBS, mixed with 500 μl of hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100, 100 μg/ml RNAse, and 50 μg/ml PI) and analyzed by flow cytometry after 60 min incubation. Cells that had lost part of their DNA due to the DNA fragmentation were detected as a population of cells with sub-G1 DNA contents (apoptotic cells).

2.7. Immunoblotting

The U1810 cell clones were cultivated in 6-well plates. At the stage of 70–90% confluence, cells were treated with 10 μM etoposide for indicated times, washed with PBS, resuspended in the Laemmli's loading buffer, harvested and heated at 95 °C for 4 min. Twenty microliters of each sample were resolved on 15% SDS–polyacrylamide gel and electroblotted onto nitrocellulose membranes. Membranes were blocked with PBS supplemented with 5% non-fat dry milk on a rocker for 1 h at room temperature and then incubated overnight at 4 °C with 1:10 000 mouse monoclonal anti-phospho-H2AX (Ser139) antibody (Upstate Biotech #05–636) or 1:2500 rabbit anti-human PrxV polyclonal antibody or with 1:3000 rabbit anti-G3PDH, followed by incubation for 1 h with 1:10 000 peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG. Bands were visualized by ECL (Pharmacia Biotech) according to the manufacturer's instructions and detected by exposure to X-ray film. Densitometry was performed on films using a GS-700 densitometer and "Quantity One" software (Bio-Rad, USA). Normalization of γ-H2AX and PrxV proteins levels was performed with G3PDH levels.

3. Results and discussion

PrxV has wide intracellular localization. It is mainly located in mitochondria and cytoplasm [8,10] but significant amount of PrxV is also present in the nucleus [8,17]. During our studies of localization of PrxV in formaldehyde-fixed human cells, besides the patterns of mitochondrial, cytoplasmic and homogeneous nuclear staining, we reproducibly observed distinct nuclear bodies containing this protein resembling in size and number of Cajal bodies. Marker protein for Cajal bodies is p80 protein coilin, and double labeling of untreated U1810 or HeLa cells with anti-PrxV and anti-coilin antibodies showed that the majority of nuclear PrxV colocalize with coilin-positive nuclear bodies (Fig. 1). Since Cajal bodies are known to play a critical role in preassembly of transcription complexes [20], we believe that PrxV may be involved in regulation of transcription through its interaction with transcription complexes. This may potentially explain the presence of significant amount of PrxV (both diffuse and punctate) in the nucleus despite the absence of known nuclear import signals in this protein. Interestingly, PrxV protein sequence contains potential nuclear export signal (NES), (**Val**GlyAspAla**Ile**Pro-Ala**Val**Glu**Val**) which fits very well to the consensus NES

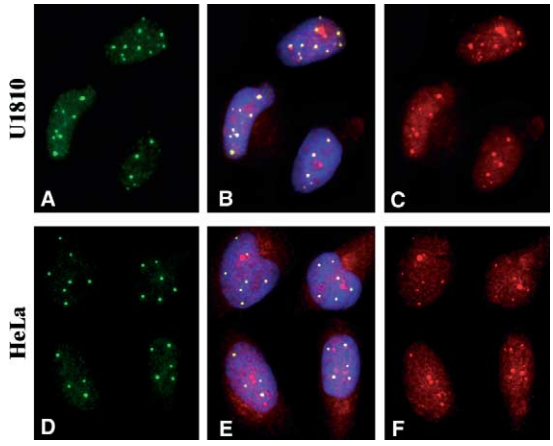

Fig. 1. Immunocytochemical staining of PrxV (red) and coilin (green) in U1810 cells after Triton X-100 extraction and in HeLa cells without the extraction (for details see Section 2). Counterstaining with Hoechst 33342 was performed (blue signal). Bar 10 μm.

[21,22]: X–R(2–4)–X–R2–X–R–X, where X is leucine, isoleucine or valine and R represents any amino acids. PrxV-associated NES may regulate transport of non-complexed nuclear PrxV to the cytoplasm. Interestingly, PrxI, which like PrxV lacks canonical nuclear localization signal, can be targeted to the nucleus because of its direct interaction with c-Abl protein [23].

To get insight into possible functions of PrxV, stable clones of human cells with impaired PrxV activity were isolated. First, we have constructed plasmid with replacement in full PrxV cDNA of redox-active cysteine (Cys-100) for serine. This has been done by primer extension of mutant oligonucleotide on a PrxV cDNA plasmid template and selection of resulting bacterial clones by hybridization. Mutant cDNA fragment from selected plasmid was then subcloned into bacterial (pQE-30) and mammalian (pIRES-Neo) expression vectors and examination of bacterially expressed and purified protein confirmed inactivation of its peroxidase activity (Fig. 2A). Mammalian expression plasmid with C100S PrxV was then used in transfection of U1810 cells and selection of stable G418-resistant clones. In two of these clones used in subsequent studies, overexpression of redox-inactive PrxV resulted in approximately 2-fold increase of intracellular ROS, which was detected by FACS analysis with dichlorofluorescein diacetate (DCFH-DA) (Fig. 2C). This suggests that redox-inactive PrxV might downregulate expression of endogenous redox-active PrxV and compete with this protein in indicated clones (named Redox Negative, RN-clones), thus suppressing its normal antioxidant function. Overall amount of PrxV protein detected in RN-clones by Western blotting with polyclonal antibodies, which cannot discriminate redox-inactive from endogenous PrxV, was found to be increased (Fig. 2B).

To isolate human PrxV KD clones expressing PrxV siRNA, a vector with U6 RNA gene promoter (mU6-pro) was used [24] into which we subcloned NEO gene and oligonucleotides with inverted repeats from PrxV gene (see Section 2). The silencing activity of resulting plasmid towards PrxV was confirmed by transient transfections of human 293 cells in which a decrease of PrxV protein was detected by Western blotting (not shown). This plasmid was introduced into U1810 cells and G418-resistant clones were selected and analyzed for PrxV content by Western blotting. Strong decrease of PrxV protein was detected in stable KD clones of U1810 cells (Fig. 2B), indicating that siRNA from the selected segment of PrxV gene efficiently induces degradation of PrxV mRNA. In KD-clones,

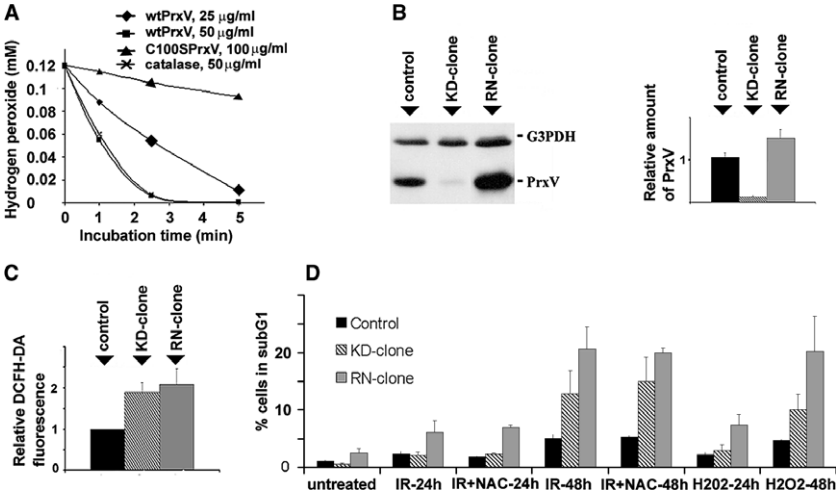

Fig. 2. Characterization of U1810 cell clones with impaired PrxV activity. (A) Peroxidase activity of recombinant wtPrxV, C100SPrxV and bovine catalase. The reaction mixture (200 μl) contained 50 mM HEPES–NaOH, pH 7.4, 2 mM DTT, 0.12 mM H_2O_2 and proteins. At the indicated times, the remaining concentration of H_2O_2 was measured in 20 μl aliquots of reaction mixture with ferrous ammonium sulfate/potassium thiocyanate [30] and compared with standards. Data are the means of three experiments. (B) Expression of PrxV protein in the U1810 clones. Western blotting with antibodies against PrxV was performed with cell lysates (30 μg of total protein) prepared from the KD, RN and control U1810 clones. Antibodies against G3PDH were used for protein loading control. Quantitation of results from three experiments is present on the right side. (C) Fluorescence intensities of DCFH probe in the U1810 clones. Bars represent mean values of cell fluorescence relative control U1810 clone obtained from three independent experiments. (D) Cells were irradiated (8 Gy) or treated with 500 μM hydrogen peroxide and percent of the subG1 cells was measured after 24 and 48 h. All values are means from three independent experiments. Concentration of NAC was 5 mM and this agent was added just before irradiation.

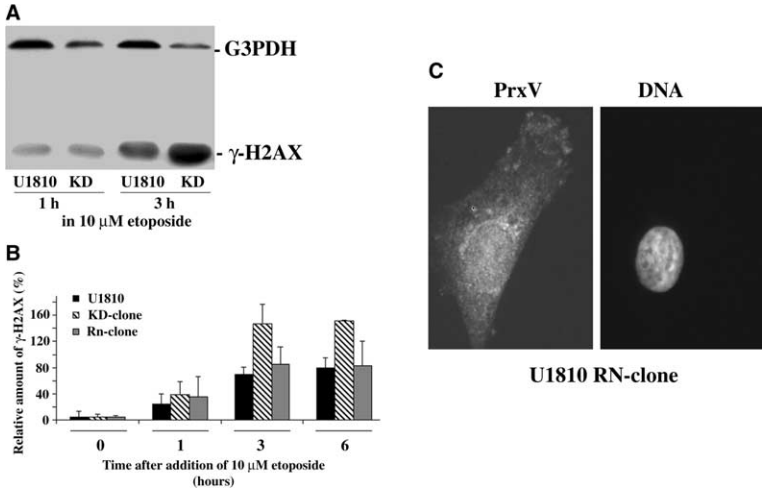

Fig. 3. Induction of DSBs in genomic DNA in the U1810 clones after treatment with 10 μM etoposide. (A) The U1810 cell clones were treated with etoposide at indicated time and formation of DSBs was analyzed by Western blotting using monoclonal anti-γ-H2AX antibodies. (B) Bars represent mean values of γ-H2AX/G3PDH densitometry ratios ± S.D. obtained from three experiments. (C) Immunocytochemical staining of PrxV in U1810 RN clone (left image). The right image is DNA staining of the same cell with Hoechst 33342.

downregulation of PrxV expression resulted in approximately 1.9-fold increase of intracellular ROS (Fig. 2C).

For further characterization of isolated KD- and RN-clones, their response to treatment with oxidative agents was studied. Phenotypic consequences of downregulation of PrxV were analyzed using measurement of the DNA fragmentation after treatment with 500 μM hydrogen peroxide or γ-irradiation (8 Gy). Fragmentation of DNA, indicator of the late events of apoptosis, results in the appearance of cells containing subG1 level of DNA that can be detected by flow cytometry analyses of propidium iodide-stained cells [19]. Both KD- and RN-clones contained significantly increased fraction of sub-G1 cells 48 h after γ-irradiation (8 Gy) or treatment with 500 μM H_2O_2 compared to control cells (Fig. 2D), indicating that PrxV suppresses radiation- and hydrogen peroxide-induced apoptosis in agreement with earlier reports [11,12]. However, efficiency of induction of apoptosis was higher in RN-clone compared to KD-clone and clearly seen in RN-clone even 24 h after cell damage (Fig. 2D). Since both RN- and KD-clones show approximately the same increase of ROS (Fig. 2C), this result suggests that not only antioxidant activity of PrxV may be responsible for suppression of apoptosis by this protein. Confirming this view was our finding that the reducing agent *N*-acetyl cysteine (NAC, 5 mM), which significantly decreased the amount of intracellular ROS, did not effect radiation-induced apoptosis in U1810 cells (Fig. 2D).

To investigate the possible role of PrxV in protection of cells against non-oxidative DNA damage, we used well-known anticancer agent, etoposide, which kills tumor cells by induction of DNA double-strand breaks, DSBs [25]. This drug inhibits religation of topoisomerase II-induced DNA breaks and formed topo II cleavage complexes that can be then converted to DSBs through DNA unwinding during replication or transcription [25]. Topo II is required for transcription elongation on chromatin template to relieve arising superhelical tension [26]. We analyzed etoposide-induced DSBs in RN- and KD-clones by Western blotting using antibodies against phosphorylated histone H2AX (γ-H2AX), which is known to be formed at megabase chromatin domains near DSBs [27,28] and which presence strongly correlates with unrepaired DSBs in vivo [29]. γ-H2AX can be detected in cells as early as 1 h after addition of 10 μM etoposide and maximal amount of γ-H2AX was found at 3–6 h after treatment with this drug (Fig. 3B). Significant increase of etoposide-induced γ-H2AX was observed in KD-clone at 3 and 6 h compared to parental U1810 cells (Fig. 3A and B), suggesting that decreased amount of PrxV in KD cells stimulated conversion of topo II cleavage complexes to DSBs. Under similar experimental conditions about the same amount of γ-H2AX as in parental cells was detected in RN-clone (Fig. 3B), suggesting that suppression of the antioxidant activity of PrxV does not affect etoposide-induced DSBs. The absence of PrxV-mediated protection against etoposide-induced DSBs in RN-clone is not a consequence of changed localization of this protein and PrxV can be easily detected in the nucleus using indirect immunofluorescence (Fig. 3C). Taken together, these results suggest that PrxV-dependent suppression of conversion of etoposide-induced cleavage complexes into DSBs in human cells is not associated with the antioxidant activity of PrxV but depends on another functional activity of this protein in the nucleus. Since PrxV co-localized with Cajal bodies and since PrxV gene has been originally cloned as potential repressor of RNA polymerase III-dependent transcription in vitro [8], it is likely that the ability of PrxV protein to suppress formation of etoposide-induced DSBs (Fig. 3) is associated with its role in regulation of transcription in vivo. Further studies of transcription in KD-clones and interactions of PrxV with transcription complexes may be helpful to get insight into possible functions of this protein in the nucleus.

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