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Role of the PLA_2-independent peroxiredoxin VI activity in the survival of immortalized fibroblasts exposed to cytotoxic oxidative stress

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Abstract Peroxiredoxin VI (PrxVI) is a bifunctional enzyme with non-selenium glutathione peroxidase and Ca^{2+}-independent acidic phospholipase A_2 activities. We demonstrate that transfection-mediated PrxVI overexpression protects immortalized human WI-38 and murine NIH3T3 fibroblasts against cytotoxic doses of *tert*-butylhydroperoxide and H_2O_2. Mutants for either glutathione peroxidase or phospholipase A_2 activity show that glutathione peroxidase but not phospholipase A_2 activity is required to promote cell survival after stress. Also, ectopic PrxVI overexpression does not protect telomerase-stabilized WI-38 fibroblasts against stress-induced premature senescence.

Key words: Peroxiredoxin; Oxidative stress; Fibroblast; Telomerase; Premature senescence

1. Introduction

Peroxiredoxins are peroxidases that reduce hydrogen peroxide and alkyl hydroperoxides to water and alcohol respectively, with the use of reducing equivalents provided by thiol-containing molecules [1–4]. Peroxiredoxin VI (PrxVI), also termed antioxidant protein-2 or 1-cys peroxiredoxin, contains a single redox-active cysteine (Cys^{47}) and is a cytosolic bifunctional enzyme with peroxidase and phospholipase A_2 (PLA_2) activities [5–7]. Macrophages derived from $PrxVI^{-/-}$ mutant mice produce higher H_2O_2 levels and display increased susceptibility to paraquat, H_2O_2 and *tert*-butylhydroperoxide (*t*-BHP). $PrxVI^{-/-}$ knock-out mice display significantly lower survival rates, severe tissue damage and higher protein oxidation levels [8]. Antisense oligonucleotides against PrxVI trigger accumulation of lipid hydroperoxides and promote apoptosis in L2 rat epithelial cells [9]. Stable expression of a green fluorescent protein-PrxVI fusion protein in highly stress-sensitive lung-derived H441 cells lacking endogenous PrxVI leads to increased degradation of H_2O_2 and *t*-BHP and decreased sensitivity to oxidative stress [10]. Lipid hydroperoxides are hydrolyzed by PLA_2 to free fatty acid hydroperoxides and then converted into a variety of toxic end-products such as aldehydes and epoxides. Thus PrxVI could promote restoration of membrane function and integrity by cleavage and elimination of peroxidized phospholipids by combining its peroxidase and PLA_2 enzymatic activities.

In this study we demonstrate that ectopic overexpression of PrxVI in SV40-immortalized WI-38 human fibroblasts and murine NIH3T3 fibroblasts increases cell survival after cytotoxic *t*-BHP and H_2O_2 stress. Mutants for either peroxidase or PLA_2 activity show that the protective effect is PLA_2-independent. Using a cell-based double transgenic model, we show that telomerase-stabilized WI-38 human diploid fibroblasts (HDFs) overexpressing PrxVI do not escape premature senescence induced by subcytotoxic concentrations of *t*-BHP and H_2O_2, strengthening previous preliminary data obtained with retrovirally transfected primary HDFs [11].

2. Materials and methods

2.1. Cell culture and media

WI-38 SV40 human fibroblasts (American Type Culture Collection, USA) were cultured in modified essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. NIH3T3 Flp-In cells (Invitrogen, Carlsbad, CA, USA) were grown in D-MEM high glucose supplemented with 10% donor calf serum, 1% penicillin/streptomycin, 2 mM L-glutamine and 100 μg/ml zeocin. WI-38 h-TERT cells [12] were cultivated in MEM supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine, and non-essential amino acids.

2.2. Expression of human PrxVI in WI-38 SV40 and h-TERT cells

WI-38 SV40 cells were transfected (CalPhos Mammalian Transfection kit, BD Bioscience Clontech, Palo Alto, CA, USA) with a plasmid containing either the PrxVI cDNA under the control of the pCMV promoter (pCR3.1/PrxVI, gift of A. Fisher, University of Pennsylvania) or the control vector pCR3.1. At 48 h after transfection, cells were selected for 2 weeks with 400 μg/ml G418 (Gibco, Paisley, UK).

2.3. Expression of human PrxVI and the peroxidase and PLA_2 mutants in mouse NIH3T3 cells

Stable expression cell lines were generated by Flp recombinase-mediated integration (Flp-In System, Invitrogen). The cDNAs corresponding to wild-type PrxVI, PrxVI C47 (Cys^{47} to Ser) or PrxVI S32 (Ser^{32} to Ala) mutants were polymerase chain reaction (PCR)-amplified from pET21b plasmids (gift of A. Fisher) using Pfu DNA polymerase (Promega, Madison, WI, USA), forward primer 5′ CACCATGCCCGGAGGTCTGCTTC and reverse primer 5′ AGGCTGGGGTGTGTAGCG. The 675-bp amplicons were cloned into the pEF5/FRT/V5-D-TOPO downstream of the human EF1α promoter using the Directional TOPO Expression Kit (Invitrogen). As the native PrxVI stop codon was not included in the PCR fragment, PrxVI and mutants were expressed as fusion proteins with a vector-derived C-terminal V5 epitope for detection. All constructs were sequenced full length. NIH3T3 Flp-In cells were then transfected with expression plasmids together with pOG44 for transient expression of

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Flp recombinase. At 48 h after transfection, cells were plated in medium containing 200 μg/ml hygromycin B (Invitrogen) for polyclonal selection.

2.4. Analysis of PrxVI overexpression by Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline, pH 7.4 (PBS) and lysed using 0.8 ml M-PER solution (Pierce, Rockford, IL, USA) containing protease inhibitor mixture (Complete tablets, Roche, Mannheim, Germany). Classical electrophoresis and transfer were performed. Membranes were incubated overnight either with a rabbit polyclonal antibody directed against PrxVI (gift from A. Fisher, University of Pennsylvania) or with the anti-V5 mouse monoclonal antibody (Invitrogen), both diluted in Tris-buffered saline plus Tween 0.1% (TBS-T) with milk and for 1 h with horseradish peroxidase-conjugated IgGs. Bound antibodies were detected by chemoluminescence (ECL Advanced Detection kit, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The level of α-tubulin (anti-α-tubulin monoclonal antibody, Innogenex, San Ramon, CA, USA) was used as reference for semi-quantification (ImageMaster TotalLab, Pharmacia, Uppsala, Sweden).

2.5. Immunofluorescence microscopy

Cells on glass coverslips were rinsed in PBS and fixed 10 min with PBS plus 4% paraformaldehyde (Merck, Darmstadt, Germany). The fixed cells were washed three times with PBS and permeabilized with a solution of PBS-Triton X-100 1% (Sigma, St. Louis, MO, USA). After three washing steps with PBS+bovine serum albumin (BSA) 3% (Sigma, Bornem, Belgium), cells were incubated at 4°C overnight with the anti-PrxVI or anti-V5 antibody (dilution 1/1000). Cells were washed three times with PBS+BSA 3%. Secondary antibodies conjugated to Alexa fluorochrome (488) (dilution 1/500, Molecular Probes, Leiden, The Netherlands) were added for 1 h. The cells were washed three times with PBS+BSA 3%. For nucleus labelling, cells were incubated with Topro3 (dilution 1/80, Molecular Probes), mounted in Mowiol and observed under a confocal microscope (Leica, Wetzlar, Germany).

2.6. Cytotoxic stress with t*-BHP, H_2O_2, ethanol and UVB*

WI-38 SV40 or NIH3T3 Flp-In cells were cultured in 6-well dishes at 60–80% confluence. Cells were exposed for 2 h with increasing concentrations of *t*-BHP (0–500 μM; Merck), H_2O_2 (0–500 μM; Merck) or ethanol (0–8%, v/v; Merck) diluted in medium containing 1% serum. After stress, cells were rinsed twice and supplemented with complete medium. Cell survival was monitored at 24 h after stress using the MTT method [13–15]. Alternatively, cells were rinsed with PBS and exposed to UVB (0 and 5 J/cm^2) in a thin layer of PBS using three TL 20W/01 lamps (Philips, Eindhoven, The Netherlands) at room temperature at a target distance of 30 cm. The emitted light was checked using a UVR radiometer with a UVB sensor (Fisher Bioblock Scientific, Illkirch, France). After a 2 h stress, cells were rinsed with PBS and supplemented with complete medium.

2.7. Stress-induced premature senescence

WI-38 h-TERT HDFs were exposed for 2 h to 30 μM *t*-BHP or 30 μM H_2O_2 diluted in MEM plus 1% FCS, rinsed twice with MEM and incubated in complete medium for 96 h. Control cultures (no stress) were processed similarly, without stressing agent. At 96 h after stress, the proportion of mitotic (morphotypes I, II and III) and post-mitotic (morphotypes IV, V and VI) HDFs was recorded in non-confluent cells [16], and the percentage of HDFs positive for senescence-associated β-galactosidase (SA β-galactosidase) was determined as described [17]. The results were expressed as mean value ± S.D. of three independent experiments in each of which 400 cells were considered.

3. Results and discussion

3.1. Overexpression of human PrxVI in WI-38 SV40 cells

WI-38 SV40 cells were transfected with the pCR3.1/PrxVI plasmid for stable overexpression of native PrxVI. After selection, several clones produced high levels of PrxVI as compared with the cells transfected with vector pCR3.1 (Fig. 1A). A 3.7–5.7-fold increase in PrxVI expression level was found among the clones tested (Fig. 1B). Clone 11b was selected for

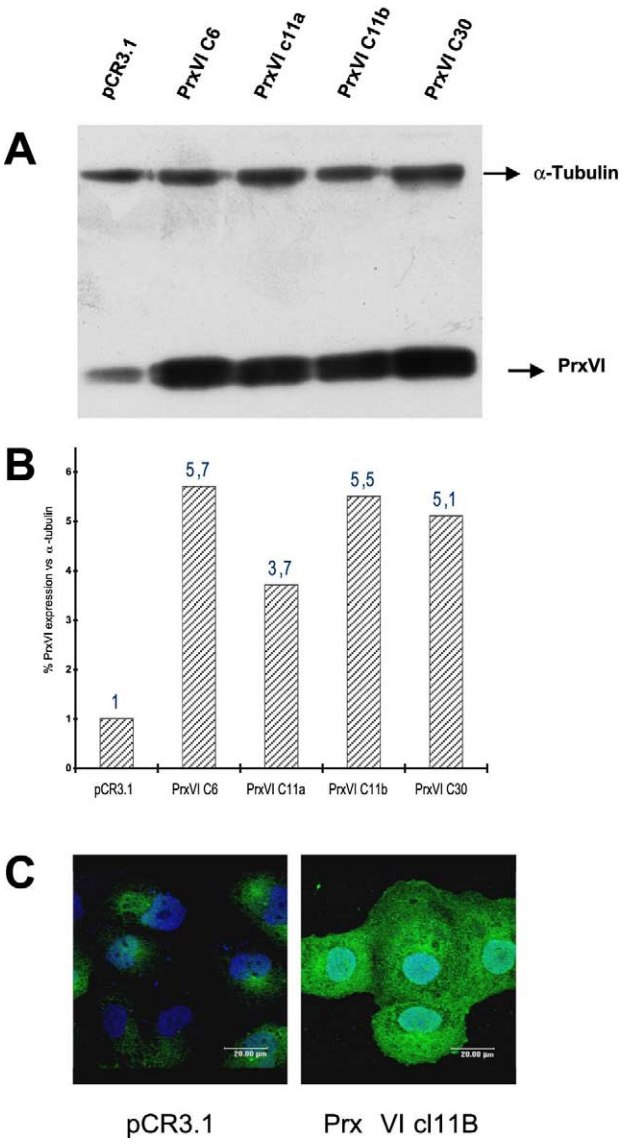

Fig. 1. Expression of human PrxVI in WI-38 SV40 cells. A: Immunoblotting analysis of PrxVI in WI-38 SV40 clones isolated after G418 selection of stably transfected cells. B: Quantification of PrxVI overexpression with α-tubulin level as reference. C: Immunofluorescence analysis of PrxVI overexpression in clone 11b using confocal microscopy (left: WI-38 SV40 transfected by pCR3.1, right: WI-38 SV40 transfected by pCR3.1/PrxVI).

further analysis. Immunofluorescence confirmed overexpression of PrxVI in the cytosol of the transfected cells (Fig. 1C). Also unexpected nuclear localization was detected. Immunohistochemical analysis of PrxVI in rat tissues previously demonstrated stronger staining in some nuclei than in the cytosol [18]. Both rat and human PrxVI share the PDKKLKL peptide closely related to the single cluster of basic amino acids for nuclear targeting. We hypothesize that this sequence close to the nuclear targeting signal consensus might target some PrxVI to the nucleus in overexpressing conditions. In addition, it should also be noted that peroxiredoxin I has also been observed in the cytoplasm and the nucleus after transfection of its cDNA in NIH3T3 fibroblasts. Although PrxI lacks a canonical nuclear localization signal, it could be transported to the nucleus in association with other proteins such as c-Abl or enter passively thanks to its small size [19].

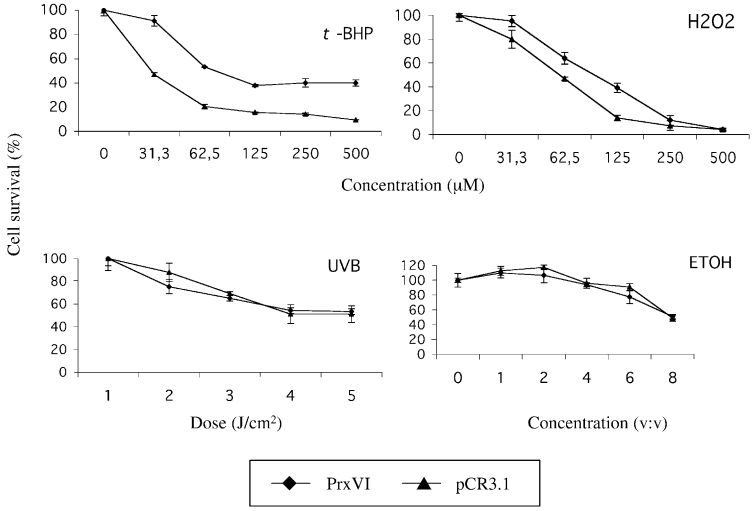

Fig. 2. Effects of PrxVI overexpression on cell survival against cytotoxic stress with *t*-BHP, H_2O_2, ethanol (EtOH) and UVB. Cells transfected with pCR3.1/PrxVI or by pCR3.1 alone were exposed to increasing doses of stressors for 2 h. Survival was estimated at 24 h after stress (MTT assay). Results are expressed as percentages of survival of unstressed cells and represent the mean values of three independent experiments ± S.D.

3.2. *Increased survival after cytotoxic exposures to* t-*BHP and* H_2O_2 *but not ethanol and UVB*

WI-38 SV40 cells overexpressing PrxVI as well as cells transfected with the pCR3.1 control plasmid were exposed for 2 h to increasing concentrations of *t*-BHP, H_2O_2, ethanol and increasing doses of UVB. Cell survival was determined at 24 h after stress. PrxVI-overexpressing cells were more resistant to *t*-BHP (0–500 μM) and H_2O_2 (31.25, 62.5, 125 μM; Fig. 2). Protection against *t*-BHP was dramatic since an increase in survival of 20–40% was found. Given its hydrophobicity, *t*-BHP critically affects biological membranes, causing peroxidation of membrane lipids and subsequent cell death. H_2O_2 is hydrophilic, has a higher reactivity than *t*-BHP and induces damages to many more cellular components than membranes. Hydrophobicity could explain why protection of PrxVI against H_2O_2 cytotoxicity is lower than against *t*-BHP. No protective effect on cell survival was observed after exposure to ethanol (0–8%, v/v) or UVB (0–5 J/cm^2).

3.3. *Peroxidase but not* PLA_2 *activity is essential for protection of NIH3T3 cells against* t-*BHP*

Mutation of Ser^{32} to Ala (S32A) abolishes the PLA_2 activity of PrxVI and does not affect the glutathione peroxidase activity, while the reverse is observed with mutation of the conserved Cys^{47} to Ser (C47S) [5]. Intracellular peroxidase activity of PrxVI is well documented in NIH3T3 fibroblasts [7]. Overexpression of PrxVI in these cells prevents the increase of H_2O_2 level triggered by incubating the cells with platelet-derived growth factor (PDGF). This PDGF-induced increase of H_2O_2 level is not inhibited with the C47S mutant, consistent with the loss of its peroxidase activity in NIH3T3 and confirming biochemical assays [5]. This justifies why NIH3T3 cells were chosen for this part of the study. Also a Flp-In-compatible line of NIH3T3 cells was available, allowing polyclonal selection of transfected cells.

We investigated whether inactivation of either peroxidase or PLA_2 activity still allowed a protective effect of PrxVI overexpression against *t*-BHP cytotoxicity. The cDNA corresponding to wild-type PrxVI as well as the C47S and S32A mutants were transfected in NIH3T3 Flp-In cells. Western blots (Fig. 3A) and immunofluorescence microscopy (Fig. 3B) showed that the transfected cells overexpressed PrxVI and the C47S and S32A mutants at similar levels. Cells were exposed for 2 h to increasing concentrations of *t*-BHP (0–200 μM) and allowed to recover in complete medium. After 24 h, we found that overexpression of wild-type PrxVI also protected NIH3T3 fibroblasts against *t*-BHP cytotoxicity at all concentrations tested. When the peroxidase activity was abolished by mutation, the survival advantage of PrxVI overexpression vanished. Nevertheless, overexpression of the PLA_2-deficient PrxVI still allowed increased survival after exposure to *t*-BHP. Unexpectedly, at 150 and 200 μM *t*-BHP, protection was even better with the mutant than with wild-type PrxVI (Fig. 4). This finding supports the hypothesis that PLA_2 activity might be deleterious and involved in oxidant-induced cell death at high *t*-BHP concentrations, while peroxidase activity is absolutely required to promote survival after stress.

3.4. *PrxVI overexpression in WI-38 h-TERT HDFs does not protect against* t-*BHP- or* H_2O_2*-induced premature senescence*

Exposure of HDFs to different types of subcytotoxic stress such as *t*-BHP and H_2O_2 triggers acute stress-induced premature senescence or SIPS. SIPS can be defined as the long-term effects of subcytotoxic stress on proliferative cell types, including appearance of many features of replicative senescence, such as irreversible growth arrest, a dramatic shift to post-mitotic morphology and an increase in the proportion of cells positive for SA β-galactosidase activity [20].

A major disadvantage of transfection of a cDNA in cells with limited proliferative lifespan is that the selection process consumes a significant part of the proliferative potential of those cells, which potentially affects results about premature senescence. Therefore, we tried to induce SIPS in SV40-immortalized WI-38 cells, but we could not detect any senescence-like morphological change or SA β-galactosidase activity at 96 h after exposure to subcytotoxic concentrations of

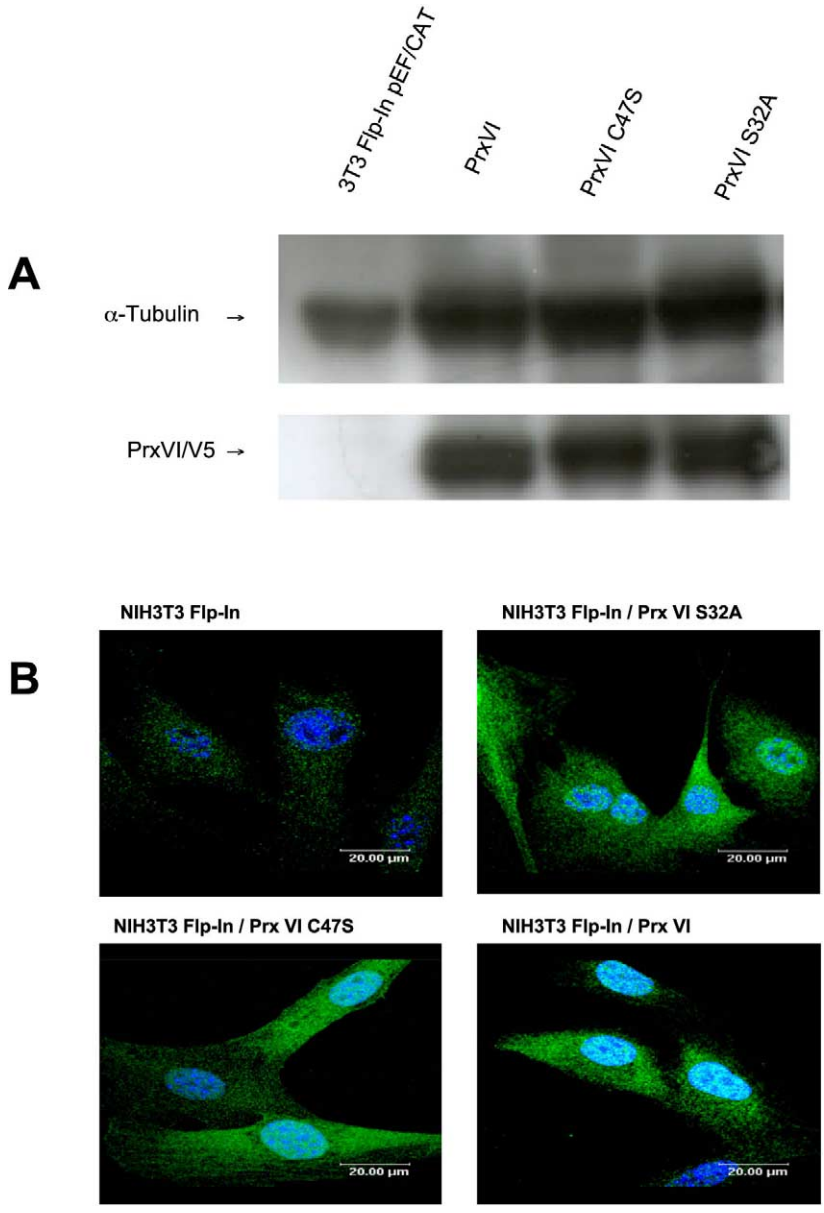

Fig. 3. Expression of wild-type PrxVI, S32A and C47S mutants in NIH3T3 Flp-In cells. A: Expression of V5-tagged PrxVI and mutants was tested in total cell lysates of stably transfected NIH3T3 Flp-In cells. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by Western blotting using an anti-V5 monoclonal antibody. B: Immunofluorescence analysis of normal and mutant PrxVI overexpression in transfected or untransfected cells using confocal microscopy. The monoclonal anti-V5 antibody was used as primary antibody.

t-BHP or H_2O_2. This was probably due to the fact that immortalization by SV40 large T antigen involves binding and degradation of p53 and pRb [21,22]. pRb is an essential mediator of SIPS [22]. Fortunately, we knew that similar proportions of parental and telomerase-expressing fibroblasts of at least two strains (WI-38 ± h-TERT, BJ ± h-TERT) prematurely senesce when exposed to subcytotoxic stress with *t*-BHP and H_2O_2 [12,23]. Ectopic h-TERT expression stabilizes cells in culture by preventing telomere shortening, allowing extra-proliferative life span. This does not interfere directly with known pathways involved in SIPS.

PrxVI cDNA was therefore transfected in WI-38 h-TERT HDFs. After selection, clones were isolated and tested individually for PrxVI overexpression. Clone 4 demonstrated a 4.6-fold overexpression (Fig. 5). Cells of this clone were subjected to a single subcytotoxic 2 h stress with 30 µM *t*-BHP or H_2O_2. SA β-galactosidase activity and morphotypes were used as markers of senescence. A single exposure of WI-38 h-TERT pCR3.1 (empty vector) or WI-38 h-TERT PrxVI to subcytotoxic concentrations of *t*-BHP or H_2O_2 led to a similar increase in the proportion of SA β-galactosidase-positive cells (Fig. 6A) and a shift from mitotic to post-mitotic morphotypes (Fig. 6B,C). These observations suggest that PrxVI overexpression does not protect WI-38 h-TERT HDFs against premature senescence, thereby strengthening previous results obtained with parental WI-38 cells [11].

The data obtained in this work are stronger than the previously published work [11] since we were able to demonstrate overexpression at the protein level, which was not possible in the previous preliminary work. This suggests that the over-

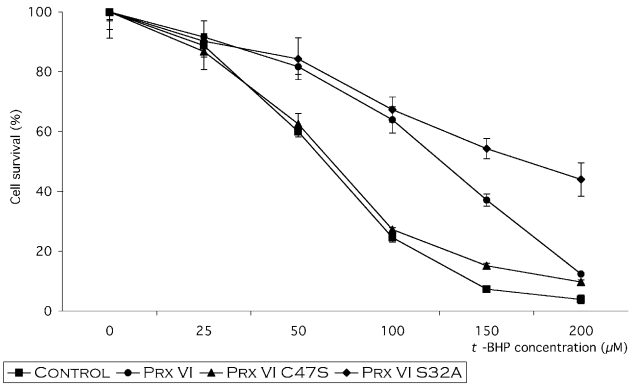

Fig. 4. Cytotoxicity of *t*-BHP in NIH3T3 Flp-In cells overexpressing wild-type PrxVI, S32A or C47S mutants. Transfected or control cells were exposed to increasing concentrations of *t*-BHP for 2 h. Survival was estimated at 24 h after stress (MTT assay). Results are expressed as percentages of survival of unstressed cells and represent the mean values of three independent experiments ± S.D.

expression of PrxVI was very weak and probably explains the lack of protection against H_2O_2 cytotoxicity in this previous work. Also, the previous work was performed on parental WI-38 cells which displayed a very high resistance to H_2O_2 when compared to the cell lines used herein, which might also have obscured any potential protective effect of overexpression.

In conclusion, we demonstrated that intracellular PrxVI can protect fibroblasts of various origins against *t*-BHP and H_2O_2 stress while it has no effect on stress-induced premature senescence. These data give clues on the in vivo role of PrxVI.

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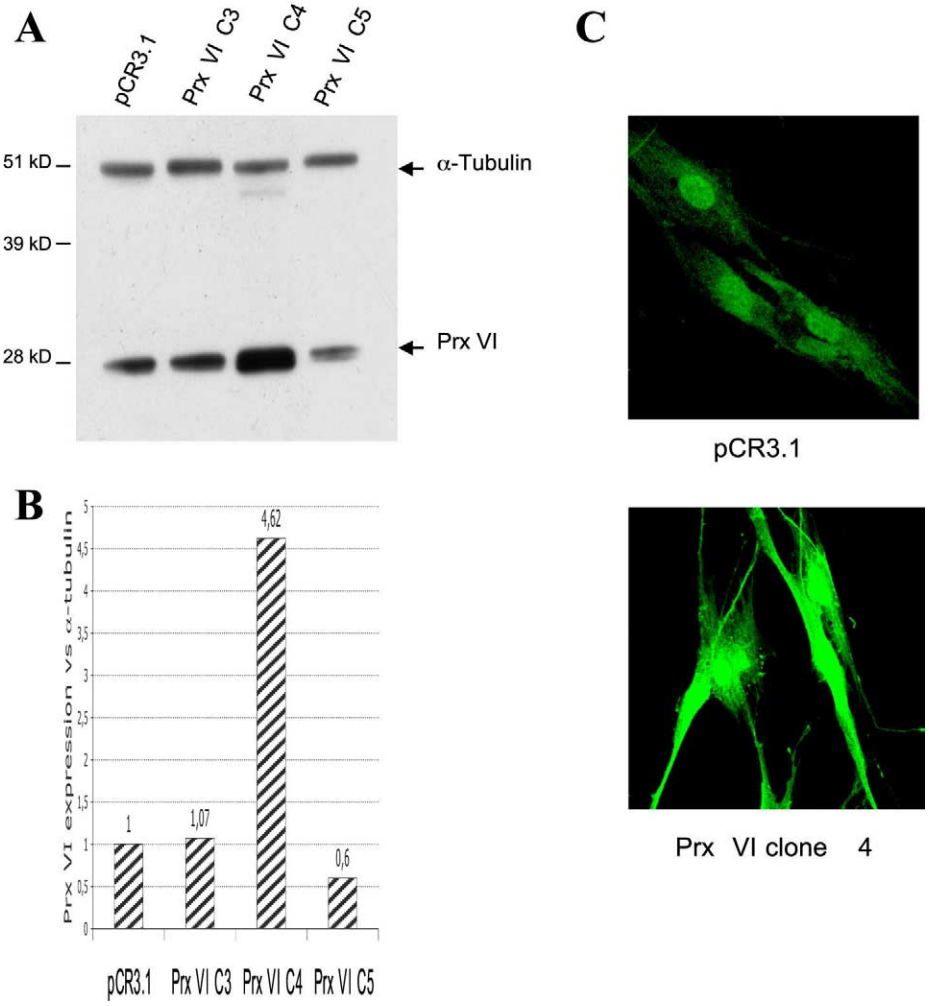

Fig. 5. Expression of human PrxVI in WI-38 h-TERT cells. A: Immunoblotting analysis of PrxVI in three different clones of WI-38 h-TERT cells isolated after selection of transfected cells (C3, C4, C5). B: Quantification of PrxVI overexpression using α-tubulin as reference. Clone C4 was selected for further testing. C: Immunofluorescence analysis of PrxVI overexpression in clone 4 using confocal microscopy (upper panel: WI-38 h-TERT transfected by the empty vector pCR3.1, lower panel: WI-38 h-TERT transfected by pCR3.1/PrxVI clone 4).

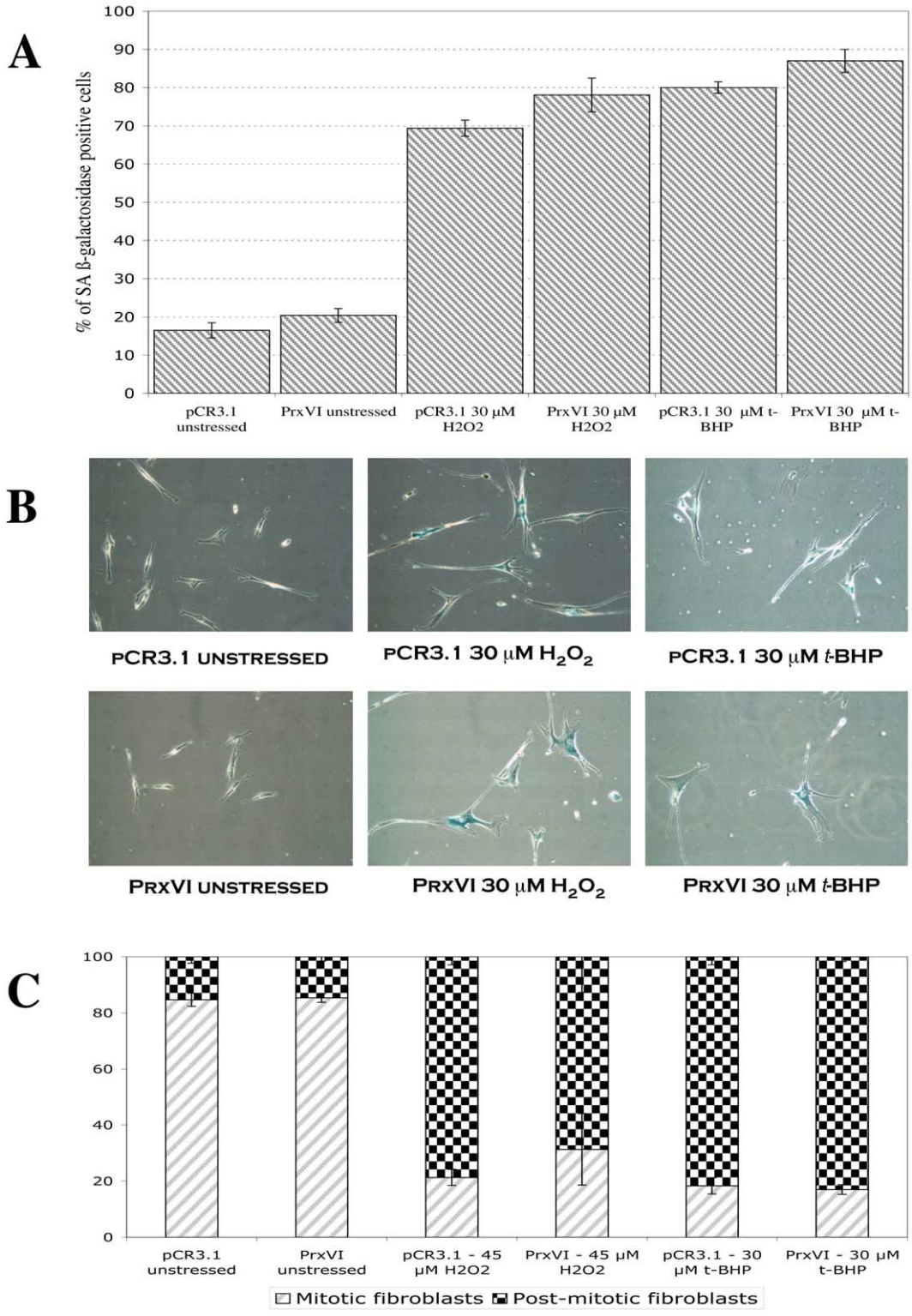

Fig. 6. Overexpression of PrxVI does not decrease the percentage of SA β-galactosidase-positive cells and post-mitotic morphotypes after induction of premature senescence. WI-38 h-TERT/pCR3.1 and WI-38 h-TERT/PrxVI were exposed to 30 μM *t*-BHP or H_2O_2 stress for 2 h. A: SA β-galactosidase histochemistry was performed at 96 h after stress. The percentage of SA β-galactosidase-positive cells was recorded in at least 400 cells per condition. B: Cells photographed in each condition (40× magnification). C: The percentages of post-mitotic fibroblasts showing a large, flat, senescent-like morphology (morphotypes IV, V, VI) versus mitotic (morphotypes I, II, III) were scored. Experiments were repeated three times.

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