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Research paper

Transcription factor GABP/NRF-2 controlling biogenesis of mitochondria regulates basal expression of peroxiredoxin V but the mitochondrial function of peroxiredoxin V is dispensable in the dog

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ABSTRACT

Peroxiredoxins (PRDXs) represent a conserved family of six antioxidant proteins which are widely expressed in different organisms. Human PRDX5 is detected in the cytosol and nucleus and can also target peroxisomes and mitochondria. However, it remains unknown if mitochondrial localization of PRDX5 is essential for its functions. Here we studied whether the known regulator of mitochondrial biogenesis, transcription factor GABP/NRF-2, is required for the basal expression of the human PRDX5 gene and what the significance is of the mitochondrial targeting of the PRDX5 protein. It was found that mutation-mediated inactivation of all potential binding sites for GAPB in the PRDX5 promoter lead to ~80% inhibition of its basal activity in a reporter gene assay. Co-transfection of plasmids expressing GABP-alpha and GABP-beta stimulated activity of the non-mutated PRDX5 promoter but had no effect on the mutated promoter, suggesting that basal expression of the human PRDX5 gene is regulated by GABP. We found that the dog c-Myc-tagged PRDX5 did not target the mitochondria of human cells. Endogenously expressed PRDX5 also showed no association with mitochondria in the dog cells. It appears, therefore, that during evolution the dog PRDX5 gene lost its upstream ATG codon and mitochondrial targeting signal without major functional consequences.

1. Introduction

Mammalian antioxidant proteins peroxiredoxins (PRDXs) reduce hydrogen peroxide and organic hydroperoxides by electron transfer from thioredoxins, glutathione, or cyclophilins [1]. Peroxiredoxin proteins are present in most living species requiring oxygen. Mammalian cells express six isoforms (PRDX1 to PRDX6) that are encoded by different nuclear genes. They are important in antioxidant defense and in hydrogen peroxide-mediated signalling [2–4]. Furthermore, the importance of mammalian PRDXs was shown in other cellular processes, including apoptosis, cell proliferation and differentiation [5,6]. PRDX1 is mainly located in the cytoplasm and knockout of its gene in mice leads to haemolytic anaemia and a shortened life span [7]. PRDX2 was found to bind to integral membrane proteins or cell membranes via its C-terminal region and knockout of its gene in mice also leads to haemolytic anaemia [8]. PRDX3 is located in mitochondria and targeting of its gene in mice results in the accumulation of reactive oxygen species (ROS) in macrophages and increased lung sensitivity to inflammation-inducing agents [9]. PRDX4 is present as a secretory protein in most tissues. In sexually mature testis it is anchored to the endoplasmic reticulum membrane of spermatogenic cells via an uncleaved N-terminal hydrophobic peptide. PRDX4 knockout results in elevated spermatogenic cell death via oxidative stress [10]. PRDX6 was identified as a secretory antioxidant protein of the olfactory epithelium [11] and PRDX6 gene knockout mice show lung pathology and increased mortality with hyperoxia [12].

PRDX5 is a thioredoxin peroxidase, which is highly expressed in many tissues. Human PRDX5 (hPRDX5) contains N-terminal mitochondrial and C-terminal peroxisome targeting (PTS1) signals, allowing its localization to mitochondria [13,16] and peroxisomes [13,14,16]. A significant amount of PRDX5 is also present in the cytosol and in the nucleus [15]. Similar to other 2-Cys peroxiredoxins, PRDX5 requires a thioredoxin [16] or cyclophilin A [8] as a reducing partner. The peroxidase function of PRDX5 *in vivo* was

Abbreviations: ETS, a family of transcription factors; EBS, ETS binding site; PRDX, peroxiredoxin; NRF-1, nuclear respiratory factor 1; GABP, GA-binding protein; aTIS, alternative translation initiation sites; GFP, green fluorescent protein; dPRDX5, dog PRDX5; hPRDX5, human PRDX5; PBS, phosphate-buffered saline.

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demonstrated in experiments in which transient expression of this protein in NIH 3T3 cells inhibited H_2O_2 accumulation and c-Jun NH2-terminal kinase activation induced by tumour necrosis factor-α [16]. In mammalian cells, PRDX5 reduced alkyl peroxides as well as hydrogen peroxide [13]. PRDX5 suppresses p53-dependent apoptosis [17], promotes differentiation and reduces apoptosis in mouse muscle cells [18]. It has also been shown that PRDX5 inhibits the formation of etoposide-induced DNA double-strand breaks [19] and suppresses oxidation of mitochondrial and nuclear DNA [20,21].

Human PRDX5 mRNA (NM_012094.3) has two in-frame AUG codons [13]. The sequences around these AUG codons correspond to Kozak consensus sequences for translation initiation. Translation from the first AUG resulted in a 24 kDa long form of the protein (L-PRDX5), and the predicted molecular mass of the protein translated from the second AUG (which is 52 amino acids shorter) was 17 kDa (S-PRDX5). The 52 amino acid residues at the NH2 terminus of the longer polypeptide were shown to constitute a mitochondria localization signal that is capable of importing the PRDX5 protein into mitochondria [13,25,37]. In human tissues and cell lines only the 17 kDa PRDX5 is usually detected by immunoblotting [16], indicating that mitochondrial PRDX5 is synthesized in the cytosol from the first initiation site of PRDX5 mRNA as a precursor protein and imported into mitochondria, where it is converted into the mature form with a size that is indistinguishable from that of the cytosolic and peroxisomal enzymes [16]. Cytosolic and peroxisomal PRDX5 are most likely translated from the second initiation site of PRDX5 mRNA. Recently, three transcription initiation sites for the PRDX5 mRNA were identified in human hepatocytes. The two first sites are upstream of the sequence coding for the mitochondrial targeting signals whereas the third one is within the sequence but upstream of the second AUG [22], suggesting that non-mitochondrial PRDX5 may be translated from the shortest PRDX5 mRNA variant.

PRDX5 is constitutively expressed at a high level in different mammalian cell lines and normal tissues, although transcription factors responsible for a high basal expression of the PRDX5 gene have not been identified. It has been shown that one or more regulatory elements localized in the human PRDX5 promoter may interact with transcription factors, such as AP-1, NF-κB or GRE, influencing the expression of PRDX5 [22]. The level of PRDX5 is also appeared to be highly dependent on c-Myc [23] and NAD kinase [24]. Recently, we identified conserved binding motifs for nuclear transcription factors controlling the biogenesis of mitochondria – nuclear respiratory factor 1 (NRF-1) and nuclear respiratory factor 2 (NRF-2/GA-binding protein, GABP) – in the promoter of the hPRDX5 gene [25].

In the present study we investigated in more detail the functions of the NRF1 and GABP transcription factors in the regulation of PRDX5 expression and, using the reporter gene assay, confirmed the activity of GABP by transient overexpression of GABP and mutagenesis of the binding sites for this factor in the hPRDX5 promoter. We also investigated localization of *Canis familiaris* (dog) PRDX5 (dPRDX5) and showed that this protein, unlike human PRDX5, does not target mitochondria.

2. Materials and methods

2.1. Plasmids

The −1145PRDX5 and −609PRDX5 luciferase reporter plasmids were constructed as follow. The −1145 to +100 and −609 to +100 fragments of PRDX5 promoter (relative to the transcription start site) were PCR-amplified from human genomic DNA using the primers: 5′-gacgctcgagccctctatcacttccacctgcggg-3′ (forward for −1145 to +100), 5′-ggtgctcgagcacatgcgagctcagcagattgtggg-3′ (forward for −609 to +100) and 5′-cggaagcttccactccgcctcctg-3′ (reverse for both reactions). The primers contained restriction sites for endonucleases the *Xho*I (CTCGAG) and *Hin*d III (AAGCTT). The PCR products were cleaved and subcloned into *Xho*I/*Hin*dIII restricted pGL3-Basic vector (Promega).

Mutations in GABPA and NRF1 sites on the −609PRDX5 construct (Table 1) were generated by the overlap extension method [39].

To generate the GABPA and GABPB expression plasmids (pRc/CMV-GABPA and pRc/CMV-GABPB), total RNA from A549 cells was isolated using Trizol according to the manufacturer's instructions (Invitrogen). A full length of human GABPA (NM_002040) and GABPB (NM_016654) cDNAs were prepared by RT-PCR using the following primers: 5′-gaaagcttgccaccatgactaaaagagaagcag-3′, 5′-gcatctagagggctcaattatccttttccg-3′ (GABPA), 5′-ccgcggccgccaccatgtccctggtagatttggg-3′, 5′-cggtctagacaattaaacagcttctttattag-3′ (GABPB). Primers contain sites for restrictases *Hin*dIII (AAGCTT), *Not*I (GCGGCCGC) and *Xba*I (TCTCAGA). After amplification the products were digested and inserted at *Hin*dIII/*Xba*I (GABPA) or *Not*I/*Xba*I (GABPB) sites of pRc/CMV vector (Invitrogen).

The plasmids for expression of dPRDX5-c-Myc and ATG-dPRDX5-c-Myc were constructed as follows. The dPRDX5 cDNA was PCR-amplified from MDCK cell total RNA using the primers: 5′-ccgtgaattccgggggtcgggccgtggt-3′ (forward for dPRDX5-c-Myc), 5′-ccgtgaattccgggggatgggccgtggt-3′ (forward for ATG-dPRDX5-cMyc), 5′-tggcccggatcctcacagatcctcttctgagatgagtttttgttcgaggatgttgggggccaggctgca-3′ (reverse for both reactions). The primers contained restriction sites for endonucleases *Eco*RI (GAATTC) and *Bam*HI (GGATCC). The PCR products were cleaved and subcloned into *Eco*RI/*Bam*HI restricted pIRES-Neo vector (Clontech).

Table 1
Constructs with mutated GABPA- and NRF1-binding sites and sequences of used mutagenic primers.

Construct	Mutated site (location)	Primer sequences[a]
−609PRDX5GABPAα	GABPAα (−513/−504)	5′-tcctccgctgcctcacgcacggggatgctccact-3′ 5′-agtggagcatccccgtgcgtgaggcagcggagga-3′
−609PRDX5GABPAβ	GABPAβ (−468/−459)	5′-atagccaggagaaccaagagtggcgaacttgct-3′ 5′-agcaagttcgccactcttggttctcctggctat-3′
−609PRDX5GABPAγ	GABPAγ (−382/−373)	5′-tcacgcgccgctaccaagagcgtctcagcagga-3′ 5′-tcctgctgagacgctcttggtagcggcgcgtga-3′
−609PRDX5GABPAδ	GABPAδ (−39/−30)	5′-cgaggcgtgggtcccaagagctctgttctgcg-3′ 5′-cgcagaacagagctcttgggacccacgcctcg-3′
−609PRDX5GABPAαβγδ	Above-listed	Above-listed
NRF1	NRF1 (−9/+3)	5′-tgcgggtggccgctcatacctgcgcagtggagg-3′ 5′-cctccactgcgcaggtatgagcggccacccgca-3′
−609PRDX5GABPAαβγδNRF1	Above-listed	Above-listed

[a] Mutated bases are underlined.

All constructions were confirmed by DNA sequencing (ABI PRISM® 3130xl Genetic Analyzer; Applied Biosystems).

2.2. Cell culture

Human embryonic kidney HEK293 cells, human adenocarcinoma HeLa cells, human SCLC U1810 and A549 cells, and canine kidney MDCK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and cultured at 37 °C in a 5% CO_2 humidified incubator.

2.3. Transient transfection and luciferase assay

HEK293 cells were seeded at 10^5 cells per well in 24-well plate. One day later the cells were transfected with 0.5–1 μg plasmid DNA using Lipofectamine 2000 (Invitrogen). To normalize for transfection efficiency, the 5 ng pRL-TK vector (Promega), which contains the *Renilla reniformis* luciferase gene under the herpes simplex virus thymidine kinase promoter, was included in the transfections. Two days after transfection, the cells were harvested and lysed. The luciferase activity in the lysates was measured with the dual-luciferase reporter assay system (Promega) using a DLReady TD20/20 luminometer (Turner Designs). All transfections were performed in duplicate and repeated three times.

2.4. Immunofluorescence analysis

Cells grown on microscope slides were stained with 100 nM MitoTracker Red (Invitrogen M-7512) in growth medium for 30 min at 37 °C. They were washed then with phosphate-buffered saline (PBS) and fixed for 15 min with 4% formaldehyde. After permeabilization with 0.1% Triton X-100 in PBS for 10 min, cells were incubated overnight at +4 °C with affinity-purified monoclonal mouse anti-c-Myc IgG (Clontech, Cat#3800-1) or polyclonal rabbit anti-PRDX5 IgG (Abcam, ab16823-100) diluted 1:100 in PBS containing 5% BSA. After rinsing four times for 5 min each in PBS, the samples were incubated for 1 h with Alexa Fluor® 488 donkey anti-rabbit (1:400, Molecular probes, A-21206) and Alexa Fluor® 488 donkey anti-mouse IgG (1:400, Molecular probes, A-21202) antibodies in PBS containing 5% BSA and l μg/ml of DAPI. After a final rinse (four times for 5 min each) in PBS, the samples were mounted on glass slides using Pro-Long mountain medium (Molecular Probes) and analysed using a confocal microscope.

2.5. Immunoblotting

Cells were washed with PBS, resuspended in Laemmli's loading buffer, harvested and heated at 95 °C for 4 min. Twenty microliters of each sample were resolved on 15% SDS-polyacrylamide gels and electroblotted onto the nitrocellulose membranes, which were blocked with PBS supplemented with 5% non-fat dry milk powder for 1 h at room temperature and incubated overnight at 4 °C with affinity-purified monoclonal mouse anti-c-Myc IgG (Clontech, Cat#3800-1) or polyclonal rabbit anti-PRDX5 IgG (Abcam, ab16823-100) 1:2500, 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.

2.6. Isolation of liver mitochondria

The liver was minced with scissors on ice, resuspended in 50 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.5) supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon pestle. Before homogenization, the buffer was changed 2–3 times to get rid of as much blood as possible (pieces of liver go down to the bottom and the buffer can be easily decanted). Homogenates were centrifuged at 600 g for 8 min at 4 °C. The supernatant was decanted and recentrifuged at 5,500 g for 15 min to form a mitochondrial pellet that was resuspended in MSH buffer without EDTA and centrifuged again at 5500 g for 15 min. The final mitochondrial pellet was resuspended in MSH.

2.7. Computer analysis

To predict protein localization we used TargetP server (http://www.cbs.dtu.dk/services/TargetP/) [40] and the PSORT server (http://wolfpsort.org/) [41].

3. Results

3.1. Transcription factor GABP regulates PRDX5 promoter activity in HEK293 cells

To examine whether the GABP complex functionally regulates the PRDX5 promoter, HEK293 cells were transiently co-transfected with GABP expression plasmids and the promoter-luciferase reporter plasmid −1145PRDX5. We found that overexpression of one GABP subunit (alpha or beta) does not influence the PRDX5 promoter activity (Fig. 1). Only in the case of co-transfection with both GABP subunits was a ~1.6-fold increase in PRDX5 promoter activity was observed.

Using computer analysis we had previously shown that the hPRDX5 promoter has four potential GABPA-binding sites which were called α, β, γ and δ, and one NRF-1 binding site [25]. To prove that these sites are essential for PRDX5 transcription, we made reporter constructs with −609 to +100 promoter fragments in which the GABPA and NRF1 binding sites were mutated. The single mutation in GABPA binding sites α, β or δ decreased luciferase activity, while mutation of the γ site lead to slight activation of the reporter gene expression (Fig. 2). The mutation of all four GABPA binding sites resulted in a 77% reduction of luciferase activity. No change was observed in luciferase production when the binding site for NRF-1 was mutated. Likewise, there was no change in luciferase activity in the case of transfection of the −609PRDX5GABPAαβγδNRF1 construct in comparison with the transfection of the −609PRDX5GABPAαβγδ construct.

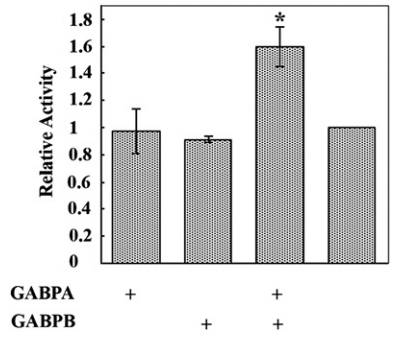

Fig. 1. The PRDX5 promoter is activated by GABP. HEK293 cells were transfected with a 0.5 μg −1145PRDX5 construct, 5 ng pRL-TK and 0.25 μg of pRc/CMV-GABPA and/or pRc/CMV-GABPB expression vectors, as indicated. Each sample was transfected with the additional empty vector pRc/CMV to bring the amount of transfected DNA in each well to 1 μg. Activity is expressed relative to that of the −1145PRDX5 construct without overexpression of GABP. Data are given as mean ± SD from five independent measurements. *$P < 0.05$ versus control (GABPA -, GABPB -).

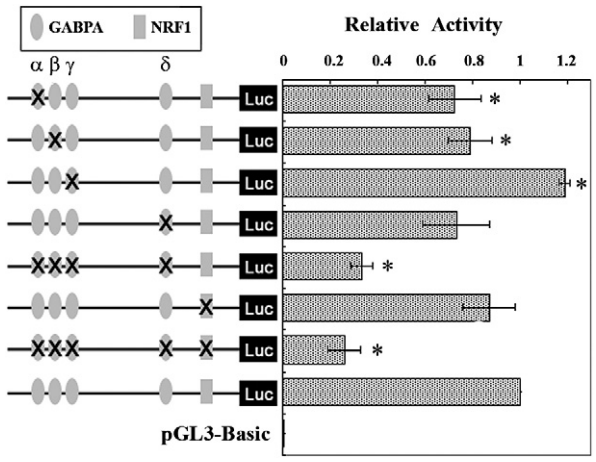

Fig. 2. Mutation of the GABPA sites modifies activity of the PRDX5 promoter. HEK293 cells were transfected with 0.5 μg −609PRDX5 wild-type or mutant constructs and 5 ng pRL-TK. The pGL3-Basic vector was used as a negative control. The grey ellipse and rectangle indicate GABPA and NRF1 binding sites, respectively. Activity is expressed relative to that of the wild-type promoter. Data are given as mean ± SD from five independent measurements. $^{*}P < 0.05$ versus control (wild type promoter).

Finally, we co-transfected the mutant construct −609PRDX5GABPAαβγδ with GABPA and GABPB cDNAs subcloned into an expression vector. The −609PRDX5 plasmid with the wild-type promoter of the human PRDX5 gene was used as control. Here, we applied the shorter fragment of the PRDX5 promoter, which did not change the results of the overexpression (Fig. 3). The introduction of mutations into all GABPA-binding sites completely abolished activation of luciferase activity by GABP expression plasmids. Altogether, these results indicated that basal expression of the human PRDX5 gene is regulated by transcription factor GABP/NRF-2, which is a major factor controlling biogenesis of mitochondria [26].

3.2. *C. familiaris PRDX5 does not targeted to mitochondria*

GeneBank contains information about the PRDX5 protein from *C. familiaris* (dPRDX5), which does not contain a mitochondrial targeting signal. Its mRNAs (XM_533241.2) are translated from the AUG initiation site, which corresponds to the second AUG of the *Homo sapiens* PRDX5 (NM_012094.3) and has no other AUG sites in the same frame. To investigate whether an absence of mitochondrial

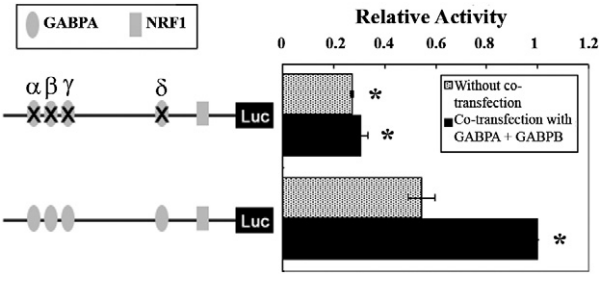

Fig. 3. GABP activates the PRDX5 promoter in HEK293 cells. HEK293 cells were transfected with 0.5 μg −609PRDX5 wild-type or mutant constructs, with 5 ng pRL-TK in the absence or presence of 0.25 μg each of expression vectors for GABPA and GABPB. Each sample was transfected with the additional empty vector pRc/CMV to equalize the amount of transfected DNA in each well to 1 μg. The grey ellipse and rectangle indicate GABPA and NRF1 binding sites, respectively. Data are given as mean ± SD from five independent measurements. $^{*}P < 0.05$ versus control (wild-type promoter without cotransfection).

targeting signals prevents dPRDX5 from targeting mitochondria, the longest dPRDX5 mRNA (from the EST database – CX015028.1) was analysed for the presence of alternative translation initiation sites (aTIS). The supposed aTIS gggccgggcc**gtg**gtgcgcg was found near the 5′ end of the mRNA but this site was not a part of the main reading frame. Taking into account possible mistakes in the CX015028.1 sequence, the mRNA was cloned into the mammalian expression vector pIRES-Neo and two constructs for determination of dPRDX5 localization were prepared. In the first construct the C-terminal peroxisome targeting signal sequences SQL of the dPRDX5 were changed to c-Myc-tag (dPRDX5-c-Myc). In the second construct in addition to the c-Myc-tag at the C-end, the ATG initiation codon was added near the 5′-end of the mRNA in a corresponding reading frame (ATG-dPRDX5-c-Myc). Similar constructions which express human L-PRDX5 and S-PRDX5 targeted by the c-Myc epitope (h-L-PRDX5-c-Myc and h-S-PRDX5-c-Myc) were used as controls. Human HeLa cells were transiently transfected with these plasmids and localization of the proteins was studied by immunofluorescent staining using anti-c-Myc antibodies. The results presented in Fig. 4A demonstrate that dPRDX5-c-Myc without its peroxisome targeting signal (PTS1) targeted the cytosol and nucleus and ATG-dPRDX5-c-Myc almost exclusively targeted the nucleus. Transfection of HeLa cells with a plasmid encoding the h-L-PRDX5-c-Myc without its PTS1 almost exclusively targeted the protein in mitochondria (which was co-labelled with Mitotracker Red). Expression of the h-S-PRDX5-c-Myc prevented accumulation of this protein in mitochondria and promoted the localization of hPRDX5 in the cytosol and nucleus, which is consistent with the idea that the N-terminus of L-PRDX5 is responsible for mitochondrial targeting by a fraction of this protein [13,37]. The same results were obtained using the human non-small cell lung carcinoma (NSCLC) cell line U1810 (not shown).

Western blot analysis of ATG-dPRDX5-c-Myc and dPRDX5-c-Myc expression in HeLa cells with anti-c-Myc antibodies revealed two bands: 27 kDa and 17 kDa, respectively (Fig. 4B lines 1 and 2), which means that the pre-sequence of the ATG-dPRDX5-c-Myc is not removed during maturation of the protein in contrast to the mitochondrial targeting signal peptide of the h-L-PRDX5-c-Myc, which is removed during transport of this protein to mitochondria (Fig. 4B, line 3). A minor protein band of 25 kDa (Fig. 4B, line 3) was the unprocessed c-Myc-tagged L-PRDX5 protein.

In addition, untransfected human HeLa cells and the *C. familiaris* cell line MDCK were stained with polyclonal anti-PRDX5 antibodies. The results presented in Fig. 5 demonstrate that human PRDX targets the mitochondria, cytosol and nucleus, whilst dPRDX5 does not target mitochondria.

To confirm these data, mitochondria were isolated from HeLa and MDCK cell lines and from rat and canine livers. Western blot analysis with polyclonal antibodies to PRDX5 revealed that, in contrast to rat and human PRDX5, *C. familiaris* PRDX5 does not target mitochondria (Fig. 6). The molecular size of PRDX5 in all tissues and cell lines, as well as in the two subcellular fractions, was examined and found to be identical (17 kDa). These observations, together with the results obtained with ATG-dPRDX5-cMyc, suggest that dPRDX5 only translated from the AUG site annotated in GeneBank (XM_533241.2).

4. Discussion

In our previous study using computational analysis we found binding sites for transcription factors Sp1, NRF1 and GABP in the promoter region of the PRDX5 gene [25]. Specifically, four GABPA binding elements were established: α, β, γ and δ. Two of them (β and γ) perfectly matched with the consensus sequence, while site α and site δ had one and two mismatches, respectively. Using

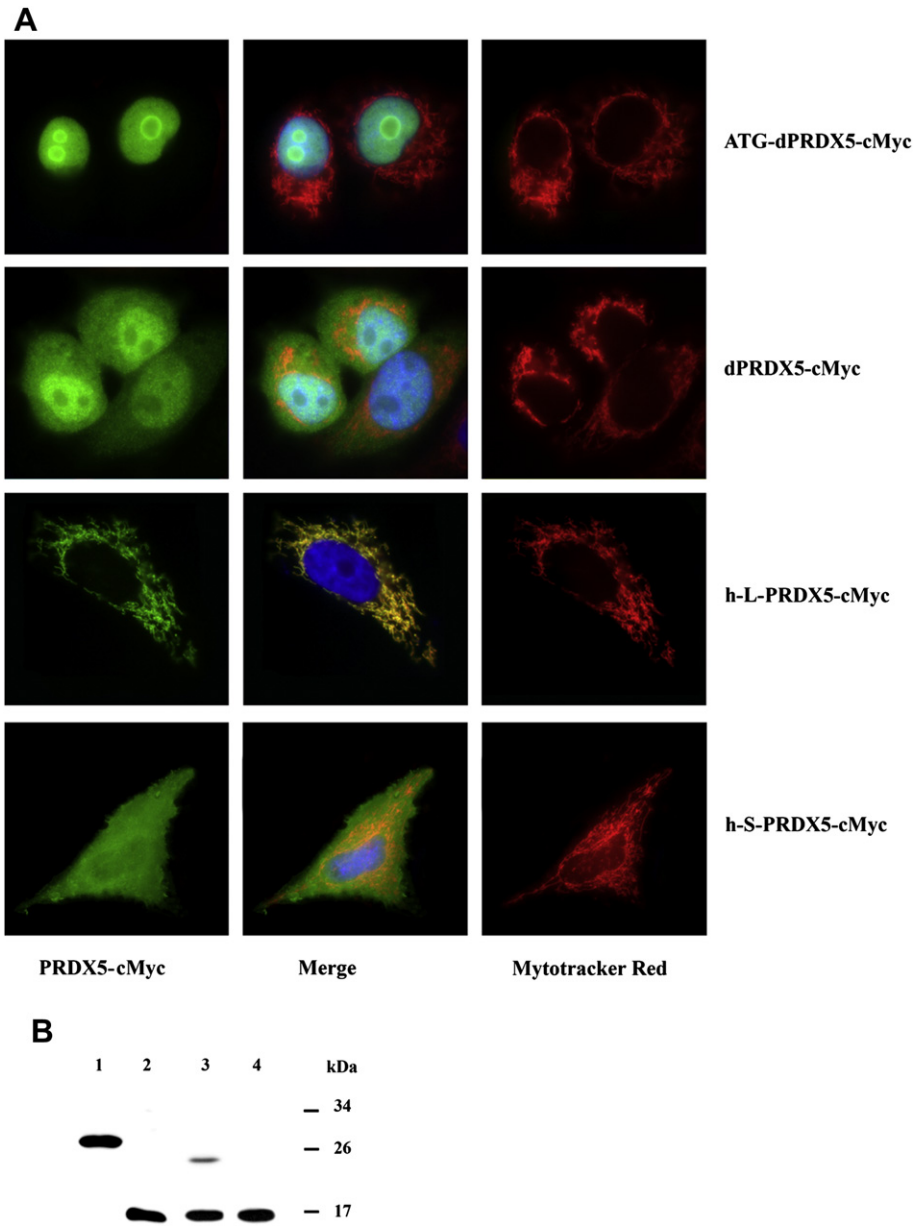

Fig. 4. A. Intracellular distribution of dPRDX5-cMyc, ATG-dPRDX5-cMyc, h-L-PRDX5-cMyc and h-S-PRDX5-cMyc transiently expressed in the HeLa cell line. Monoclonal antibodies against c-Myc were used. Mitochondria were stained with Mitotracker Red. B. Western blot analysis of the ATG-dPRDX5-c-Myc (line-1), dPRDX5-c-Myc (line-2), h-L-PRDX5-c-Myc (line 3) and h-S-PRDX5-c-Myc (line-4) transiently expressed in HeLa cells.

luciferase reporter constructs containing PRDX5 gene promoter deletion mutants, we showed that the above mentioned binding motifs are responsible for most of the PRDX5 basal promoter activity. In the present study, we investigated in more details the functions of the NRF-1 and GABP transcription factors in the regulation of PRDX5 expression. First, we analysed the influence of GABP complex overexpression on luciferase transcription driven by the −1145 to +100 fragment of the PRDX5 promoter. Neither GABPA nor GABPB subunits alone were found to modify luciferase activity. It is known that GABP is a unique protein among the ETS family of transcription factors because it is the only obligate multimeric factor. The GABPA subunit contains a DNA binding domain, while the transcriptional activation domain presents in the GABPB subunit. The functional GABP complex is a heterotetramer. When two GABPA subunits bind to appropriate DNA elements, two GABPB subunits are recruited [27]. This explains why activation of luciferase transcription is observed only in the case of the combined overexpression of both GABP subunits.

To verify a significance of GABPA and NRF-1 binding sites, we sequentially mutated each of these in the reporter construct containing the −609 to +100 fragment of the PRDX5 promoter. Mutations in any of the GABPA binding sites led to a modulation of the promoter activity. The most substantial decrease of luciferase expression was found when all GABPA binding sites were mutated. It is remarkable that the mutation in the γ site led to slight activation of the reporter gene activity. Although nothing is known about possible transcription repression activity of the GABP complex, some of the ETS family transcription factors, e.g. Yan, Erf, Net and Tel, may act as transcriptional repressors. Besides, in some cases interaction of transcription factors with other proteins can minimize or eliminate their transactivating effects. In other words, the activity of transcription factors depends on the cell and the

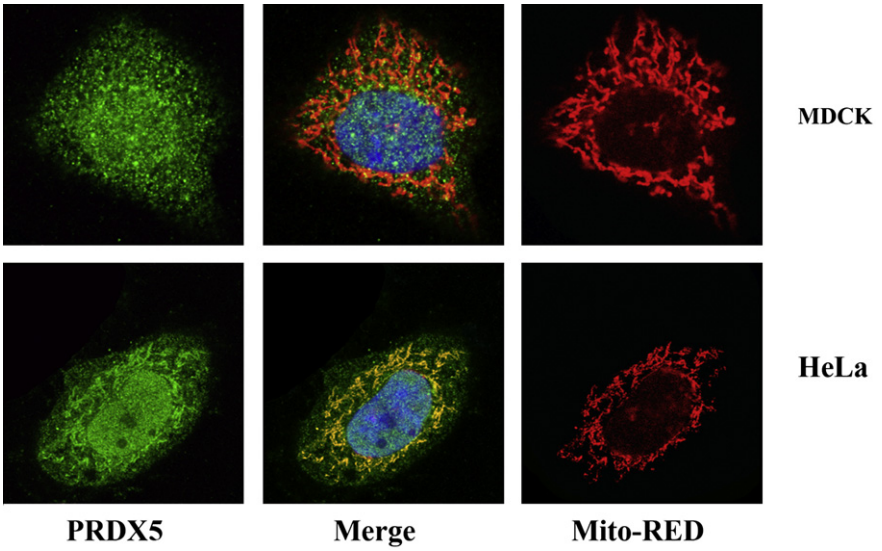

Fig. 5. Intracellular distribution of endogenous PRDX5 (green) in formaldehyde-fixed human HeLa and canine MDCK cells. Polyclonal antibodies against PRDX5 were used. Mitochondria were stained with Mitotracker Red.

promoter context [28,29]. A so-called 'pointed domain' of the GABPA factor serves for multiple protein-protein interactions [27].

No considerable changes in the promoter activity were observed when the NRF-1 binding site was mutated. It seems that this factor does not participate in the regulation of the PRDX5 gene, at least in HEK293 cells, but it is possible that the mutation, which we introduced into NRF-1 site, did not substantially suppress the binding of this factor to the PRDX5 promoter.

Lastly, overexpression of the GABP complex did not influence the activity of the promoter with the mutated GABPA binding sites. This proves that the increase in the PRDX5 promoter activity in the case of GABP overexpression is provoked by the binding of GABPA to the corresponding sites.

It has been shown recently that Ets1 and Ets2 transcription factors could bind to the ETS binding site (EBS) located at −39 to −30 bp of the PRDX5 gene promoter in human prostate cancer PC3 cells [30]. This has the same motif as the GABPA binding site δ. All transcription factors of the ETS family bind to similar DNA sequences centred over a GGAA/T core motif [31]. Thus, the Ets-1 binding site consensus (NCMGGAWGYN) is similar to the GABPA binding site consensus (ACCGGAAGNS). Therefore, diverse ETS family factors can potentially interact with the same DNA sequence. For example, both GABP and Ets-1 can bind to an EBS located at −96 bp on the rat prolactin promoter [32]. GABP and another ETS transcription factor PU.1 activate the interleukin-7 receptor α promoter in developing B cells by interacting with a highly conserved EBS [33]. GABP competes with Ets-like factor-1 (Elf-1) for binding to the *cis*-element in the 5′-region of the human Fc receptor γ-chain gene [34]. Moreover, two ETS-related factors can also cooperate with each other. Thus, GABP binds to one of three EBSs in the human α4 integrin gene promoter. A higher level of expression is achieved in cooperation with protein complexes which bind to other EBS and contain Ets-1 [35]. Regulation of β2 leucocyte integrin gene expression is dependent on the cooperative interactions of GABP with PU.1 [36]. It is possible that competition or cooperation of GABP with Ets-1 occurs in the case of the PRDX5 gene.

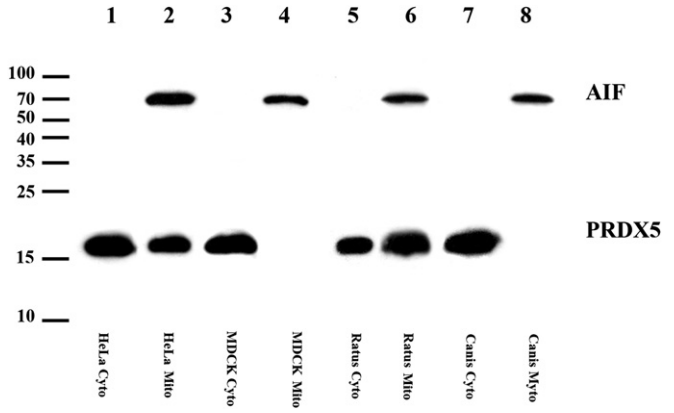

Fig. 6. Western blot analysis of cytosolic and mitochondrial fractions from human HeLa cells (lines 1 and 2), canine MDCK cells (lines 3 and 4), rat liver (lines 5 and 6) and dog liver (lines 7 and 8). Polyclonal antibody against PRDX5 was used. A polyclonal antibody against apoptosis-inducing factor (AIF) was used as marker for mitochondrial fraction.

The importance of the mitochondrial localization of PRDX5 was suggested in several studies. In Chinese hamster ovary cells, overexpression of hPRDX5 in either the cytosolic, mitochondrial or nuclear compartments significantly reduced cell death, with more effective protection by overexpression of peroxiredoxin 5 in mitochondria confirming that this organelle is a major target of peroxides [37]. Expression of the redox-negative h-L-PRDX5 in human NSCLC U1810 cells affected the mitochondrial pathway of apoptosis, as assessed by the cytochrome *c* release assay. Impairment of the PRDX5 enzymatic function also affected the transmembrane potential and calcium loading capacity of mitochondria, as well as mitochondrial morphology [42].

Human PRDX5 targets mitochondria because this protein can be translated from the upstream AUG in its mRNA with a specific targeting pre-sequence [25]. The antioxidant function of PRDX5 is enabled by its short 17 kDa form, which is usually present in the cytoplasm, nuclear, mitochondria and peroxisomes and can neutralize ROS in these intracellular compartments. Regulation of basal expression of mammalian PRDX5 genes by nuclear transcription factor GABP controlling the biogenesis of mitochondria may be required to adjust the cellular PRDX5 level to a level that is sufficient for the number of mitochondria producing most of the cellular ROS.

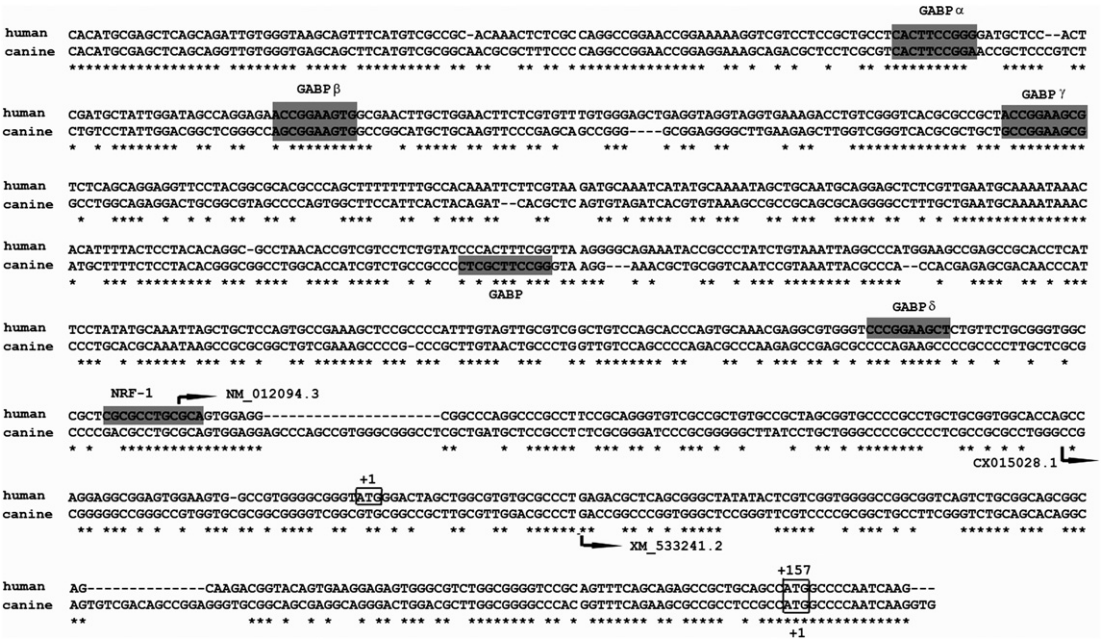

Fig. 7. CLUSTAL W alignments of human and canine promoter regions of PRDX5 genes. Transcription sites are indicated by black arrows. ATG translation initiation codons are surrounded by a rectangle. The GABP and NRF-1 transcription factor binding sites are indicated in grey.

The promoter of the *C. familiaris* PRDX5 gene was found to be well conserved with a human promoter (Fig. 7). It also contains four GABPA binding elements and three of them are located in the same positions as in the human PRDX5 promoter (GABPA α, β, γ), which suggests that the known regulator of mitochondrial biogenesis, transcription factor GABP, may be required for the basal expression of not only of the human PRDX5 gene but also the dPRDX5 gene. However, this question was not addressed in our work and will be investigated separately. We also showed that dPRDX5 does not contain a mitochondrial targeting sequence and does not target mitochondria. Apparently, the mitochondrial targeting pre-sequence was lost in the PRDX5 gene during evolution of the dog lineage but retained in primate and rodent lineages. We propose that the loss of PRDX5 function in dog mitochondria may be compensated by an increased expression of PRDX3. Indeed, silencing of PRDX3 and PRDX5 genes in human neuroblastoma cells using RNA interference suggested that the products of these genes can substitute for each other [38].

Interestingly, *Sus scrofa* PRDX5 mRNA only has one AUG translation initiation site, which corresponds to the second initiation site of the hPRDX5. The longest *S. scrofa* PRDX5 mRNA (CX056471.1) does not contain other AUG sites upstream but it has a sequence ctcagcctctaggtgggggtggag in the correct reading frame, which may act as an aTIS. Nevertheless, computational analysis of the protein sequence translated from the aTIS showed a very low score for mitochondrial localization of the protein (TargetP − 0.234; PSORT − 2.5 as compared with hPRDX5 − 0.83 and 9.0, respectively). The actual intracellular localization of the *S. scrofa* PRDX5 remains to be established. Further studies of these genes and their mitochondrial pre-sequences in different species are required to clarify the mechanism of their transport into, and functions in, mitochondria.

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