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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.