Renal peroxiredoxin 6 interacts with anion exchanger 1 and plays a novel role in pH homeostasis

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Peroxiredoxin 6 (PRDX6) is one of the six members of the PRDX family, which have peroxidase and antioxidant activity. PRDX6 is unique, containing only one conserved cysteine residue (C47) rather than the two found in other PRDXs. A yeast two-hybrid screen found PRDX6 to be a potential binding partner of the C-terminal tail of anion exchanger 1 (AE1), a Cl^-/HCO_3^- exchanger basolaterally expressed in renal α-intercalated cells. PRDX6 immunostaining in human kidney was both cytoplasmic and peripheral and colocalized with AE1. Analysis of native protein showed that it was largely monomeric, whereas expressed tagged protein was more dimeric. Two methionine oxidation sites were identified. *In vitro* and *ex vivo* pull-downs and immunoprecipitation assays confirmed interaction with AE1, but mutation of the conserved cysteine resulted in loss of interaction. *Prdx6* knockout mice had a baseline acidosis with a major respiratory component and greater AE1 expression than wild-type animals. After an oral acid challenge, PRDX6 expression increased in wild-type mice, with preservation of AE1. However, AE1 expression was significantly decreased in knockout animals. Kidneys from acidified mice showed widespread proximal tubular vacuolation in wild-type but not knockout animals. Knockdown of PRDX6 by siRNA in mammalian cells reduced both total and cell membrane AE1 levels. Thus, PRDX6-AE1 interaction contributes to the maintenance of AE1 during cellular stress such as during metabolic acidosis.

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Anion exchanger 1 (AE1), also known as Band 3, is a sodium-independent Cl^-/HCO_3^- exchanger member of the SLC4 gene family expressed in erythrocytes (eAE1) and at the basolateral surface of intercalated cells in the kidney (kAE1). AE1 has a crucial role in intracellular and systemic pH regulation, and in the maintenance of intracellular Cl^- levels and cell volume.[1] Compared with eAE1, human kAE1 lacks the N-terminal 65 amino acids.[2] This truncation is known to preclude kAE1 protein binding to some known eAE1 partners (such as aldolase and ankyrin), but it does not interfere with anion exchange.[3–7] The C-terminus of AE1 (AE1(C)), containing residues 876–911, has been proven to be interesting, with the elucidation, first, of AE1(C) mutations that result in distal renal tubular acidosis, including a truncating mutation (AE1(C)Δ11) and a point mutation (M909T) causing aberrant kAE1 membrane targeting, and, second, C-terminal protein binding partners including GAPDH, the sodium pump's β1 subunit, kinesin 3B, carbonic anhydrase, and AP1.[8–17]

To investigate the protein binding of AE1(C), a yeast two-hybrid screen was designed using both intact AE1(C)WT and AE1(C)Δ11 against a human kidney cDNA library.[13] A number of candidate binding partners were identified, including peroxiredoxin 6 (PRDX6), which we investigated in this work.

The peroxiredoxins, a family of at least six members in mammals, have peroxidase activity, catalyzing H_2O_2 to H_2O and acting as antioxidant proteins. While maintaining these functions, PRDX6 is also uniquely capable of phospholipase A2 (PLA2) activity, hydrolyzing the *sn*-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids.[18] PRDX6 has only one conserved cysteine (C47) that is buried within the protein's tertiary structure,[19] whereas the other PRDX family members all contain two conserved cysteine residues and typically form homodimers.[20] The crystal structure of PRDX6 reveals the exposed catalytic triad for PLA2 activity (His26, Ser32, Asp140) and the buried conserved cysteine (C47), which is the catalytic site for peroxidase activity;[18] other structural work indicates that heterodimerization with glutathione accompanies PRDX6's activity.[21]

Northern and western blotting have shown significant tissue expression of PRDX6 in the brain, heart, kidney, lung, and testis,[22] and PRDX6 expression has recently been

identified in all sections of the nephron.[23] Here, we report the interaction between AE1 and PRDX6 in the kidney, and demonstrate a role for renal PRDX6 in pH homeostasis.

RESULTS

PRDX6 identified as a potential binding partner for AE1(C)

Initial evidence of an interaction between AE1 and PRDX6 from yeast two-hybrid screening was validated by obtaining colonies after specific mating of both the AE1(C)WT and AE1(C)Δ11 bait strains, but not p53 vector strain alone, with the PRDX6 strain (Figure 1a). This indicates that the interaction takes place upstream of the R901 truncation site within AE1's 36-residue C-terminal cytoplasmic domain.

PRDX6 is present in human and mouse kidneys

Localization of the two proteins in human kidney was examined using dual immunostaining. As expected, kAE1 was constrained to the basolateral surface of intercalated cells (Figure 1b–f, red), whereas PRDX6 (green) was observed in all cells and its distribution was more widespread in both the cytoplasm and at the cell periphery. Clear colocalization of PRDX6 and kAE1 was observed (Figure 1f). The absence of staining when primary antibodies were omitted confirmed specificity (Figure 1c). Further confirmation of the PRDX6 antibodies' specificity was demonstrated on *Prdx6*$^{+/+}$ and *Prdx6*$^{-/-}$ kidney sections and lysates (Supplementary Figure 1 online).

Analysis of native PRDX6 expression in human and mouse kidney under non-reducing conditions demonstrated largely monomeric expression as a doublet (~26 kDa) with a small amount of dimer (~52 kDa) (Figure 2a). Reducing conditions yielded an expected single monomeric band. Given the embedded location of the cysteine, any *in vivo* dimerization is most likely via noncovalent bonding.[19]

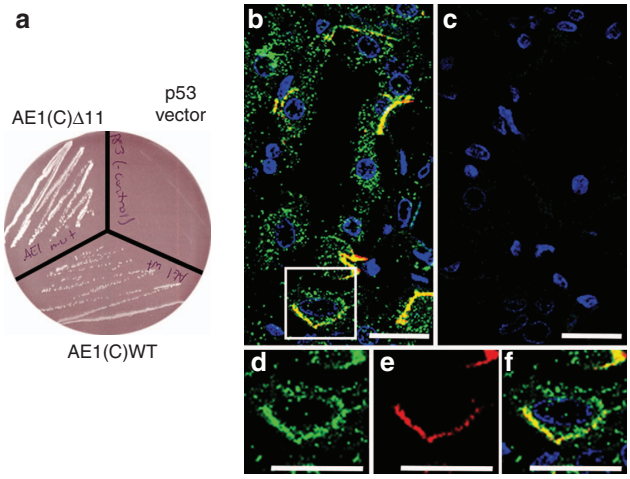

Figure 1 | Confirmation of peroxiredoxin 6 (PRDX6) as a binding candidate of kAE1, and co-staining of intercalated cells. (**a**) Yeast mating test of both AE1(C)WT and AE1(C)Δ11 with PRDX6 in yeast cells shows protein potential interaction indicated by the blue colonies on selection plates with no cell growth of the negative control (p53 empty vector). (**b**–**f**) Immunofluorescence in human renal collecting duct. PRDX6 (green) and AE1 (red) colocalize in a number of collecting duct cells (**b**) with the absence of staining when primary antisera were omitted (**c**). An enlarged image of an intercalated cell shows widespread PRDX6 expression (**d**) and basolateral AE1 (Bric 170) expression (**e**). Merged panel (**f**) indicates colocalization of PRDX6 and AE1 at the basolateral surface (Scale bars = 20 μm). AE1, anion exchanger 1; WT, wild type.

PRDX6 co-immunoprecipitates with kAE1

In vivo confirmation of the kAE1-PRDX6 association was provided by co-immunoprecipitation of the two from human kidney membrane preparations. The completed IP was probed with Bric 170 (anti-AE1 antibody), and a band was observed in the bead-bound anti-PRDX6 lane (Figure 2b) but not in either of the two control lanes (beads alone and a bead-bound extraneous precipitating antibody (PI3K)). However, although these results supported an *in vivo* interaction between kAE1 and PRDX6, they could not differentiate between direct and indirect linkage between the two proteins.

Protein expression and HisPRDX6 mutagenesis

Expressed and purified N-terminal histidine-tagged PRDX6 (HisPRDX6) run on a coomassie gel under non-reducing conditions yielded approximately equal ratios of monomeric (~26 kDa) and dimeric (~52 kDa) states (Figure 2c), in contrast to the behavior of the untagged protein (where monomer prevailed; Figure 2a). Mass spectrometry of all four bands revealed the purified tagged protein in four different oxidation states via the two methionine residues per molecule (Supplementary Figure 2 online); this likely also accounts for the doublets observed in Figure 2a.

The single conserved cysteine (C47) was next mutated to alanine (HisPRDX6-C47A), and here, monomer prevailed over dimer (Figure 2c). *In vitro* dimerization is potentially influenced by both noncovalent bonding between two HisPRDX6 proteins and by the interaction between N-terminal His tags.[24]

Binding of PRDX6 to AE1 confirmed by pull-down

To further support the interaction between AE1 and PRDX6, a pull-down using purified bead-bound HisPRDX6 to precipitate kAE1 from a human kidney membrane protein fraction was also performed, showing that HisPRDX6 precipitates kAE1. When mutagenized HisPRDX6-C47A was used, a loss of binding to kAE1 was demonstrated (Figure 2d). Beads alone provided a negative control.

Direct protein–protein interaction

The direct interaction between purified HisPRDX6 and expressed and purified N-terminal maltose-binding protein-tagged AE1(C) (MBP-AE1(C)) was assessed by pull-down using the two recombinant proteins under non-reducing conditions. Bead-bound MBP-AE1(C) or MBP alone were incubated with HisPRDX6, and precipitated proteins were analyzed by western blotting using anti-PRDX6. The same blot was reprobed for MBP as a loading control. As with the previous *in vitro* assay, HisPRDX6 was seen largely in its dimeric form (Figure 2e). Again, mutagenized HisPRDX6-C47A showed