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Binding sites for interaction of peroxiredoxin 6 with surfactant protein A

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ABSTRACT

Peroxiredoxin 6 (Prdx6) is a bifunctional enzyme with peroxidase and phospholipase A_2 (PLA_2) activities. This protein participates in the degradation and remodeling of internalized dipalmitoylphosphatidylcholine (DPPC), the major phospholipid component of lung surfactant. We have shown previously that the PLA_2 activity of Prdx6 is inhibited by the lung surfactant-associated protein called surfactant protein A (SP-A) through direct protein-protein interaction. Docking of SPA and Prdx6 was modeled using the ZDOCK (zlab.bu.edu) program in order to predict molecular sites for binding of the two proteins. The predicted peptide sequences were evaluated for binding to the opposite protein using isothermal titration calorimetry and circular dichroism measurement followed by determination of the effect of the SP-A peptide on the PLA_2 activity of Prdx6. The sequences $_{195}$EEEAKKLFPK$_{204}$ in the Prdx6 helix and $_{83}$DEELQTELYEIKHQIL$_{99}$ in SP-A were identified as the sites for hydrophobic interaction and H^+-bonding between the 2 proteins. Treatment of mouse endothelial cells with the SP-A peptide inhibited their recovery from lipid peroxidation associated with oxidative stress indicating inhibition of Prdx6 activity by the peptide in the intact cell.

1. Introduction

Peroxiredoxin 6 (Prdx 6) is expressed widely in tissues but is especially enriched in lung where it is present in alveolar type II cells, alveolar macrophages, bronchiolar epithelium, and endothelial cells [7,17,26]. Subcellular fractionation of lungs and cells has indicated the presence of Prdx6 in the lamellar body, lysosomal, and cytosolic fractions [1] and we have identified a specific amino acid sequence that is required for organellar targeting of Prdx6 [27,28]. Lung lamellar bodies are lysosomal related organelles (LROs) that, similar to lysosomes, maintain an acidic internal pH that is necessary for basal PLA_2 activity of Prdx6 [3]. This activity of Prdx6 has been called $aiPLA_2$ as it is measured *in vitro* under acidic, calcium independent conditions [1,17,18]. The important role of $aiPLA_2$ in the metabolism of lung dipalmitoylphosphatidylcholine (DPPC), the lipid component of the lung surfactant that is primarily responsible for its biological activity, has been demonstrated by the study of mice with 'knock-out' or overexpression of Prdx6 [13,14] and by use of a competitive transition state analog inhibitor of $aiPLA_2$ activity (MJ33) in intact rats, isolated perfused rat lungs, isolated rat alveolar epithelial type II cells, and isolated lung lamellar bodies [8,9].

Lung surfactant protein A (SP-A) is the major specific protein associated with the lung surfactant [29]. Our initial studies of SP-A interaction with PLA_2 proteins demonstrated its binding to Habu snake venom PLA_2 and inhibition of its enzymatic activity; SP-A did not bind to or inhibit activity of several other snake venoms that were tested concurrently [11]. Subsequently, we showed specific inhibition of lung $aiPLA_2$ activity by SP-A [10]; the protein expressing this PLA_2 activity was later identified as Prdx6 [5,17,18]. Addition of SP-A to rat lung homogenate, isolated lamellar bodies, or isolated rat alveolar type II cells inhibited both $aiPLA_2$ activity and the degradation of DPPC [10] while SP-A "knock-out" resulted in increased lung $aiPLA_2$ activity [16]. Thus, $aiPLA_2$ activity can be modulated by the presence of SP-A. This inhibiting effect of SP-A on the $aiPLA_2$ activity of Prdx6 occurs through direct protein-protein interaction [32]. Since SP-A and Prdx6 are present in the same acidic compartments of the lung (the LROs), their interaction could be important in the regulation of $aiPLA_2$ activity in those organelles with a consequent effect on lung phospholipid metabolism.

The present study was designed to determine the molecular sites for interaction of the 2 proteins. We first determined potential interaction sites between SP-A and Prdx6 using the Z-DOCK program and then evaluated these theoretical sites for their interaction with the native protein using synthetic peptides based on the *in silico* analysis.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl, *sn*-glycerol-3-phosphocholine (DPPC), egg phosphatidylcholine (PC), phosphatidylglycerol (PG), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). 1-palmitoyl, 2-[9, 10-^{3} H]-palmitoyl, *sn*-glycero-3-phosphocholine (^{3}H –DPPC) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Triton X-100 was obtained from Roche Diagnostics (Indianapolis, IN), NaCl, Mg

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Cl_2, and ATP from Fisher Scientific (Pittsburgh, PA), EDTA from GIBCO (Grand Island, NY), DMEM with isopropyl β-D-1-thiogalactopyranoside (IPTG) from Denville Scientific (Metuchen, NJ), extracellular-signal regulated kinase 2 (Erk 2) from Upstate (Millipore, Billerica, MA), Chariot™ protein delivery reagent from ActiveMotif (Carlsbad, CA), and His-Bind resin from Novagen (EMD, San Diego, CA). A549 lung epithelial cells (CCL-185), a human lung carcinoma cell line, were obtained from the American Type Culture Collection (Manassas, VA). Pulmonary microvascular endothelial cells (PMVEC) that were isolated following enzymatic digestion of mouse lungs are maintained in our laboratory [22,23]; cells were used at passages 8–13.

2.2. Design of peptides

SP-A and Prdx6 peptides were designed with Protein docking software (http://zlab.bu.edu/zdock) using structures/sequences of SP-A and Prdx6 that were obtained from the Protein Data Bank. These analyses were based on the published crystal structures of Prdx6 [6] and of a truncated SPA molecule (the carbohydrate recognition domain, CRD) with the site of truncation between the hydrophobic neck region and the collagen-like domain [24]. Initial stage docking was modeled using ZDOCK, followed by refinement stage docking and finally clustering of sequences/structures to predict sequences that might mediate interaction between SPA and Prdx6 proteins (Fig. 1). For further study, we picked the highest scoring sequences that were $_{83}$DEELQTELYEIKHQIL$_{99}$ for the SP-A sequence and $_{195}$EEEAKKLFPK$_{204}$ for the Prdx6 sequence. A decapeptide corresponding to the proposed Prdx6 sequence and a scrambled peptide using the same amino acids with one extra Ala (PAEKLKAFEKE) were synthesized by GenScript (Piscataway, NJ). A 16 amino acid SPA peptide corresponding to the SP-A sequence and a scrambled peptide using the same amino acids (LELDEEITEYQKQLHI) were synthesized by Proteintech (Chicago, IL).

2.3. Generation and isolation of proteins

Recombinant human Prdx6 with a C-terminal His tag was cloned in pET21b and expressed in *Escherichia coli*; his-tagged Prdx6 was purified with a Ni^{2+} column (His-Bind resin) as described previously [5,19]. Prdx6 was phosphorylated *in vitro* by incubation with Erk2, ATP, and $MgCl_2$ as described previously [25]. Human SP-A was isolated by density gradient centrifugation of cell-free bronchoalveolar lavage (BAL) fluid obtained by therapeutic lavage of alveolar proteinosis patients at the Hospital of the University of Pennsylvania as described previously [2]. SP-A was extracted from whole surfactant using 1-butanol and octyl glucopyranoside [15,30].

Human Prdx6 cDNA, cloned into pET21b plasmid, was the starting plasmid for production of Prdx6 deletion proteins [5]. Deletions of aa 210–225 (Prdx6Δ210–225) and aa 195–225 (Prdx6Δ195–225) were introduced by PCR. The forward primer for both deletions was:

5′-ATGCCAT**ATG**CCCGGAGGTCTGCTTCTCG-3′. This primer has an NdeI site (underlined) containing the start codon (bolded). The reverse primer for generating Prdx6 Δ195–225 had the sequence:

5′-ATCGCTCGAG**AGG**GATGGTTGGAAGGACCATCAC-3′. This primer contains an XhoI site (underlined) and the last codon of the coding region (bolded). The PCR product contains amino acids 1–194; thus, this construct removed the putative SP-A binding region (amino acids 195 to 204) as well as the remainder of the carboxy terminus of Prdx6. The reverse primer for the 210–225 deletion had the sequence: 5′-ATCGCTCGAGTTTGGTGAAGACTCCTTTCGG-3′; it also contains an XhoI site (underlined). The PCR product that was obtained (Prdx6Δ210–225) was depleted of the carboxy terminus region but retained the putative binding site and was used as a positive control for binding. The PCR products for each deletion were cleaved with NdeI and XhoI and cloned into the original vector from which the full-length Prdx6 had been removed by cleavage with the same enzymes. This cloning strategy allowed an in-frame fusion with the His-tag and production of the two His-tagged proteins in which different lengths of the carboxy terminus had been deleted. Deletion constructs were purified on a Ni^{2+} column as described above for recombinant human Prdx6.

2.4. Rationale for choice of buffer

SP-A is a secreted protein that is stored prior to secretion in the lung lamellar bodies. Prdx6 is a cytoplasmic protein that also localizes to lamellar bodies through a specific transport mechanism [27,28]. As the lamellar bodies (or possibly lysosomes) are the only known cellular site for co-localization of these two proteins, we reasoned that this compartment would be the likely site of physiologically relevant interaction. Since the luminal pH of lamellar bodies is acidic [3], similar to lysosomes, we carried out our studies of protein/peptide interaction at pH 4.

2.5. Isothermal titration calorimetry (ITC)

ITC titrations were performed using a VP-ITC 200 Micro Calorimeter (Micro Cal, Northampton, MA) to determine interaction of the proteins/peptides. Samples were degassed for 5 min with gentle stirring under vacuum. The reaction cell was filled with 11 μM recombinant Prdx6 or native SP-A and the injection syringe was filled with either 200 μM of SP-A peptide, Prdx6 peptide, scrambled peptide, or truncated Prdx6 protein. Titration was performed at 25 °C in 50 mM acetate buffer at

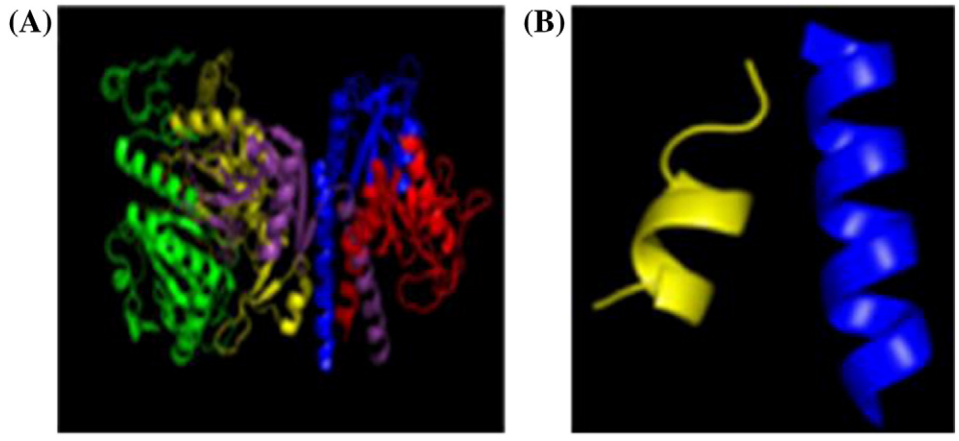

Fig. 1. Modeling of the SP-A:Prdx6 interaction with the Z Dock program. (A) Ribbon model of SP-A–Prdx6 complex. Green, blue and purple show the monomers of SP-A; yellow and red show homodimeric Prdx6. (B) Amplified view of interacting α-helices. Yellow—Prdx6 ($_{195}$EEEAKKLFPK$_{204}$), blue—SP-A. ($_{83}$DEELQTELYEIKHQIL$_{99}$).