Peroxiredoxin 6 translocates to the plasma membrane during neutrophil activation and is required for optimal NADPH oxidase activity

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Neutrophils provide the first line of defense against microbial invasion in part through production of reactive oxygen species (ROS) which is mediated through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase generating superoxide anion (O2−). The phagocyte oxidase (phox) has multiple protein components that assemble on the plasma membrane in stimulated neutrophils. We recently described a protein in neutrophils, peroxiredoxin 6 (Prdx6), which has both peroxidase and phospholipase A2 (PLA2) activities and enhances oxidase activity in an SDS-activated, cell-free system. The function of Prdx6 in phox activity is further investigated. In reconstituted phox-competent K562 cells, siRNA-mediated suppression of Prdx6 resulted in decreased NADPH oxidase activity in response to formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol myristate acetate (PMA). In neutrophils stimulated with PMA, Prdx6 translocated to plasma membrane as demonstrated by Western blot and confocal microscopy. Translocation of Prdx6 in phox competent K562 cells required both p67phox and p47phox. In addition, plasma membrane from PMA-stimulated, oxidase competent K562 cells with siRNA-mediated Prdx6 suppression contained less p47phox and p67phox compared to cells in which Prdx6 was not decreased. Cell-free oxidase assays showed that recombinant Prdx6 did not alter the Km for NADPH, but increased the Vmax for O2− production in a saturable, Prdx6 concentration-dependent manner. Recombinant proteins with mutations in Prdx (C47S) and phospholipase (S32A) activity both enhanced cell-free oxidase activity.

1. Introduction

Neutrophils provide the host with a first line of defense against microbial invasion. These cells migrate to the site of infection and ingest microorganisms [1–5]. As the phagosome forms, fusion of specific and azurophilic granules releases contents of these organelles into the phagolysosome; and activation of the respiratory burst generates superoxide anion (O2−) [2] and other reactive oxygen species (ROS) [6,7]. The focus of ROS dependent and independent antimicrobial systems on the ingested microbe leads to its death and dissolution [6,7]. The neutrophil respiratory burst involves activation of the NADPH oxidase composed of multiple components including gp91phox and gp91phox, forming the flavocytochrome b558, and regulator proteins designated as p47phox, p67phox and p40phox, and Rac2 [6,7]. In unstimulated neutrophils, the latter four proteins reside in the cytosol while flavocytochrome b558 is associated with plasma membrane and specific granules [8,9]. With activation, cytosolic phox components translocate to the plasma membrane and NADPH oxidase activity is expressed reducing oxygen to O2− [8–10]. Chronic granulomatous disease (CGD), an inherited disorder of bactericidal activity, illustrates the importance of the phagocyte NADPH oxidase activity and ROS in host defense [6]. A deficiency of any one of these essential phox proteins leads to an inactive oxidase and impaired ROS production; afflicted patients suffer from recurrent, life-threatening infections with catalase positive microbes and inflammatory diseases.

Abbreviations: fMLP, formyl-methionyl-leucyl-phenylalanine; H2O2, hydrogen peroxide; KRPPD, Krebs Ringers phosphate with dextrose; NADPH oxidase, NADPH oxidase 2; O2−, superoxide anion; PMA, phorbol myristate acetate; Prdx, peroxiredoxin; Prdx6, peroxiredoxin 6; phox, phagocyte oxidase; PLA2, phospholipase A2; rh, recombinant human; RLUs, relative light units; ROS, reactive oxygen species; siRNA, short interfering RNA; SOD, superoxide dismutase

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Peroxiredoxins (Prdxs) are a group of peroxidases that reduce H$_2$O$_2$ with concomitant oxidation of active site cysteine residues [11]. These proteins may serve to protect against the damaging effects of ROS or in a signaling role by controlling levels of H$_2$O$_2$ [11]. We recently demonstrated the presence of Prdx6 in neutrophils [12] as a 29 kDa protein (originally designated p29) with amino acid sequence identical to Prdx6 and with Prdx enzyme activity and showed that it binds to p67phox, exhibits a phospholipase A2 (PLA2) activity, and enhances NADPH oxidase activity [12]. In the following studies we investigate further the role of Prdx6 in regulation of NADPH oxidase activity and show that its translocation to the membrane in stimulated cells is linked with assembly and activation of the oxidase.

2. Materials and methods

2.1. Materials

SDS, cytochrome c, PIPES and TRIS buffers, NADPH, imidazole, Dextran, Ficoll-Hypaque, EGTA, sucrose, glycine, ethanolamine, PMA, IMLP, leupeptin, DFP, phenylmethanesulfonyl fluoride (PMSF), horse- radish peroxidase, o-phenylenediamine, DTT, and Tween 20 were purchased from Sigma (St. Louis, MO). Ficoll-Hypaque, HRP-labeled anti-rabbit IgG and enhanced chemiluminescence (ECL) reagents were obtained from GE Healthcare (Piscataway, NJ). Donkey anti-goat IgG was from Rockland Immunocchemicals (Gilbertsville, PA). Nickel affinity resin, RPMI, Cy5 and Alexa 488 (FITC), and labeling kits were from Invitrogen (Carlsbad, CA). Heparin was purchased from APP Pharmaceuticals (Schaumburg, IL); Nunc plates (96 well) were obtained from GE Healthcare (Piscataway, NJ). Donkey anti-rabbit IgG was used to raise a polyclonal antibody in rabbits. The initial immunization was completed with the recombinant protein in Freund's adjuvant, and was followed by two subsequent immunizations. Pre-immune serum and serum after two subsequent immunizations were used for antibody purification. Antibodies to Prdx6 were affinity purified on the recombinant protein in Freund's adjuvant, and was followed by two subsequent immunizations. Pre-immune serum and serum after two subsequent immunizations were used for antibody purification.

2.2. Isolation of neutrophils and preparation of subcellular fractions

Heparinized (10 units/ml) peripheral blood was obtained from healthy adult volunteers after signed consent which was approved by the Combined Institutional Review Board at the University of Colorado Denver. Neutrophils were isolated by the standard technique of Dextran sedimentation, Ficoll-Hypaque density gradient centrifugation and hypotonic lysis of red cells [13,14]. Neutrophils were incubated with buffer or PMA (1 μg/ml) for 5 min at 37 °C; and subcellular fractions were isolated by sucrose density gradient centrifugation after disruption of the cells with nitrogen cavitation in the presence of DFP (1 mM), leupeptin (1 μg/ml) and PMSF (0.2 mM) as previously described [8,13–15].

For subcellular fractions from K562 cells, 10° cells/ml were suspended in cavitation buffer and sonicated in a Branson sonicator (Heat Systems Ultrasonics, Plainview, NY) with three, 15 s bursts [16,17]. After centrifugation (250 g) to remove unbroken cells, purified plasma membranes and cytosol were collected on a sucrose gradient as previously described [16,17]. Plasma membrane and other subcellular fractions were immediately frozen at −70 °C. Protein concentration was determined by BCA protein assay.

2.3. Recombinant proteins

Using standard molecular biology techniques, a previously described [12] p29 (Prdx6) ORF with an N-terminal extension that included a poly-His tag was moved to pBlueBacHis 2B (Invitrogen). Mutant versions of this plasmid with substitution of serine for cysteine at amino acid 47 and an alanine for serine at amino acid 32 (numbering relative to untagged protein) were produced using QuickChange Mutagenesis (GE Healthcare) [13,18]. For all expression plasmids, the correct sequence was confirmed by DNA sequencing. Recombinant human (rh) Prdx6 (or rh-wt) and the two mutants (rh-C47S and rh-S32A) were produced by expression in SF-9 cells and purified by Ni$^{2+}$ affinity chromatography [12].

The peroxidase and PLA2 activity of rh-wt protein have been described by us and others [11,12,18–20]. The deletion of peroxidase activity by mutating the cysteine at position 47 has been described [19,21,22]. We confirmed this effect in our C47S mutant with a glutamine synthetase protection assay [12]. The C47S mutant exhibited 8.6 ± 6.4% protection compared to 85.4 ± 4.9% protection by hr-wt (p < 0.0001) two-tailed t-test. The contribution of serine to the active catalytic site for PLA2 enzymes is well defined in the literature [23,24] and has been demonstrated for Prdx6 [21,25].

2.4. Polyclonal Prdx6 and other antibodies

Purified rh-Prdx6 [12] (1 mg/ml) was used to raise a polyclonal antibody in rabbits. The initial immunization was completed with the recombinant protein in Freund’s adjuvant, and was followed by two subsequent immunizations. Pre-immune serum and serum after two subsequent immunizations were used for antibody purification. Antibodies to Prdx6 were affinity purified on the recombinant protein in Freund’s adjuvant, and was followed by two subsequent immunizations. Pre-immune serum and serum after two subsequent immunizations were used for antibody purification.

2.5. SDS activated cell-free system of oxidase activity

Production of O$_2^-$ in a cell-free system of NADPH oxidase activity was performed as previously described [8,12–14], but with further optimization to provide maximal oxidase activity as follows: 100 nM p67phox, 100 nM p47phox. 32 nM constitutively active Rac1 plus or minus varying amounts of rh-Prdx6 or buffer were combined in buffer to 100 μl. After 10 min incubation at RT, 1 μg of plasma membrane from unstimulated neutrophils and SDS (112.5 μM) were introduced. After 3 min, NADPH (varying concentrations in 15 μM) were introduced. After 3 min, NADPH (varying concentrations in 15 μM) was added to give a total assay volume of 150 μl and the initial rate of O$_2^-$ production was determined as SOD-inhibitable cytochrome c reduction in an ELISA reader at 550 nm as previously reported [12–14]. In one series of experiments, rh-Prdx6 (0, 0.27, 0.55, 1.10, and 2.20 μM) was added to the reaction mixture. The contribution of serine to the active catalytic site for PLA2 enzymes is well defined in the literature [23,24] and has been demonstrated for Prdx6 [21,25].

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rh-Prdx6. In a third set of studies, the Prdx6 mutants, rh-C47S and rh-S32A, were added to the standard assay mix and production of O$_2^-$ determined.

2.6. Confocal microscopy

For confocal microscopy studies, neutrophils were incubated with buffer or PMA (1 µg/ml) for 5 min, fixed to poly-l-lysine coated coverslips with 3% paraformaldehyde, washed and incubated with primary antibody to CD45 and a secondary Cy3 labeled rabbit anti-lg. The cells were then permeabilized with lysophosphatidylcholine (100 µg/ml) for 5 min and incubated with anti-p67phox IgG labeled with FITC and anti-p29 Prdx6 IgG labeled with Cy5 for 1 h at room temperature. After washing, cells were imaged with a Leica DMRXA digital fluorescence microscope under a 100× lens, and the images analyzed with an open-source image analysis tool, IMAJIN_COLOC developed in PERL (v. 5.8, build 806) using the PERL Magick libraries (Image-Magick) [26]. Using a pixel-by-pixel analysis algorithm, this program analyzes antigen expression and can measure all possible combinations of co-localization for up to three distinct fluorescent probes. Background fluorescence for each experiment was obtained with cell preparations exposed to Cy3 labeled goat anti-mouse, Cy5 rabbit anti-human and FITC mouse anti-human lgG, processed as noted above and subtracted from fluorescence obtained with primary antibodies using the analysis program. For all analyses, colors were arbitrarily assigned, green for CD45, blue for p67phox, and red for Prdx6.

2.7. K562 cells for siRNA knockdown of Prdx6 and for translocation studies

NADPH oxidase-competent K562 cells were grown at 37 °C in 5% CO$_2$ with RPMI and 10% fetal calf serum [16,17,27]. These cells were stably transduced with p47phox, p67phox, gp91phox (K+++) grown as above and transfected with the fMLP receptor (FPR); this reconstituted line has sufficient levels of endogenous p22phox, p40phox, and Rac proteins to support NADPH oxidase activity in response to stimulation by PMA or fMLP [17]. Small interfering RNA (siRNA) molecules were uniquely targeted to hybridize 19-nucleotide sense-strand sequences and Nsi (Qiagen), UUC UCC GAA CGU GUC ACG UdT dT. A si1 (Qiagen), UUG GUG AAG ACU CCU UUC GGG; si2 (Ambion), GCA UUC AAG ACA UUC Ctc; si3 (Ambion), UGG GUU GAG GAU AGA CAG Ctt; and Nsi (Qiagen), UUC UCC GAA CGU GUC ACG UdT dT. A plasmid expressing GFP assessed transfection efficiency. Transfections were conducted with Amaxa Nucleofector technology (Lonza, Basel, Switzerland). Transgenic K562 cells (5 × 10$^6$, passage < 12), grown for 24 h, were resuspended in 100 µl Solution L. GFP plasmid (provided by Lonza to assess transfection efficiency), si1, si2, si3, and Nsi, were added at various concentrations (0.5, 1, 2, and 5 µM) to optimize the knockdown and transfection completed with the T2020 protocol. RPMI with 10% (500 µl) FCS was added and the cells were incubated for 15 min at 37 °C. Cells were transferred to larger plates, brought up in 2 ml RPMI with 10% FCS, and cultured for various times before being harvested for assays. Cell viability for all studies was determined by trypan blue exclusion and for all experiments was ≥90%.

For translocation studies, K+++ cells, the parent K562 cell line and cell lines stably transduced with all other phox proteins except p47phox (K67/91) or p67phox (K47/91) [16,17] grown as above were exposed to PMA (1 µg/ml) or buffer for 5 min and subcellular fractions prepared as noted above. Cell membranes were prepared in si experiments after 48 h in culture with 1.5 × 10$^7$ cells at a concentration of 10$^8$ cells/ml. Plasma membrane preparations were evaluated for Prdx6 and other phox proteins by Western blotting.

2.8. Oxidase activity in reconstituted K562 cells

O$_2^-$ production was determined by two techniques. Transgenic K+++, p47phox-competent cells (10$^6$/ml in KRPD) were stimulated with PMA (200 ng/ml) in 150 µl volume and O$_2^-$ production was measured as SOD-inhibitable cytochrome c reduction [12–14]. Alternatively, 10$^6$ cells/ml in KRPD were added to 30 µl Diogenes and 1 µM IMLP to start the reaction in an assay volume of 150 µl. Chemiluminescence was measured in a FB12 Luminometer (Berthold Detection Systems, Pforzheim, Germany), as relative light units (RLUs). The maximum RLUs at 30 s was used to measure the output of the respiratory burst; >95% of this chemiluminescence was SOD-inhibitable indicating the reactivity was O$_2^-$.

2.9. Cell lysates

Cell lysates were made with the addition of 10% Triton X-100 and 3 cycles of freeze/thaw (room temperature to −70 °C). Lysates were stored at −70 °C.

2.10. Western blots

Western blots of subcellular fractions or protein preparations were completed as previously described [8,12–15]. Protein from plasma membrane (2.5–25 µg, depending on cell type), recombinant protein preparations (1 µg) or cell lysates (5–10 µg) were separated on 12.5% SDS-PAGE, transferred to nitrocellulose and probed with the relevant antibodies. For translocation studies in K562 cells, the antibody to the amino-terminal end of p67phox was used. Analysis was completed with the appropriate horseradish peroxidase labeled secondary antibody (donkey anti-goat IgG or goat anti-rabbit IgG), and ECL detection ware, GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. siRNA knockdown of Prdx6 in transgenic K562 cells

Our previous studies demonstrated enhanced oxidation activity by rh-Prdx6 (p29) in the cell-free system [12]. We extended these findings with studies using intact cells. Because neutrophils are terminally differentiated, short-lived and difficult to transfect, we employed a K562 cell model for our siRNAs experiments. Production of genetically modified cultured cells has provided powerful strategies to study the activity of the oxidase [16,17,27,33]. The K+++, cells used are a clonally-derived K562 cell line stably transduced with retroviral p47phox, p67phox, gp91phox and were shown to express endogenous p22phox and Rac [16,17,27]. They were subsequently transfected with the fMLP receptor (FPR) [17]. Three siRNA molecules were developed specifically to degrade the mRNA of Prdx6 and decrease expression of this protein in these cells. A non-silencing siRNA, (Nsi) which did not interfere
with any specific mRNA in the cell served as a control. Transfection with the active siRNA (si1, si2, and si3) and Nsi was achieved with Amaxa Nucleofector technology (protocol T020 and solution I). Transfections containing 5×10⁶ K⁺⁺ cells and 2 μM si or Nsi, followed by 48 h in culture gave maximal knockdown of Prdx6, with reversal of suppression occurring another 72 h later (data not shown) and were judged ≥90% efficient based on separate experiments in which a GFP expressing plasmid was transfected and cells monitored for fluorescence.

Quantitation of Prdx6, phox proteins and actin was performed by Western blotting after transfection of K⁺⁺ cells with si or Nsi molecules. A representative experiment is shown in Fig. 1A. Expression of Prdx6 was reduced by si1, si2, and si3 as compared to Nsi. The phox proteins including p67phox, p47phox, p40phox, or p22phox were not decreased; and actin, used as a loading control, was also unaffected. gp91phox was also not decreased (data not shown). Fig. 1B summarizes quantification of these proteins by Western blot densitometry. Prdx6 was reduced to 26–43% depending on the specific si used and the results were significant (p<0.05, unpaired t-test). O₂⁻ production was determined in the si treated K562 cells after stimulation with PMA (200 ng/ml) as cytochrome c reduction or with fMLP (1 μM) using chemiluminescence. Fig. 1C shows O₂⁻ production in response to PMA. These data summarize results for four separate experiments. The response to PMA in Prdx6-targeted, si-treated cells was decreased to 50–65% of the Nsi control cells and this was significant (p<0.025, paired t-test). The suppressive effects of Prdx6 knockdown on oxidase activation were greater when using fMLP as an agonist (Fig. 1D), where O₂⁻ production for each Prdx6-targeted si was 16–38% of Nsi control (p<0.025, paired t-test). The insert demonstrates results of fMLP-stimulated oxidase kinetics from one experiment, with the main graph summarizing results from all experiments. These studies demonstrate the requirement of Prdx6 for optimal oxidase activity in intact cells.

3.2. Translocation of Prdx6 with neutrophil activation

Stimulation of neutrophils results in translocation of p67phox, p47phox, p40phox and Rac2 to the plasma membrane to bind to cytochrome b₅₅₈ (gp91phox and p22phox) to form an active oxidase complex [8–10]. If Prdx6 binds to p67phox and enhances oxidase activity, one would expect translocation of this protein as well. To evaluate translocation, subcellular fractions from unstimulated and PMA-stimulated neutrophils were prepared as described in Methods (Section 2.2). Proteins from unstimulated and PMA-stimulated plasma membrane were separated by SDS-PAGE, blotted to nitrocellulose and Prdx6 was detected by Western blotting. Representative blots are shown in Fig. 2A. PMA stimulation of neutrophils results in a dramatic increase in Prdx6 associated with the plasma membrane. Results for p67phox and p47phox are included for comparison. In eight separate preparations, densitometry of Western blots indicated that the PMA-stimulated plasma membranes had a 1.5–8 fold increase in Prdx6 (p<0.010 by paired t-test) over simultaneously blotted plasma membranes from unstimulated cells. In comparison, p67phox was 1.5–10 and p47phox 2.3–11 fold increased (both p<0.005 by paired t-test) in PMA-stimulated plasma membranes. When plasma membrane from PMA stimulated neutrophils was immunoprecipitated with antibody against p67phox, Prdx6 was pulled down with p67phox (Fig. 2B) providing further evidence of their interaction.

In a second approach, confocal microscopy was performed with unstimulated and PMA-stimulated cells imaged for Prdx6, p67phox and a membrane/cell surface marker CD45 with fluorescently labeled antibodies and analyzed using IMAJIN-COLOC software [26]. Fig. 3A shows an unstimulated neutrophil with images for CD45 (green), p67phox (blue), and Prdx6 (red) separately; a merge of all three; and a panel demonstrating only pixels with all three colors (white, RGB colocalization). In unstimulated cells, Prdx6 and p67phox were localized together in the cytosol (purple). Although small amounts
of Prdx6 and p67phox were seen in the membrane (white, merged and RGB colocalization) consistent with the Western blots, Prdx6 and p67phox remained largely distinct from CD45. In PMA-stimulated cells, more Prdx6 and p67phox became associated with CD45 and the plasma membrane (Fig. 3B, merged and RGB colocalization) consistent with the Western blots, Prdx6 and p67phox were seen in the membrane (white, merged and RGB colocalization panels). (B) Immunoprecipitation of plasma membrane from PMA-stimulated neutrophils with antibody for p67phox. Representative blot of two separate experiments shows Prdx6 co-immunoprecipitating with p67phox.

Further analysis was completed using IMAJIN_COLOC software [27]. The confocal microscopy images of individual cells were evaluated for the number of p67phox, Prdx6 and CD45 pixels within them. The first analysis determined colocalization of two fluoros, CD45 and Prdx6 or p67phox (Fig. 3C); pixels containing CD45 and Prdx6 expressed as a percent of total Prdx6 pixels (gray bars) and pixels with both CD45 and p67phox expressed as a percent of total p67phox are shown in open bars. Results for unstimulated cells are presented on the left for a percent of total Prdx6 pixels (gray bars) and pixels with both CD45 and Prdx6 as a percent of total p67phox (open bars). The same cells were analyzed as in (C). Most Prdx6 and p67phox were colocalized to the same extent in unstimulated and stimulated cells.

In an additional analysis, pixels containing all three fluoros were expressed as percent of total p67phox pixels in the cell to measure p67phox colocalization with membrane. A similar evaluation for Prdx6 pixels established colocalization for this protein in the plasma membrane. As seen in Fig. 3D, p67phox and Prdx6 colocalization in the plasma membrane increased when neutrophils were stimulated from 23% to 49% (open bars) and 17% to 37% (gray bars) respectively. This was significant, p<0.005 by unpaired t-test. Fig. 3E shows the amount of Prdx6 associated with p67phox as a percentage of total Prdx6 (gray bars) or p67phox associated with Prdx6 as a percentage of total p67phox (open bars). There was no difference between either of these measurements in unstimulated or stimulated neutrophils. In both unstimulated and stimulated neutrophils however, the percentage of Prdx6 associated with p67phox, as a percentage of total Prdx6, was less than p67phox associated with Prdx6 as a percentage of total p67phox (p<0.05, unpaired t-test). About 60% of Prdx6 was associated with p67phox (gray bars), but more p67phox (75–80%) was associated with Prdx6 (open bars) suggesting that Prdx6 is present in excess relative to p67phox and may serve other functions. These studies demonstrate cotranslocation of Prdx6 and p67phox to the neutrophil plasma membrane after stimulation with PMA and confirm Western blot results.

3.3. Involvement of p67phox and p47phox in Prdx6 translocation

K⁺⁺ cells, cells without p67phox (K47/91) or p47phox (K67/91), and non-tranduced K562 cells were treated with PMA (1 μg/ml) for 5 min, and plasma membranes isolated. Western blotting for Prdx6, p47phox and p67phox was performed on the membranes. Fig. 4A shows a representative Western blot for each cell type and protein, and Fig. 4B summarizes quantitation of these proteins as a ratio between the amounts in stimulated and unstimulated membranes. Efficient translocation of all three proteins was documented in K⁺⁺⁺ cells after PMA stimulation. The highest ratio for Prdx6 was observed in these cells with a 3-fold increase in Prdx6 in the membrane fraction (p<0.005, paired t-test). No significant increase in the ratio above 1 was seen in cells deficient in p67phox (K47/91) or p47phox (K67/91). Increases in the ratio for p47phox or p67phox are demonstrated only in K⁺⁺⁺ cells (p<0.005, paired t-test). A small amount of Prdx6 is seen in unstimulated plasma membranes (Fig. 4A), particularly those from K47/91 cells, suggesting that by binding Prdx6, p67phox may play a role in restricting the distribution of Prdx6 to the cytosol until activation of NADPH oxidase occurs. A less robust translocation of Prdx6 was associated with PMA stimulation of K562 cells lacking p47phox and p67phox (Fig. 4A, B). However, this is significantly less than that seen in the K⁺⁺⁺ cells (p<0.05, paired t-test). Previous studies have demonstrated the requirement of the interaction of p47phox with p67phox and cotranslocation of both to form an active oxidase suggesting that p47phox acts as an adaptor linking p67phox to the membrane oxidase complex [16,17]. Our results demonstrate that both p47phox and p67phox are expressed.
and p67phox promote more efficient translocation of Prdx6 to the membrane. The possibility of independent associations of Prdx6 with the membrane in the absence of phox proteins, perhaps through interactions with its lipid substrates, is raised by these studies.

3.4. Effects of rh-p29 Prdx6 on the kinetics of oxidase activity in the SDS activated cell-free system

Previous studies demonstrated that rh-Prdx6 enhanced production of $O_2^-$ and oxygen consumption by a cell-free system of oxidase activity using neutrophil plasma membrane and recombinant cytosolic phox proteins [12]. We complemented our current results with intact cells by additional studies in the cell-free system to determine the characteristics and specificity of the enhancement by Prdx6. First, with several different concentrations of Prdx6 added to the reaction mixture, the rate of $O_2^-$ production was determined at various concentrations of NADPH. Results from these studies are shown in Fig. 5A. All the rh-Prdx6 concentrations increased the rate of $O_2^-$ generation but did not change the concentration of NADPH at half maximal rate. Kinetic parameters of the oxidase were calculated with these data and the results are presented in Table 1. Additions of Prdx6 at concentrations up to 2.2 $\mu$M had no effect on the $K_m$ for NADPH. However, the $V_{max}$ was significantly increased at concentrations above 0.55 $\mu$M Prdx6.

In the second set of experiments, assays were completed using a wider range of rh-Prdx6 concentrations and 200 $\mu$M NADPH. The difference in rate of $O_2^-$ production between the system with and without the recombinant protein was calculated for each concentration of rh-Prdx6 added. The results are shown in Fig. 5B. rh-Prdx6 produces a saturable, concentration-dependent increase in oxidase activity. Thus, enhanced production of $O_2^-$ in the cell-free system appears to be due to a specific effect on the oxidase rather than increasing $O_2^-$ production by another means such as chemical ROS generation.

The ability of Prdx6 to increase the $V_{max}$ of the oxidase suggests that this protein increases the catalytic efficiency of the active oxidase complex and/or increases the total amounts of oxidase proteins retained within an active complex. To further explore these possibilities, we used si1 to suppress Prdx6 in K+++ cells and Nsi as a control as previously described (Section 2.7). After 48 h, cells were incubated with PMA (1 $\mu$g/ml) or buffer for 5 min, disrupted and plasma membranes collected on sucrose gradients after-centrifugation. Membrane proteins were separated on SDS-PAGE and oxidase proteins p67phox, p47phox, and gp91phox identified and quantified as previously described (Section 2.10). The ratio of the specific proteins in stimulated membranes compared to resting membranes was calculated for cells treated with si and Nsi. Results for two separate preparations of cells are shown in Fig 6. Knockdown of Prdx6 was achieved as previously noted (data not shown). In K+++ cells with Prdx6 not suppressed, both p47phox and p67phox demonstrated higher stimulated/unstimulated ratios (p<0.05 and p<0.001, paired t-test) compared to results for cells in which Prdx6 was reduced by siRNA. gp91phox remained unchanged in all experiments (data not shown). This data suggests that the presence of Prdx6 results in increased amounts of cytosolic phox proteins retained in the active oxidase complex.
3.5. Mutation of Prdx6 and activity in the SDS, cell-free system of oxidase activity

Cysteine 47 of Prdx6 is critical for the Prdx activity of this protein and serine 32 is required for the PL2 activity \cite{19,21,34}. Mutant Prdx6 proteins (C47S and S32A) were expressed and purified from baculovirus-infected sf-9 cells, as described in Materials and methods. Mutant recombinant Prdx6 proteins, C47S and S32A, appeared identical to rh-Prdx6 (WT) on SDS-PAGE and Western blotting (Fig. 7A). These three proteins were individually added to the cell-free system of oxidase activity at a concentration of 1 μM, and the effect on O₂⁻ production evaluated. WT, C47S, and S32A proteins all enhanced the generation of O₂⁻ by the cell-free system between 2 and 2.5 fold and no difference between any of the proteins was noted (Fig. 7B). These studies imply that neither the Prdx nor PL2 activities of Prdx6 is responsible for increasing O₂⁻ production in the cell-free system and the observed enhancement is related to a distinct effect on NADPH oxidase activity.

4. Discussion

Our studies were initiated with the observation that Prdx6 in neutrophils is found in association with the NADPH oxidase component, p67phox \cite{12}. The interaction was defined by immunoprecipitation and confirmed with yeast-two hybrid studies. A cDNA for Prdx6 was isolated from neutrophils and the recombinant protein generated from this cDNA was a 1-Cys peroxiredoxin by sequence homology and H₂O₂ reducing activity and exhibited PLA2 activity with an optimal activity at low pH and no requirement for calcium, as others have shown \cite{12,27–29}. Finally, in a cell-free system of NADPH oxidase activity, Prdx6 enhanced both O₂ consumption and O₂⁻ production \cite{12}.

The current investigations extend our understanding of the physiologic relevance of Prdx6 in oxidase activity in intact cells. While mature neutrophils are difficult to maintain in culture or use in transfection studies for specific proteins, transduced cells reconstituted with oxidase components have been employed to explore the biochemistry of the NADPH oxidase system \cite{16,17,27,33}. We combined the use of phox-competent K562 (K⁺++) cells stably transfected with the FMLP receptor along with siRNA targeting Prdx6 to suppress this protein and explore its effects on NADPH oxidase activity. Three siRNA molecules specifically silencing expression of Prdx6 were compared with a nonsilencing siRNA probe. The silencing molecules decreased the detectable levels of Prdx6 to 23–40% of the Nsi control. No significant effect was demonstrated on any of the phox components or actin. Production of O₂⁻ in response to FMLP or PMA stimulation was decreased in cells with reduced Prdx6 compared to non-silenced control cells. Results with K562 cells are consistent with our previous data from the cell-free system \cite{12} which indicated that Prdx6 enhances the activity of NADPH oxidase.

In resting neutrophils, the oxidase complex lies dormant with some components (p40phox, p47phox, p67phox and Rac2) residing in the cytosol and the cytochrome b₅₅₈ (gp91phox and p22phox) contained in the plasma membrane or membrane of the specific granules \cite{8–10}. With stimulation, the cytosolic components translocate to specific granules or to the plasma membrane, assembling the active oxidase complex \cite{6,8–10,35}. In this study, two techniques, Western blotting plasma membrane after subcellular fractionation and analysis

<table>
<thead>
<tr>
<th>Prdx6 (μM)</th>
<th>Kₘ for NADPH (μM)</th>
<th>Vₖₐₛ (nmol O₂⁻/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>68 ± 20</td>
<td>100 ± 1.0</td>
</tr>
<tr>
<td>0.27</td>
<td>49 ± 11</td>
<td>115 ± 0.9</td>
</tr>
<tr>
<td>0.55</td>
<td>46 ± 14</td>
<td>13.4 ± 1.4</td>
</tr>
<tr>
<td>1.10</td>
<td>65 ± 15</td>
<td>18.9 ± 1.7*</td>
</tr>
<tr>
<td>2.20</td>
<td>56 ± 13</td>
<td>21.0 ± 1.5*</td>
</tr>
</tbody>
</table>

* Numbers represent mean ± SEM of 4 separate experiments at each concentration.

b Significantly different from no rh-p29 Prdx6 added p < 0.05 by unpaired t-test.

Fig. 6. Ratio of phox components in membranes from PMA stimulated compared to unstimulated K562 oxidase competent cells with and without suppression of Prdx6. Closed bars represent results from Nsi treated control cells and open bars represent si treated cells with suppression of Prdx6. Results are shown for p47phox and p67phox. Bars and brackets show mean ± SEM for 9 experiments with two separate preparations. Ratio for p47phox is decreased in si suppressed cells, p < 0.05, and for p67phox, p = 0.001, paired t-test.

Fig. 7. Effects of Prdx6 mutations on cell-free oxidase activity. (A) SDS-PAGE (Coomassie blue staining, top) and Western blot analysis for rh-Prdx6 (WT), C47S, and S32A proteins (probed with polyclonal antibody to Prdx6), bottom. (B) Activity of rh-wt, C47S, and S32A in the SDS-activated cell-free assay of oxidase activity. Proteins were added at 1 μM. O₂⁻ production was measured as described in Materials and methods expressed as fold increase over the system without Prdx6 protein. Results are mean ± SEM for 5 experiments for rh-wt and 6 each for C47S and S32A.
of intact neutrophils with confocal microscopy, demonstrated Prdx6 assembled with the p47phox and p67phox components in the plasma membrane after stimulation of oxidase activity. Immunoprecipitation studies with plasma membrane from PMA stimulated neutrophils further supports evidence of interaction between p67phox and Prdx6. In the oxidase-reconstituted K562 cell model, optimal Prdx6 translocation required the presence of oxidase components, specifically the presence of both p47phox and p67phox. This is consistent with binding of Prdx6 to p67phox and previous studies demonstrating the requirement of p47phox for p67phox assembly into the active NADPH oxidase complex [12,16,17]. Translocation of Prdx6 further expands the relationship of these proteins in assembly and activation of NADPH oxidase. The association of a small amount of Prdx6 with plasma membrane of PMA stimulated K562 cells without p67phox and p47phox as well as confocal studies in neutrophils demonstrating that some Prdx6 is not associated with p67phox opens the possibilities of other roles of this protein in myeloid function involving ROS scavenging or phospholipase activity.

In an additional series of experiments, recombinant Prdx6 had specific effects in supporting higher levels of oxidase activity in vitro. Although it was not required for baseline activity of the cell-free system, which requires soluble amphiphiles (SDS or arachidonate) in place of the normal physiological signaling events that direct assembly of NADPH oxidase components in whole cells [36–40], Prdx6 was found to enhance the rate of O2− production in a concentration-dependent manner. In kinetic studies of NADPH oxidase activity, Prdx6 did not change the Vmax for NADPH, but did increase the Vmax, exhibiting a hyperbolic relationship that was consistent with saturable binding to the oxidase. The data suggest Prdx6 enhances the rate at which the NADPH oxidase generates O2−, either by enhancing the flow of electrons through the complex or by stabilizing the oxidase complex or active conformations of its components. Studies demonstrating increased ratios of p47phox and p67phox in membranes of stimulated cells whose Prdx6 is not suppressed in comparison to low ratios in cells with Prdx6 knocked down suggests that this protein not only enhances activity throughout activation (Fig. 1D) but also supports retention of the components in the active complex.

In the cell-free system, production of O2− was enhanced to the same extent by rh-Prdx6 and mutant proteins with deficient Prdx or PLA2 activity [12,19,21,34]. This implies that Prdx6 may have a distinct function with the oxidase in the cell-free system separate from its Prdx or PLA2 activity. However, the cell-free system circumvents signaling and modifications that may be critical to protein function in intact cells. Thus, Prdx, PLA2 activity, or even phosphorylation may be important for the intracellular function of Prdx6 in oxidase activity, whereas these activities are not required for in vitro oxidase reconstitution in the cell-free system.

Peroxiredoxins are a new class of proteins that catalytically degrade H2O2 [11]. Several classes exist based on the number of active cysteines and their activity. Prdx6 falls into a class of 1-Cys peroxiredoxins with one active cysteine and exhibits an additional activity, that of a PLA2 [12,19,21,34]. Like other peroxiredoxins, Prdx6 is thought to protect against excess ROS generation, particularly H2O2 that of a PLA2 [11,19,21,34]. Like other peroxiredoxins, Prdx6 is a member of a class of 1-Cys peroxiredoxins with one active cysteine and exhibits an additional activity, that of a PLA2 [12,16,17]. Translocation of Prdx6 further expands the relationship of these proteins in assembly and activation of NADPH oxidase. The association of a small amount of Prdx6 with plasma membrane of PMA stimulated K562 cells without p67phox and p47phox as well as confocal studies in neutrophils demonstrating that some Prdx6 is not associated with p67phox opens the possibilities of other roles of this protein in myeloid function involving ROS scavenging or phospholipase activity.

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5. Conclusion

Our investigations with neutrophils, the cell-free system of NADPH oxidase activity and reconstituted K562 cells expressing NADPH oxidase activity demonstrate that Prdx6 translocates to the plasma membrane requiring other cytosolic phox components, enhances the activity of the oxidase, and supports retention of phox proteins in the active complex. The enhancement effect is saturable and specific; and, although not essential for baseline oxidase activity, appears to play a role in optimal NADPH oxidase activity. This protein has the potential for dramatic effects on myeloid function. Additional work is needed to explore the mechanisms for these effects on NADPH oxidase and its relationship to biochemical activities of Prdx6.

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References


