Characterization of a Mutation in the Phox Homology Domain of the NADPH Oxidase Component p40\textsuperscript{phox} Identifies A Mechanism for Negative Regulation of Superoxide Production*  

Received for publication, May 30, 2007, and in revised form, August 7, 2007 Published, JBC Papers in Press, August 13, 2007, DOI 10.1074/jbc.M704416200  

Jia Chen\textsuperscript{1, 1}, Rong He\textsuperscript{1}, Richard D. Minshall\textsuperscript{1, 2}, Mary C. Dinauer\textsuperscript{1, 2}, and Richard D. Ye\textsuperscript{1, 2}  

From the \textsuperscript{4}Department of Pharmacology, College of Medicine, University of Illinois, Chicago, Illinois 60612 and \textsuperscript{5}Herman B. Wells Center for Pediatric Research, Riley Hospital for Children, Indiana University School of Medicine, Indianapolis, Indiana 46020  

The phagocyte oxidase (Phox) protein p40\textsuperscript{phox} contains a Phox homology (PX) domain which, when expressed alone, interacts with phosphatidylinositol 3-phosphate (PtdIns (3)P). The functions of the PX domain in p40\textsuperscript{phox} localization, association with the cytoskeleton, and superoxide production were examined in transgenic COS-7 cells expressing gp91\textsubscript{phox}, p22\textsubscript{phox}, p67\textsubscript{phox}, and p47\textsubscript{phox} (COSphox cells). Full-length p40\textsuperscript{phox} exhibited a cytoplasmic localization pattern in resting cells. Upon stimulation with phorbol 12-myristate 13-acetate or fMet-Leu-Phe, p40\textsuperscript{phox} translocated to plasma membrane in a p67\textsubscript{phox}- and p47\textsubscript{phox}-dependent manner. Heterologous expression of p40\textsuperscript{phox} markedly enhanced superoxide production in phorbol 12-myristate 13-acetate- and fMet-Leu-Phe-stimulated COSphox cells. Unexpectedly, mutation of Arg-57 in the PX domain to Gln, which abrogated PtdIns (3)P binding, produced a dominant inhibitory effect on agonist-induced superoxide production and membrane translocation of p47\textsuperscript{phox} and p67\textsuperscript{phox}. The mutant p40\textsuperscript{phox} (p40R57Q) displayed increased association with actin and moesin and was found enriched in the Triton X-100-insoluble fraction along with p67\textsuperscript{phox} and p47\textsuperscript{phox}. The enhanced cytoskeleton association of p67\textsuperscript{phox} and p47\textsuperscript{phox} and the dominant inhibitory effect produced by the p40R57Q were alleviated when a second mutation at Asp-289, which eliminated p40\textsuperscript{phox} interaction with p67\textsuperscript{phox}, was introduced. Likewise, cytochalasin B treatment abolished the dominant inhibitory effect of p40R57Q on superoxide production. These findings suggest a dual regulatory mechanism through the PX domain of p40\textsuperscript{phox}; its interaction with the actin cytoskeleton may stabilize NADPH oxidase in resting cells, and its binding of PtdIns (3)P potentiates superoxide production upon agonist stimulation. Both functions require the association of p40\textsuperscript{phox} with p67\textsuperscript{phox}.  

Phox homology (PX)\textsuperscript{3} domains are evolutionarily conserved protein modules of 120–140 amino acids that bind phosphoinositides. Initially named for their presence in the two cytosolic factors of NADPH oxidase, p47\textsuperscript{phox} and p40\textsuperscript{phox} (1), PX domains have been identified in more than 150 eukaryotic proteins including the sorting nexins (SNX1–15), vacuolar sorting and morphogenesis proteins (Vam7p, Vps5p, and Vps17p), yeast bud-emergence proteins (Bem1p and Bem3p), and phospholipase D2 (2, 3). The PX domains from these proteins interact with a variety of phosphoinositides. Published studies have shown that the PX domain in p40\textsuperscript{phox} binds phosphatidylinositol 3-phosphate (PtdIns (3)P), and the PX domain in p47\textsuperscript{phox} preferentially interacts with phosphatidylinositol 3,4-phosphates (4–6). A proposed function of the PX domain is membrane targeting of proteins containing this structural module. In studies using a green fluorescence protein (GFP)-fused PX domain of p40\textsuperscript{phox}, membrane localization was observed in a phosphatidylinositol 3-kinase-dependent manner (4, 5). Membrane binding of the PX domains involves electrostatic interaction as well as membrane penetration by hydrophobic residues in the PX domain-containing proteins (7). Structural analysis of the PX domain of p40\textsuperscript{phox} reveals a positively charged binding pocket for the negatively charged PtdIns (3)P (4, 5). Binding of p40\textsuperscript{phox} to the phosphoinositide requires three conserved arginine residues (Arg-57, Arg-58, and Arg-105) that stabilize a critical lipid binding loop within the PX domain (8). Mutation of any one of the three arginines can cause a significant reduction in binding of PtdIns (3)P (4, 5).  

Studies have been conducted for the function of p40\textsuperscript{phox} in NADPH oxidase activation since its initial discovery as a p67\textsuperscript{phox}-associated protein (9–11). These studies have resulted in different and sometimes conflicting observations. Evidence supporting a positive regulatory role of p40\textsuperscript{phox} came from studies using both cell-free reconstitution and whole-cell assays. The possible mechanisms for p40\textsuperscript{phox}-mediated potentiation of NADPH oxidase include increasing the affinity of p47\textsuperscript{phox} for flavocytochrome b\textsubscript{558} (12), binding to membrane-associated PtdIns (3)P through its PX domain (5) and cooperation with p67\textsuperscript{phox} for membrane translocation of the cytosolic complex (13). Other investigators, using essentially the same cells and cell-free reconstitution assays, found p40\textsuperscript{phox} to be a negative regulator for NADPH oxidase. The negative regulatory mechanisms include SH3 domain-mediated interference receptor; CL, chemiluminescence; GFP, green fluorescence protein; PMA, phorbol 12-myristate 13-acetate; RT, room temperature; CPS, counts/s; PBS, phosphate-buffered saline.
Regulation of NADPH Oxidase by the PX Domain of p40<sub>phox</sub>

of p40<sub>phox</sub> association with other cytosolic factors (14) and inhibition of p67<sub>phox</sub> membrane translocation (15). More recent studies have examined the roles of p40<sub>phox</sub> in NADPH oxidase activation using transfected cells and mouse models. Suh et al. (16) reported that p40<sub>phox</sub> is required for FcγR receptor-mediated superoxide generation after phagocytosis, a function that was lost when critical residues for PtdIns (3)P binding were mutated. Ellson et al. (17) found that neutrophils from p40<sub>phox</sub> knock-out mice displayed defective oxidant production in response to several types of stimuli. Moreover, replacement of the mouse p40<sub>phox</sub> gene with one that contains a Arg-58 to Ala mutation caused embryonic lethality in homozygous offspring, with the heterozygous mice displaying compromised ability to kill Staphylococcus aureus (18). These findings demonstrate a physiological function of p40<sub>phox</sub> in regulating NADPH oxidase activation that involves its PX domain.

Despite recent progress in p40<sub>phox</sub> research, the function of p40<sub>phox</sub> in resting cells remains undefined. p40<sub>phox</sub> was originally discovered as a p67<sub>phox</sub>-associated protein (9–11). In unprimed neutrophils, p40<sub>phox</sub> forms a complex with p67<sub>phox</sub>, whereas p47<sub>phox</sub> was not a part of the complex (19). Neutrophils from chronic granulomatous disease (CGD) patients who lack p67<sub>phox</sub> contain very little p40<sub>phox</sub> (11, 15), suggesting that interaction between the two cytosolic factors helps to stabilize their structures. Moreover, p40<sub>phox</sub>, like the other cytosolic factors, associates with the actin cytoskeleton in resting neutrophils and with membrane skeleton in activated neutrophils (20). One of the proteins that helps to mediate protein association with the actin cytoskeleton is moesin, which interacts with the PX domain of p40<sub>phox</sub> (21). Based on these findings, we speculate that the PX domain in p40<sub>phox</sub> may have dual regulatory functions through its interaction with the actin cytoskeleton and with PtdIns (3)P. In the current study, we employed a COS-7-based whole-cell reconstitution system (22) to examine the effects of a full-length p40<sub>phox</sub> and a PX domain mutant on NADPH oxidase activity. We observed that expression of the wild type p40<sub>phox</sub> could enhance superoxide generation in response to both PMA and fMet-Leu-Phe (fMLF), a finding consistent with recent publications suggesting that p40<sub>phox</sub> enhances NADPH oxidase activation (16–18). Surprisingly, an Arg to Gln mutation at position 57 (R57Q), which abolishes p40<sub>phox</sub> interaction with PtdIns (3)P through its PX domain (4), switched p40<sub>phox</sub> to a different mode of action. It not only abrogated the potentiation effect but also produced a dominant inhibitory effect on superoxide generation. We found an increased association of p40R57Q with actin and moesin compared with the wild type p40<sub>phox</sub>. In cells expressing p40R57Q, more cytosolic factors were targeted to the Triton X-100-insoluble fraction than in cells expressing the wild type p40<sub>phox</sub>. The dominant inhibitory effect of p40R57Q was eliminated when the cells were treated with cytochalasin B, which prevents actin polymerization, or when the association of p40<sub>phox</sub> with p67<sub>phox</sub> was eliminated. These intriguing findings suggest that p40<sub>phox</sub> can positively and negatively regulate NADPH oxidase through its PX domain interaction with PtdIns (3)P and with the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMA, FMLF, isoluminol, cytochalasin B, antimoesin, and anti-FLAG monoclonal antibodies were purchased from Sigma-Aldrich. Horseradish peroxidase was obtained from Roche Applied Science. The anti-p67<sub>phox</sub> (against amino acids 317–469) and early endosome antigen 1 monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-p40<sub>phox</sub> (against a C-terminal 6-histidine-tagged full-length human p40<sub>phox</sub>) and anti-p47<sub>phox</sub> (against a glutathione S-transferase-fused full-length human p47<sub>phox</sub>) polyclonal antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Plasmid Constructs**—Preparation and characterization of the expression constructs of formyl peptide receptor (FPR), protein kinase Cδ and p40<sub>phox</sub> were described in a previous publication (23). The full-length cDNA encoding the human p40<sub>phox</sub> was subcloned in-frame with GFP in pEGFP-N1 vector (Clontech, Palo Alto, CA) to produce a p40<sub>phox</sub> protein fused to the N terminus of GFP. Point mutations of p40<sub>phox</sub> were generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide primers used for the PCR-based mutagenesis were 5′-GTAACCTCATCTACACAAGTCCGCCAGTTC-3′ and its reverse and complementary sequence for the R57Q mutant and 5′-CTGAAATTCCGGGCACGCTGAGGGGGGAG-3′ and its reverse and complement primer for the D289A mutant. Both primer pairs were used for construction of the double mutant p40R57Q/D289A. All DNA constructs were verified by automated sequencing.

**Cell Culture and Transient Transfection**—The transgenic COS<sup>p<sub>phox</sub></sup> and COS<sup>91/22</sup> cells were generated as described previously (22). COS<sup>91/22</sup> expresses gp91<sub>phox</sub> and p22<sub>phox</sub>. Subsequent transfection resulted in COS<sup>p<sub>phox</sup></sub>, which expresses p67<sub>phox</sub> and p47<sub>phox</sub> in addition to gp91<sub>phox</sub> and p22<sub>phox</sub> (22). The stable cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics for proper selection (22). Cells plated in 90-mm (diameter) tissue culture dishes (0.5–1 × 10<sup>6</sup> cells per dish) were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. A total of 6.5 μg of DNA was used in each transfection. Transient transfection efficiency of 45–50% was routinely obtained based the expression of a co-transfected GFP construct using flow cytometry.

The human myelomonoblastic cell line PLB-985 was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mm L-glutamine. The cells were grown in suspension at a density between 2 × 10<sup>7</sup>/ml and 1 × 10<sup>8</sup>/ml. Cell line Nucleofector Kit V (Amaxa Biosystems, Cologne, Germany) was used for transient transfection of 3 × 10<sup>6</sup> PLB-985 cells with 5 μg of DNA using Program C-023. Transfection efficiency was ~30% as determined by flow cytometry based on the fluorescence of a co-expressed green fluorescent protein.
Measurement of NADPH Oxidase Activity—Superoxide produced by COSphox and PBL-985 cells was determined using an isoluminol-enhanced chemiluminescence assay, as previously described (23, 24). Oxidant production was inhibited by superoxide dismutase (250 units) as reported previously (23). The assay buffer contained horseradish peroxidase (see below) to offset the possible effect of myeloperoxidase. Briefly, COSphox cells were harvested with enzyme-free cell dissociation buffer (Invitrogen). Both COSphox and PLB-985 cells were collected by centrifugation and resuspended in RPMI 1640 containing 0.5% bovine serum albumin at 1–3 x 10^6 cells/ml. Cells were incubated in the dark with 100 μM isoluminol and 40 units/ml horseradish peroxidase at room temperature for 10 min, and 200-μl aliquots were transferred into 6-mm diameter wells of a 96-well, flat-bottom, white tissue culture plate (E&K Scientific, Campbell, CA). Chemiluminescence (CL) was measured at 37 °C in a Wallac 1420 Multilabel Counter (PerkinElmer Life Sciences). The CL CPS were continually recorded at 1-min intervals for 5–15 min before and 20–40 min after stimulation with PMA (200 ng/ml) or fMLF (1 μM). The relative amount of superoxide produced was calculated based on the integrated CL during the first 20 min after agonist stimulation.

Western Blotting—Protein samples were loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were blocked with 5% nonfat dry milk in TBS/T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 2 h at RT. The blots were washed with TBS/T and incubated with primary antibodies (0.2–1 μg/ml) overnight at 4 °C. Anti-rabbit (Bio-Rad) or anti-mouse (Calbiochem) peroxidase-conjugated secondary antibodies were added to the membranes at a dilution ratio of 1:3000, and incubation was continued to 1 h at RT. The protein bands on the membrane were visualized by chemiluminescence (Pierce).

Immunoprecipitation—Twenty-four hours after transfection, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture set 1 (Calbiochem). For immunoprecipitation with moesin and actin, a buffer containing 1% sodium deoxycholate, 10 mM Tris, pH 7.4, 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture set 1 was used instead. The cell lysates were cleared of debris by centrifugation at 14,000 x g for 10 min at 4 °C. Protein content in the cell lysate was measured using a DC Protein Assay (Bio-Rad) and standardized before immunoprecipitation with the anti-FLAG monoclonal antibody (5 μg/ml) at 4 °C overnight. Protein A/G PLUS-agarose was added to the samples for 1.5 h at 4 °C. The beads were washed twice in washing buffer (20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and then once in PBS. The beads were resuspended in 50 μl of 2 × SDS-PAGE loading buffer and boiled for 5 min. The samples were analyzed by Western blotting.

Cell Fractionation—Cell fractionation was performed as described (25), with a modification in buffer composition. Briefly, 24 h after transfection, the cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, and 1% Triton X-100 at 4 °C for 20 min. Cell lysates were then centrifuged at 14,000 x g for 15 min to separate Triton X-100-soluble and -insoluble fractions. The insoluble fraction was dissolved in 500 μl of 1 × SDS-PAGE loading buffer and boiled for 5 min. The samples were analyzed by Western blotting.

Membrane Translocation Assay—Twenty-four hours after transfection, COSphox or COS91/22 cells were stimulated with or without agonists. Cells (1 x 10^7/sample) were lysed with ice-cold hypotonic buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1:50 dilution of Protease inhibitor mixture set I). The lysate was then subjected to 3 cycles of freeze/thaw in liquid nitrogen and at 37 °C. Samples were then centrifuged, and the pellets were washed twice in the hypotonic buffer and resuspended in the same buffer containing 1% Triton X-100. The samples were mixed for 30 min at 4 °C to dissociate membrane-bound proteins and then spun down at 14,000 rpm for 10 min at 4 °C. The supernatant were collected as the Triton-soluble membrane fraction. The proteins in the sample were detected by Western blotting.

Immunofluorescence Microscopy—Confocal microscopy was performed using indirect immunofluorescence. Six hours after transfection, cells were seeded on glass coverslips pre-coated with 50 μg/ml poly-l-lysine (Sigma) and grown for 18 h in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. Cells were stimulated with or without PMA (200 ng/ml) for 5 min, washed 3 times in PBS, and fixed with 3% paraformaldehyde in PBS for 15 min at RT. Cells were washed 3 more times in PBS at RT and permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT. Coverslips were blocked with 5% bovine serum albumin in PBS for 1 h at RT. Cells were washed 3 times in PBS and incubated with the primary antibodies in PBS containing 5% bovine serum albumin overnight at 4 °C. Anti-p47phox and anti-p67phox were used at 2 μg/ml each. After washing 5 times with PBST (0.2% Tween 20 in PBS) at RT, cells were incubated with rhodamine red-X-conjugated goat anti-rabbit IgG (secondary antibody; Jackson ImmunoResearch Laboratories, West Grove, PA) at 1.5 μg/ml for 1 h at RT. After additional washes with PBST and H₂O, coverslips were mounted on glass slides using the ProLong Gold antifade reagent with 4’,6-diamidino-2-phenylindole (Molecular Probes). Fluorescence images were captured with a Zeiss LSM 510 confocal microscope equipped with helium-neon, argon, and krypton laser sources.

Statistical Analysis—Data were analyzed by paired Student’s t-test using the PRISM software (Version 4.0, GraphPad, San Diego, CA).

RESULTS

Localization of the Full-length p40phox in Transfected Cells—The PX domain of p40phox is thought to preferentially bind PtdIns (3)P for its membrane targeting (4, 6, 26). Previous studies have shown that an isolated PX domain fused to a GFP was localized primarily in early endosome (4, 5), an intracellular organelle enriched with PtdIns (3)P (27). In this study we examined the full-length p40phox for its intracellular localization and
Regulation of NADPH Oxidase by the PX Domain of p40phox

Redistribution before and after agonist stimulation. A full-length p40phox fused to GFP (p40phox-GFP) was transfected into COSphox cells, a stable cell line of COS-7 that expresses gp91phox, p22phox, p47phox, and p67phox but lacks p40phox (22). Imaging analysis of the transfected COSphox cells (Fig. 1, A–F) revealed cytoplasmic localization of the GFP fluorescence in resting state (Fig. 1, A and C). Slightly more intense fluorescence was observed in the perinuclear region and in membrane ruffles. In comparison, an antibody against early endosome antigen 1 (EEA-1) stained punctate structures in unstimulated cells (Fig. 1B). There were few punctate structures with both green (p40phox-GFP) and red (anti-early endosome antigen 1) fluorescence (Fig. 1C). Upon stimulation with PMA (Fig. 1, D–F) or fMLF (data not shown), there was a marked increase in plasma membrane-associated green fluorescence (Fig. 1, I, D, F). PMA stimulation did not increase or decrease double-stained fluorescence in the periphery of the cells (Fig. 1F), indicating the absence of fusion between early endosome and the plasma membrane. To determine whether agonist-induced membrane translocation of p40phox requires p67phox and p47phox, which is consistent with the notion that p40phox translocates to plasma membrane in a complex with p67phox and p47phox. A recent study conducted by Ueyama et al. (28) showed that in the RAW267.4 macrophage cell line, which contains very low level of endogenous p67phox, PMA and fMLF were unable to induce membrane translocation of p40phox. Our result is in agreement with their observation.

An Arg to Gln Mutation of p40phox (R57Q) Produces a Dominant Negative Effect in Superoxide Production—We recently reported that NADPH oxidase activation through FPR could be reconstituted in COSphox cells through expression of FPR along with selected signaling molecules (23). Heterologous expression of p40phox significantly enhanced fMLF-induced superoxide (Fig. 2B, solid line) as compared with vector control (dotted line). The potentiation effect of p40phox was maximal at an input plasmid DNA concentration of 1.5 μg. A slight decline (~10%) from the maximal superoxide production was observed at the input DNA concentration of 2.5 μg (data not shown). The change in superoxide production followed a similar time course in the presence or absence of p40phox, indicating that p40phox

---

**FIGURE 1.** Localization of p40phox-GFP in transfected cells. COSphox cells (A–F) and COS91/22 cells (G–L) were transiently transfected with an expression construct encoding p40phox-GFP. Cells were stimulated for 5 min with vehicle control (A–C and G–I) or PMA (200 ng/ml; D–F and J–L), fixed, and stained with 4',6-diamidino-2-phenylindole for nuclei (blue) and anti-early endosome antigen 1 (red), and examined in confocal microscopy. Several experiments were conducted, and similar results were obtained. Twelve representative confocal images are shown. Scale bar, 20 μm.

**FIGURE 2.** Opposing effects of p40phox and p40R57Q in superoxide production. A, schematic representation of p40phox with the R57Q mutation indicated. COSphox cells were transiently transfected with 0.5 μg each of the expression constructs encoding FPR, Gαq, and protein kinase Cδ along with the wild type p40phox expression vector or the R57Q mutant or an empty vector. Twenty-four hours after transfection, cells were harvested, and the expression level of the wild type and mutant p40phox was determined in Western blot with an anti-p40phox antibody. The transfected cells were stimulated with either 1 μM of fMLF (B) or 200 ng/ml of PMA (C) for the indicated times. Superoxide production was recorded as described under “Experimental Procedures.” Representative traces from three-four experiments are shown. Solid lines, p40phox; dotted lines, vector control; dashed lines, p40R57Q; triangles, with superoxide dismutase (SOD; 250 units) added to the assay buffer.
Regulation of NADPH Oxidase by the PX Domain of p40phox

Increased oxidant production without altering its kinetics. COSphox cells expressing p40phox also produced more superoxide when stimulated with PMA (Fig. 2C, solid line), suggesting that p40phox regulates an NADPH oxidase activation pathway shared by fMLF and PMA. Release of superoxide was not detectable in the presence of superoxide dismutase (SOD) (Fig. 2, B and C, triangles).

Mutation of selected amino acids (Arg-57, Arg-58, and Arg-105) in the PX domain of p40phox abolishes its interaction with PtdIns (3)P (4, 6, 26). We prepared an R57Q mutation (Fig. 2A) and examined its effect in the context of a full-length p40phox. When transfected into COSphox cells, p40R57Q was expressed at a level similar to that of the wild type p40phox (Fig. 2A). However, cells co-transfected with p40R57Q failed to respond to fMLF with superoxide production (Fig. 2B, dashed line). Likewise, expression of p40R57Q markedly reduced the PMA-stimulated oxidant production (Fig. 2C, dashed line).

Given that all COSphox cells in the sample could respond to PMA and only 45–50% of the cells were transfected with the p40R57Q construct, the actual inhibition by p40R57Q in the transfected cells could be greater than shown in Fig. 2C. Mutation of Arg-58 to Gln produced a similar inhibitory effect when expressed in COSphox cells (data not shown). The R57Q mutant was further characterized in subsequent experiments.

The difficulty associated with transfecting suspension cells and the presence of endogenous p40phox in many hematopoietic cell lines prevented us from conducting an extensive investigation of the PX domain in leukocytes. To determine whether the effect produced by p40R57Q is an isolated phenomenon or is applicable to leukocytes, we used nucleofection to deliver the plasmid encoding p40phox or p40R57Q vectors used in each transfection is indicated (in µg). When necessary, empty vector was added to bring the total amount of DNA in transfection equal for all samples. A transfection efficiency of ~50% was obtained in these experiments. Twenty-four hours after transfection, cells were harvested for determination of the protein expression level using Western blotting (A), as described in Fig. 1, and for stimulation with 1 µM of fMLF (B) or 200 ng/ml of PMA (C). Superoxide production was monitored for the indicated period of time, as described under "Experimental Procedures." Representative traces from three independent experiments are shown. Solid lines, p40phox; dotted lines, p40R57Q. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

superoxide production (dashed lines). These changes were observed in both fMLF- and PMA-stimulated cells (Fig. 3, B and C). Because of the low transfection efficiency, actual inhibition in the transfected cells could be greater than what was observed.

We next examined whether p40R57Q acted as a dominant negative mutant. COSphox cells were transfected with a fixed
amount of plasmid DNA coding for the wild type p40phox and variable amounts of plasmid DNA coding for the mutant. The resulting changes in p40phox expression levels were determined with an anti-p40phox antibody (Fig. 4A). When the transfected cells were stimulated with fMLF (Fig. 4B), the R57Q mutant of p40phox could overcome the potentiation effect of the wild type p40phox. At a p40R57Q:p40phox DNA input ratio of 4:1, the mutant brought superoxide production below the level obtained without p40phox. In cells stimulated with PMA, similar changes were observed (Fig. 4C), but the magnitude of potentiation and inhibition was smaller because only half of the cell population was affected by the wild type and mutant constructs due to a ~50% transfection efficiency. These results demonstrate a dominant inhibitory effect of p40R57Q on NADPH oxidase activation.

Expression of p40R57Q Reduces Membrane Translocation of the Cytosolic Factors—A hallmark of NADPH oxidase activation is membrane translocation of the cytosolic factors and their interaction with flavocytochrome b558. We sought to determine whether the R57Q mutant of p40phox could affect this important process of NADPH oxidase activation. At resting state (Fig. 5A), there was minimal membrane association of p40phox and p47phox in vector- or p40phox-transfected COSphox cells, which was consistent with the accepted notion that p40phox is present as a cytosolic protein in unstimulated phagocytes. In COSphox cells expressing p40phox, enhanced membrane translocation of both p40phox (green fluorescence) and p47phox (red fluorescence) was observed upon PMA stimulation (Fig. 5B). In cells transfected to express p40R57Q, PMA-induced membrane translocation of the mutant p40phox was greatly impaired. Reduced membrane translocation of p47phox was also observed in cells expressing p40R57Q. We next examined whether membrane translocation of p67phox was affected by the R57Q mutation. As shown in Fig. 5C (unstimulated cells) and Fig. 5D (PMA-stimulated cells), the PMA-induced p67phox translocation (red fluorescence) was markedly impaired in cells expressing p40R57Q. This result is expected since p40phox is closely associated with p67phox and translocates to plasma membrane in a complex consisting of p67phox and p47phox.

To determine whether inhibition of p47phox and p67phox membrane translocation is a mechanism for the p40R57Q mutant to negatively regulate NADPH oxidase activation, COSphox cells transfected with wild type p40phox or p40R57Q were stimulated with either PMA or vehicle control and subjected to cellular fractionation. The membrane fraction was collected and analyzed by SDS-PAGE and Western blotting with antibodies against p40phox, p47phox, p67phox, and p22phox (a membrane marker for loading control). As shown in Fig. 6A,
PMA potently increased the level of membrane-associated p40phox. In unstimulated cells, a small amount of p47phox and p67phox was found in the membrane fraction. PMA stimulation caused an increase in membrane-associated p47phox and p67phox without p40phox. In cells expressing the wild type p40phox, PMA stimulation resulted in a more potent increase in p47phox and p67phox membrane association. Densitometry analysis of the blots were shown in Fig. 6B (p40phox), Fig. 6C (p47phox), and Fig. 6D (p67phox). Because the PMA-induced changes only occurred in about half of the cell population that were transfected with the p40phox expression plasmids and untransfected cells lacking p40phox could also respond to PMA, the actual effect of p40phox could be greater. Indeed, after normalization against the transfection efficiency, the enhancement effect of p40phox on p47phox membrane translocation became more apparent (Fig. 6C, solid bar in the fourth group). In contrast, expression of p40R57Q reduced membrane association of p47phox. When the proportion of untransfected cells, in which p47phox membrane translocation was not affected by p40R57Q, was taken into consideration, the inhibitory effect of p40R57Q was more prominent (Fig. 6C, solid bar in the last group). A similar effect on p67phox membrane translocation was observed. Whereas p40phox potentiated p67phox membrane translocation, p40R57Q reduced this response to PMA (Fig. 6D). Therefore, results from the biochemical characterization corroborate with data from imaging analysis and together support the conclusion that the R57Q mutation of p40phox has a negative effect on p47phox and p67phox membrane translocation.

**Suppression of Superoxide Production by p40R57Q Requires Its Interaction with p67phox**—Published studies demonstrate that p40phox is tightly associated with p67phox, and p40phox can be co-purified with p67phox in resting neutrophils. In CGD neutrophils lacking p67phox, there is a concomitant reduction in cellular content of p40phox (9, 10, 31, 32). The tight association between p40phox and p67phox involves their PB1 domains, and mutation of selected residues in these domains, such as Asp-289 in p40phox, can abolish this interaction (13). To determine whether the inhibitory effect of p40R57Q involves its interaction with p67phox, we prepared an R57Q/D289A double mutant of p40phox. The double mutant could be readily expressed in COSphox cells, as determined in Western blotting using cell lysate prepared from the transfected cells (Fig. 7A). However, the immunoprecipitated, FLAG-tagged double mutant failed to associate with p67phox or p47phox in co-immunoprecipitation and Western blotting assays (Fig. 7B). Interestingly, the mutation at Asp-289 abolished the dominant inhibitory effect of p40R57Q in fMLF- and PMA-induced superoxide production (Fig. 7, C and D). This result indicates that an interaction between p40phox and p67phox is required for the inhibitory effect of p40R57Q.

**Expression of p40R57Q Increases the Association of the Cytosolic Factors with Actin Cytoskeleton**—In resting cells, the p40phox/p67phox complex was primarily associated with the cytoskeleton, whereas p47phox was found in both the soluble fraction and cytoskeleton fraction (20, 33). Using Western blotting for detection of proteins in the Triton X-100-insoluble fraction, which is enriched with cytoskeletal proteins such as

![FIGURE 6. Western blot analysis of the effects of p40phox and p40R57Q on membrane translocation of p47phox and p67phox. A, COSphox cells were transiently transfected with either empty vector or expression constructs of p40phox or p40R57Q. Twenty-four hours after transfection, cells were collected, and the membrane fractions were prepared as described under “Experimental Procedures.” The relative levels of p40phox, p47phox, and p67phox in the membrane fractions were determined using antibodies against p40phox, p47phox, and p67phox, respectively. An anti-p22phox was used to detect equal loading of membrane proteins. Three independent experiments were conducted, and a representative set of blots is shown. The membrane-associated p40phox (B), p47phox (C), and p67phox (D) was quantified against p22phox based on relative intensity of the Western blot bands in A using Quantity One software (Bio-Rad, Version 4.3.1). In C and D, both raw data (unprocessed, open bars) and normalized data (processed against a 50% transfection efficiency, solid bars) are presented. In PMA-stimulated samples, only ~50% of the cells were transfected and affected by the p40phox or p40R57Q constructs.](image-url)
Regulation of NADPH Oxidase by the PX Domain of p40phox

filamentous actin, we examined the potential effect of p40R57Q on cellular distribution of the cytosolic factors. In unstimulated COSphox cells, expression of wild type p40phox slightly increased the contents of p47phox and p67phox in the Triton X-100-insoluble fraction (Fig. 8). However, in cells expressing p40R57Q, significantly more p67phox and p47phox were recovered in the Triton X-100-insoluble fraction along with the mutant p40phox. This result indicates that p40R57Q can promote cytoskeleton association of the cytosolic factors, thereby altering their cellular distribution profile.

Given that mutation at Asp-289 abolished the dominant inhibitory effect of p40R57Q (Fig. 7), we next examined whether the double mutant was able to retain the cytosolic factors in the Triton X-100-insoluble fraction. As shown in Fig. 8, the D289A mutation caused a marked decrease in the amount of p67phox and p47phox in the Triton X-100-insoluble fraction, whereas it had a smaller effect on the retention of p40phox in the Triton X-100-insoluble fraction. These results are consistent with data from the functional assay shown in Fig. 7 and together demonstrate a correlation between increased actin cytoskeleton association of the cytosolic factors and reduced NADPH oxidase activity in cells expressing the p40R57Q mutant. The above results also indicate that the interaction between p40phox and p67phox is necessary for inhibition of superoxide production as well as increased association of p67phox and p47phox with the actin cytoskeleton in the presence of p40R57Q.

The effect of the R57Q mutation on p40phox association with the actin cytoskeleton was further examined using co-immunoprecipitation of a FLAG-tagged p40phox or a similarly tagged p40phox/D289A double mutant was unable to interact with p67phox or p47phox. The transfected cells were stimulated with either 1 μM fMLF (C) or 200 ng/ml of PMA (D). Superoxide produced in a 20-min time span was recorded as described under “Experimental Procedures.” The integrated chemiluminescence (Int. CL) was shown as % change relative to vector-transfected cells (set as 100%). Data shown in C and D are the mean ± S.E. from three independent experiments.

**DISCUSSION**

In this study we attempted to characterize the PX domain in the full-length p40phox for p40phox intracellular localization, its interaction with the actin cytoskeleton, and its role in superoxide production. Mutations were introduced to abolish PtdIns(3)P binding through the PX domain and to eliminate interac-
Regulation of NADPH Oxidase by the PX Domain of p40phox

Intracellular Localization of Full-length p40phox Differs from That of the Isolated PX Domain—We have shown that a full-length p40phox construct, when expressed in transfected cells, displayed a cytoplasmic localization pattern similar to that of p67phox and p47phox (Fig. 5). Upon agonist stimulation, a portion of the cytoplasmic p40phox moved to plasma membrane (Figs. 1 and 5). This pattern of distribution is drastically different from that of the isolated PX domain, which displayed a punctate, early endosome localization profile (4, 5). PtdIns(3)P, to which the PX domain of p40phox binds, is enriched in early endosome membrane (27). The observed localization in early endosome of transfected cells suggests that the PX domain, when expressed alone, retains an open conformation for access of PtdIns(3)P in the absence of agonist stimulation. The absence of early endosome localization with the full-length p40phox suggests that its PX domain is not accessible to PtdIns(3)P. Therefore, any function of p40phox in resting cells should be independent of PtdIns(3)P binding. A recent study by Ueyama et al. (28), published during the course of this work, provides a mechanism for the lack of PtdIns(3)P binding with the full-length p40phox. Using deletion mutagenesis, the authors found that the C-terminal PB1 domain could fold back to mask the N-terminal PX domain in p40phox (28). Subsequently, a structural analysis of the full-length p40phox confirmed that the intramolecular interaction between the PB1 domain and the PX domain prevents access to membrane-associated PtdIns(3)P by the PX domain (35). Taken together, both imaging data and structural analysis confirm that in unstimulated cells, the PX domain in the full-length p40phox does not interact with PtdIns(3)P in the membrane, suggesting that its functions in resting state and activated state are regulated differently.

Ueyama et al. (28) observed differential targeting of p67phox by p40phox (to early endosome) and by p47phox (to plasma membrane). During FcγR-mediated phagocytosis, transient vesicular accumulation of GFP-p40phox and its fusion with phagosome were observed. Therefore, the early endosome-targeted p67phox, which constitutes a small fraction of the membrane translocated protein, may be available for NADPH oxidase activation. Interestingly, they have shown that arachidonic acid, but not PMA or fMLF, was able to alter the structure of full-length p40phox, allowing access of the PX domain to membrane-associated PtdIns(3)P (28). A similar finding was made in this study, in which we observed that PMA was unable to induce membrane translocation of p40phox in the absence of p47phox and p67phox (Fig. 1). The mechanism by which arachidonic acid regulates structural changes of p40phox is still undefined. Phosphorylation of p40phox at Thr-154 and Ser-315 has been reported and was thought to be a regulatory mechanism for induced structural changes of p40phox (36, 37). Lopes et al.
Regulation of NADPH Oxidase by the PX Domain of p40\textsuperscript{phox}

(38) previously reported that phosphorylation of p40\textsuperscript{phox} at Thr-154 could cause inhibition of NADPH oxidase activity in cell-free assays. However, no functional changes were observed when Thr-154 and Ser-315 were mutated to Ala in the study conducted by Ueyama et al. (28) in transfected cells. Further study will be necessary to determine how a closed conformation of p40\textsuperscript{phox} is transformed during NADPH oxidase activation.

Increased Association with the Actin Cytoskeleton Is a Possible Mechanism for Inhibition of NADPH Oxidase through p40R57Q—The potent inhibition of NADPH oxidase brought upon by the R57Q mutation was unexpected. Because p40\textsuperscript{phox} is not essential for PMA- and fMLF-induced superoxide production in reconstituted COS\textsuperscript{phox} cells (22, 23), it would not be surprising if the mutation simply eliminated the potentiation effect through a change in the PtdIns (3)P binding site. Our attention was directed to the actin cytoskeleton as previous studies have shown that p40\textsuperscript{phox}, like p67\textsuperscript{phox} and p47\textsuperscript{phox}, interacts with the actin cytoskeleton although the biological consequence of this interaction was not entirely clear. Earlier studies have shown that functional cytosolic factors are found in the Triton X-100-insoluble fraction (33, 39), suggesting a potential role of the actin cytoskeleton in the organization and redistribution of the cytosolic complex of phagocyte NADPH oxidase. Other studies have shown association of p40\textsuperscript{phox} along with p67\textsuperscript{phox} and p47\textsuperscript{phox} with the actin cytoskeleton and actin-binding proteins in unstimulated neutrophils, implying that such an association might stabilize the cytosolic factors in the resting state (20, 21, 40). Of interest is the finding that p40\textsuperscript{phox} interacts with moesin, an actin-binding protein and a member of the ezrin-radixin-moesin family, through its PX domain (21). The mutation at Arg-57 may alter the structure of the PX domain so that not only PtdIns (3)P binding is abolished but also the affinity for moesin is changed. Indeed, we observed increased association of p40R57Q with actin and moesin in co-immunoprecipitation assay as well as an enrichment of the mutant in the Triton X-100-insoluble fraction, which contains abundant polymerized actin cytoskeleton. These findings suggest the possibility that an aberrant p40\textsuperscript{phox} protein “entraps” the cytosolic complex through an enhanced interaction with the actin cytoskeleton, thereby preventing its membrane translocation and interaction with flavocytochrome b\textsubscript{558}.

Arg-57 and Arg-58 are highly conserved among the PX domains identified so far (1, 41). In p47\textsuperscript{phox}, the analogous residue of Arg-57 is Arg-42, which when mutated to a Gln results...
Regulation of NADPH Oxidase by the PX Domain of p40phox

in an autosomal recessive CGD (42). Studies of the isolated PX domain of p40phox have shown that mutations of Arg-57 (4), Arg-58 (5, 7, 8), and Arg-105 (6) abolish the interaction of the PX domain with PtdIns (3)P. However, the absence of PtdIns (3)P binding cannot explain why replacement of the mouse p40phox gene with a R58A mutant gene leads to embryonic lethality in homozygous offspring (18). Another mechanism, such as dominant inhibition of superoxide production, may be responsible for the deleterious effect in consideration of the important roles that reactive oxygen species play in organ development (43, 44). There has been no reported clinical case of CGD that results from mutation in the p40phox gene. The possibility that natural mutations at these sites lead to embryonic lethality due to defective superoxide production merits further investigation.

The Interaction between p40phox and p67phox Is Essential for Both Potentiation and Inhibitory Effects of p40phox in Superoxide Production—We showed that the R57Q mutation abolished the potentiation effect of p40phox on superoxide production as well as induced membrane translocation. This mutation, however, did not affect the interaction of p40phox with p67phox, which is mediated through a C-terminal PB1 interaction with the corresponding PB1 domain in p67phox. Therefore, although the R57Q mutation can possibly destabilize the PX domain with respect to PtdIns (3)P binding (8), this point mutation does not seem to alter the global structure of p40phox so as to weaken its interaction with p67phox. This property of p40R57Q must be considered when evaluating its functional impact on NADPH oxidase activity. Indeed, p40phox was originally identified as a cytosolic protein tightly associated with p67phox (9–11). As we have shown above (Fig. 7), when this association was disrupted by a second mutation at Asp-289, the resulting p40phox double mutant could no longer inhibit NADPH oxidase in superoxide production assays. Consistent with the functional change, dissociation of p67phox from p40R57Q due to the D289A mutation caused a significant reduction in the amount of cytoskeleton-associated p67phox and p47phox found in the Triton X-100-insoluble fraction. Kuribayashi et al. (13) previously reported that the D289A mutation could abolish the potentiation effect of the wild type p40phox, a finding confirmed in our study of PPR-reconstituted COSphox cells (data not shown). Taken together, these experimental results support the notion that both the potentiation effect and the inhibitory effect of p40phox require its association with p67phox.

We have shown that, in the resting state, p40phox as well as p40R57Q could be co-immunoprecipitated with p67phox and p47phox (Fig. 7). Upon agonist stimulation, p40phox translocated to plasma membrane rather than early endosome (Fig. 1) and colocalized with p47phox and p67phox (Figs. 5 and 6). The absence of early endosome localization could indicate that the PX domain of p40phox remains “closed” under the experimental conditions used in this study or the p47phox-directed membrane translocation of the cytosolic complex is predominant. Interaction between p40phox and p47phox may be secondary to the p40phox-p67phox interaction, as reported previously (13, 31, 32) and confirmed in this study (Fig. 7). Our findings are consistent with one of the original observations that p40phox remains associated with p67phox and p47phox in activated neutrophils (9) and together suggest that p40phox facilitates membrane targeting of p67phox. The tight association between p40phox and p67phox apparently helps to stabilize their structures, as CGD patients with diminished p67phox expression also display reduced p40phox expression (9, 11, 15). Because of the association between these two cytosolic factors, it is possible that structural changes induced by the R57Q mutation could affect the structure and function of p67phox and even p47phox, thereby prohibiting their membrane translocation. In this regard it is notable that published reports showed that p40phox dissociates from p67phox in activated membranes (20). Another published study suggested that p40phox stabilizes the resting state and should be dissociated from p67phox for maximal oxidase activity (15).

In summary, the current study examines a PX domain mutation in the context of a full-length p40phox and found that alteration of the PtdIns (3)P binding site may produce drastic changes in the way p40phox regulates NADPH oxidase. Our observations suggest that, in addition to the potentiation effect through PtdIns (3)P binding, which has been confirmed both in cultured cells and in primary neutrophils (16–18), the PX domain of p40phox may negatively regulate NADPH oxidase through stabilization of the resting state. An increased association with the actin cytoskeleton combined with a possibly direct effect on p67phox structure may contribute to the observed inhibitory effect. So far, most studies on NADPH oxidase have been focused on the activation mechanism. An understanding of how NADPH oxidase is negatively regulated may help to expand our knowledge with potential applications to the control of oxidant production.

Acknowledgments—We thank members of our laboratories for helpful discussions.

REFERENCES—We thank members of our laboratories for helpful discussions.

1. Ponting, C. P. (1996) Protein Sci. 5, 2353–2357
2. Sato, T. K., Overduin, M., and Emr, S. D. (2001) Science 294, 1881–1885
3. Xu, Y., Seet, L. F., Hanson, B., and Hong, W. (2001) Biochem. J. 360, 513–530
4. Kanai, F., Liu, H., Field, S. J., Akbary, H., Matsu, T., Brown, G. E., Cantley, L. C., and Yaffe, M. B. (2001) Nat. Cell Biol. 3, 675–678
5. Ellison, C. D., Gobert-Gosse, S., Anderson, K. E., Davidson, K., Erdjument-Bromage, H., Tempst, P., Thuring, J. W., Cooper, M. A., Lim, Z. Y., Holmes, A. B., Gaffney, P. R., Coadwell, J., Chilvers, E. R., Hawkins, P. T., and Stephens, L. R. (2001) Nat. Cell Biol. 3, 679–682
6. Ago, T., Takeya, R., Hiroaki, H., Kuribayashi, F., Ito, T., Kohda, D., and Sumimoto, H. (2001) Biochem. Biophys. Res. Commun. 287, 733–738
7. Stahelin, R. V., Burian, A., Brazik, K. S., Murray, D., and Cho, W. (2003) J. Biol. Chem. 278, 14469–14479
8. Bravo, J., Karathanassis, D., Pacold, C. M., Pacold, M. E., Ellison, C. D., Anderson, K. E., Butler, P. J., Lavenir, I., Perisic, O., Hawkins, P. T., Stephens, L., and Williams, R. L. (2001) Mol. Cell 8, 829–839
9. Wiintjes, F. B., Hsuan, J. J., Totty, N. F., and Segal, A. W. (1993) Biochem. J. 296, 557–561
10. Someya, A., Nagaoa, I., and Yamashita, T. (1993) FEBs Lett. 330, 215–218
11. Tsunawaki, S., Mizunari, H., Nagata, M., Tatsuzawa, O., and Kuratsuji, T. (1994) Biochem. Biophys. Res. Commun. 199, 1378–1387
12. Cross, A. R. (2000) Biochem. J. 349, 113–117
13. Kuribayashi, F., Nuno, H., Nakamura, K., Tsunawaki, S., Sato, K., Ito, T., and Sumimoto, H. (2002) EMBO J. 21, 6312–6320
Regulation of NADPH Oxidase by the PX Domain of p40phox

14. Sathyamoorthy, M., de Mendez, I., Adams, A. G., and Leto, T. L. (1997) J. Biol. Chem. 272, 9141–9146
15. Vergnaud, S., Paclet, M. H., El Benna, J., Pocidalo, M. A., and Morel, F. (2000) Eur. J. Biochem. 267, 1059–1067
16. Suh, C. I., Stull, N. D., Li, X. J., Tian, W., Price, M. O., Grinstein, S., Yaffe, M. B., Atkinson, S., and Dinauer, M. C. (2006) J. Exp. Med. 203, 1915–1925
17. Ellson, C. D., Davidson, K., Ferguson, G. J., O’Connor, R., Stephens, L. R., and Hawkins, P. T. (2006) J. Exp. Med. 203, 1927–1937
18. Ellson, C., Davidson, K., Anderson, K., Stephens, L. R., and Hawkins, P. T. (2006) EMBO J. 25, 4468–4478
19. Brown, G. E., Stewart, M. Q., Liu, H., Ha, V. L., and Yaffe, M. B. (2003) Mol. Cell 11, 35–47
20. El Benna, J., Dang, P. M., Andrieu, V., Vergnaud, S., Dewas, C., Cachia, O., Fay, M., Morel, F., Chollet-Martin, S., Hakim, J., and Gougerot-Pocidalo, M. A. (1999) J. Leukocyte Biol. 66, 1014–1020
21. Wientjes, F. B., Reeves, E. P., Soskic, V., Furthmayr, H., and Segal, A. W. (2001) Biochem. Biophys. Res. Commun. 289, 382–388
22. Price, M. O., McPhail, L. C., Lambeth, J. D., Han, C. H., Knaus, U. G., and Dinauer, M. C. (2002) Blood 99, 2653–2661
23. He, R., Nanamori, M., Sang, H., Yin, H., Dinauer, M. C., and Ye, R. D. (2004) J. Immunol. 173, 7462–7470
24. Dahlgren, C., and Karlsson, A. (1999) J. Immunol. Methods 232, 3–14
25. Zhan, Y., He, D., Newburger, P. E., and Zhou, G. W. (2004) J. Cell. Biochem. 92, 795–809
26. Ellson, C. D., Andrews, S., Stephens, L. R., and Hawkins, P. T. (2002) J. Cell Sci. 115, 1099–1105
27. Ellson, C. D., Anderson, K. E., Morgan, G., Chilvers, E. R., Lipp, P., Stephens, L. R., and Hawkins, P. T. (2001) Curr. Biol. 11, 1631–1635
28. Ueyama, T., Tatsuno, T., Kawasaki, T., Tsujibe, S., Shirai, Y., Sumimoto, H., Leto, T. L., and Saito, N. (2007) Mol. Biol. Cell 18, 441–454
29. Tucker, K. A., Lilly, M. B., Heck, L., Jr., and Rado, T. A. (1987) Blood 70, 372–378
30. Zhen, L., King, A. A., Xiao, Y., Chanock, S. J., Orkin, S. H., and Dinauer, M. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9832–9836
31. Tsunawaki, S., Kagara, S., Yoshikawa, K., Yoshida, L. S., Kuratsuji, T., and Namiki, H. (1996) J. Exp. Med. 184, 893–902
32. Lopack, K., Smith, S. J., Groemping, Y., and Rittinger, K. (2002) J. Biol. Chem. 277, 10121–10128
33. Woodman, R. C., Ruedi, J. M., Jesaitis, A. J., Okamura, N., Quinn, M. T., Smith, R. M., Curnutte, J. T., and Babior, B. M. (1991) J. Clin. Investig. 87, 1345–1351
34. Price, M. O., Atkinson, S. J., Knaus, U. G., and Dinauer, M. C. (2002) J. Biol. Chem. 277, 19220–19228
35. Hou, K., Minakami, R., Yuzawa, S., Takeya, R., Suzuki, N. N., Kamakura, S., Hideki, S., and Inagaki, F. (2007) EMBO J. 26, 1176–1186
36. Bouin, A. P., Grandvaux, N., Vignais, P. V., and Fuchs, A. (1998) J. Biol. Chem. 273, 30097–30103
37. Someya, A., Nuno, H., Hasebe, T., and Nagaoka, I. (1999) J. Leukocyte Biol. 66, 851–857
38. Lopes, L. R., Dagher, M. C., Gutierrez, A., Young, B., Bouin, A. P., Fuchs, A., and Babior, B. M. (2004) Biochemistry 43, 3723–3730
39. Nauseef, W. M., Volpp, B. D., McCormick, S., Leidal, K. G., and Clark, R. A. (1991) J. Biol. Chem. 266, 5911–5917
40. Grovan, A., Reeves, E., Keep, N., Winter, F., Totty, N. F., Burlingame, A. L., Hsu, J. I., and Segal, A. W. (1997) J. Cell Sci. 110, 3071–3081
41. Hiroaki, H., Ago, T., Ito, T., Sumimoto, H., and Kohda, D. (2001) Nat. Struct. Biol. 8, 526–530
42. Noack, D., Rae, J., Cross, A. R., Ellis, B. A., Newburger, P. E., Curnutte, J. T., and Heyworth, P. G. (2001) Blood 97, 305–311
43. Bedard, K., and Krause, K. H. (2007) Physiol. Rev. 87, 245–313
44. Ushio-Fukai, M. (2007) Antioxid. Redox Signal. 9, 731–739