CHARACTERIZATION OF A MUTATION IN THE PX DOMAIN OF THE NADPH OXIDASE COMPONENT \( p40^{\text{PHOX}} \) IDENTIFIES A MECHANISM FOR NEGATIVE REGULATION OF SUPEROXIDE PRODUCTION *

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Running title: Regulation of NADPH oxidase by the PX domain of \( p40^{\text{PHOX}} \)

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The phagocyte oxidase (phox) protein \( p40^{\text{phox}} \) contains a phox homology (PX) domain which, when expressed alone, interacts with phosphatidylinostol 3-phosphate (PtdIns(3)P). The functions of the PX domain in \( p40^{\text{phox}} \) localization, association with the cytoskeleton and superoxide production were examined in transgenic COS-7 cells expressing gp91phox, p22\(^{\text{phox}}\), p67\(^{\text{phox}}\) and p47\(^{\text{phox}}\) (COS\(^{\text{phox}}\) cells). Full-length \( p40^{\text{phox}} \) exhibited a cytoplasmic localization pattern in resting cells. Upon stimulation with PMA or fMet-Leu-Phe, \( p40^{\text{phox}} \) translocated to plasma membrane in a p67\(^{\text{phox}}\)- and p47\(^{\text{phox}}\)-dependent manner. Heterologous expression of \( p40^{\text{phox}} \) markedly enhanced superoxide production in PMA- and fMet-Leu-Phe-stimulated COS\(^{\text{phox}}\) cells. Unexpectedly, mutation of Arg57 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 p67\(^{\text{phox}}\) and p47\(^{\text{phox}}\). The mutant \( p40^{\text{phox}} \) (\( p40^{R57Q} \)) displayed increased association with actin and moesin, and was found enriched in the Triton X-100 insoluble fraction along with p67\(^{\text{phox}}\) and p47\(^{\text{phox}}\). The enhanced cytoskeleton association of p67\(^{\text{phox}}\) and p47\(^{\text{phox}}\) and the dominant inhibitory effect produced by the p40R57Q were alleviated when a second mutation at Asp289, which eliminated \( p40^{\text{phox}} \) interaction with p67\(^{\text{phox}}\), 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 \( p40^{\text{phox}} \): 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 \( p40^{\text{phox}} \) with p67\(^{\text{phox}}\).

Phox homology (PX)\(^4\) 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\(^{\text{phox}}\) and p40\(^{\text{phox}}\) (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 \( p40^{\text{phox}} \) binds phosphatidylinostol 3-phosphate (PtdIns(3)P), and the PX domain in p47\(^{\text{phox}}\) 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 GFP-fused PX domain of \( p40^{\text{phox}} \), membrane localization was observed in a PI3K-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 \( p40^{\text{phox}} \) reveals a positively charged binding pocket for the negatively charged PtdIns(3)P. Binding of \( p40^{\text{phox}} \) to the phosphoinositide requires three conserved arginine residues (Arg57, Arg58 and Arg105) 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).

Since its initial identification as a p67\(^{\text{phox}}\)-associated protein (9-11), \( p40^{\text{phox}} \) has been studied...
for its function in NADPH oxidase activation with different and sometimes conflicting results. Evidence supporting a positive regulatory role of p40phox came from studies using both cell-free reconstitution and whole-cell assays. The possible mechanisms for p40phox mediated potentiation of NADPH oxidase include increasing the affinity of p47phox for flavocytochrome b558 (12), binding to membrane-associated PtdIns(3)P through its PX domain (5), and cooperation with p67phox for membrane translocation of the cytosolic complex (13). Other investigators, using essentially the same cells and cell-free reconstitution assays, found p40phox to be a negative regulator for NADPH oxidase. The negative regulatory mechanisms include SH3 domain-mediated interference of p40phox association with other cytosolic factors (14), and inhibition of p67phox membrane translocation (15). More recent studies have examined the roles of p40phox in NADPH oxidase activation using transfected cells and mouse models. Suh et al reported that p40phox is required for FcγR receptor-mediated superoxide generation following phagocytosis, a function that was lost when critical residues for PtdIns(3)P binding were mutated (16). Ellson et al found that neutrophils from p40phox knockout mice displayed defective oxidant production in response to several types of stimuli (17). Moreover, replacement of the mouse p40phox gene with one that contains a Arg58 to Ala mutation caused embryonic lethality in homozygous offspring, with the heterozygous mice displaying compromised ability to kill S. aureus (18). These findings demonstrate a physiological function of p40phox in regulating NADPH oxidase activation that involves its PX domain.

Despite recent progress in p40phox research, the function of p40phox in resting cells remains undefined. p40phox was originally discovered as a p67phox-associated protein (9-11). In unprimed neutrophils, p40phox forms a complex with p67phox, whereas p47phox was not a part of the complex (19). Neutrophils from chronic granulomatous disease (CGD) patients who lack p67phox contain very little p40phox (11,15), suggesting that interaction between the two cytosolic factors helps to stabilize their structures. Moreover, p40phox, 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 p40phox (21). Based on these findings, we speculate that the PX domain in p40phox 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 p40phox and a PX domain mutant on NADPH oxidase activity. We observed that expression of the wild type p40phox could enhance superoxide generation in response to both PMA and fMet-Leu-Phe (fMLF), a finding consistent with recent publications suggesting that p40phox enhances NADPH oxidase activation (16-18). Surprisingly, an Arg to Gln mutation at position 57 (R57Q), which abolishes p40phox interaction with PtdIns(3)P through its PX domain (4), switched p40phox 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 to the wild type p40phox. In cells expressing p40R57Q, more cytosolic factors were targeted to the Triton X-100 insoluble fraction than in cells expressing the wild type p40phox. 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 p40phox with p67phox was eliminated. These intriguing findings suggest that p40phox can positively and negatively regulate NADPH oxidase through its PX domain interaction with PtdIns(3)P and with the actin cytoskeleton.

MATERIALS AND METHODS

Materials: PMA, fMLF, isoluminol, cytochalasin B, anti-moesin and anti-FLAG monoclonal antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase (HRP) was obtained from Roche (Indianapolis, IN). The anti-p67phox (against amino acids 317-469) and EEA-1 monoclonal antibodies were purchased from BD Transduction Laboratories (Lexington, KY). Anti-actin and anti-p22phox polyclonal antibodies (against amino acids 1-195) were acquired from Santa Cruz.
Biotechnology (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 GST-fused full-length human p47<sub>phox</sub>) polyclonal antibodies were obtained from Upstate (Lake Placid, NY).

**Plasmid constructs:** Preparation and characterization of the expression constructs of formyl peptide receptor (FPR), PKCδ 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 QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The following oligonucleotide primers were used for the PCR-based mutagenesis: 5'-GTACCTCATCTACCAACGCTACCGCCAGTT-3' and its reverse and complementary sequence for the R57Q mutant, and 5'-CTGAATTACCGGGCCGCTGAGGGGGATC-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>phox</sup> and COS<sup>91/22</sup> cells were generated as described previously (22). COS<sup>91/22</sup> expresses gp91<sup>phox</sup> and p22<sup>phox</sup>. Subsequent transfection resulted in COS<sup>phox</sup>, which expresses p67<sup>phox</sup> and p47<sup>phox</sup> in addition to gp91<sup>phox</sup> and p22<sup>phox</sup> (22). The stable cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS 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 DNA was used in each transfection. Transient transfection efficiency of 45-50% was routinely obtained based on the expression of a co-transfected GFP construct using flow cytometry.

The human myeloid leukemia cell line PLB-985 was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 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>5</sup>/ml and 1 × 10<sup>6</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 DNA, using Program C-023. Transfection efficiency was approximately 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 COS<sup>phox</sup> and PBL-985 cells was determined using an isoluminol-enhanced chemiluminescence assay, as previously described (23,24). Oxidant production was inhibited by superoxide dismutase (SOD, 250 U) as reported previously (23). The assay buffer contained HRP (see below) to offset the possible effect of myeloperoxidase. Briefly, COS<sup>phox</sup> cells were harvested with enzyme-free cell-dissociation buffer (Invitrogen). Both COS<sup>phox</sup> and PLB-985 cells were collected by centrifugation and resuspended in RPMI 1640 containing 0.5% BSA at 1–3 × 10<sup>6</sup> cells/ml. Cells were incubated in the dark with 100 µM isoluminol and 40 U/ml HRP 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, Boston, MA). The CL counts per second (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, Keene, NH). 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 µg/ml to 1 µg/ml) overnight at 4°C. Anti-rabbit (Bio-Rad, Hercules, CA) or anti-mouse (Calbiochem) peroxidase-conjugated secondary antibodies were added to the membranes at a dilution ratio of...
1:3,000 and incubation was continued to for 1 h at RT. The protein bands on the membrane were visualized by chemiluminescence (Pierce, Rockford, IL).

**Immunoprecipitation:** Twenty-four hours after transfection, the 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 (PMSF), and 1× protease inhibitor cocktail set I (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% NP-40, 2 mM EDTA, 1 mM PMSF, and 1× protease inhibitor cocktail set I was used instead. The cell lysates were cleared of debris by centrifugation at 14,000 × g for 10 min at 4°C. Protein content in the cell lysate was measured using a DC Protein Assay (BioRad) 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 PMSF) and then once in PBS. The beads were resuspended in 50 µl 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 × 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, COS phox or COS 91/22 cells were stimulated with or without agonists. Cells (1 × 10⁷ / sample) were lysed with ice-cold Hypotonic Buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1 mM PMSF and 1:50 dilution of Protease inhibitor cocktail 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, 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 DMEM medium supplemented with 10% heat-inactivated FBS. 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% BSA in PBS for 1 h at RT. Cells were washed 3 times in PBS and incubated with the primary antibodies in PBS containing 5% BSA 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 DAPI (Molecular Probes). Fluorescence images were captured with a Zeiss LSM 510 confocal microscope equipped with helium-neon, argon and krypton laser sources.

**Statistic 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 green fluorescence protein (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 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 (Figure 1, A-F) revealed cytoplasmic localization of the GFP fluorescence in resting state (Figure 1, A & 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 (1B). There were very few punctate structures with both green (p40phox-GFP) and red (anti-EEA-1) fluorescence (1C). Upon stimulation with PMA (1D-1F) or fMLF (data not shown), there was a marked increase in plasma membrane-associated green fluorescence (1D, 1F). PMA stimulation did not increase or decrease double-stained fluorescence in the periphery of the cells (1F), indicating absence of fusion between early endosome and the plasma membrane. To determine whether agonist-induced membrane translocation of p40phox requires p67phox and p47phox, p40phox-GFP was expressed in COS91/22 cells, a stable cell line of COS-7 expressing gp91phox, p22phox, p47phox and p67phox (22). As shown in Figure 1 (1G-1L), the GFP fluorescence remained cytoplasmic in resting state (1G, 1I) as well as following PMA stimulation (1J, 1L). This result suggests that p40phox membrane translocation requires the presence of 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 showed that, in the RAW267.4 macrophage cell line which contains very low level of endogenous p67phox, PMA and fMLF was unable to induce membrane translocation of p40phox (28). 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 formyl peptide receptor (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 (Figure 2B, solid line) as compared to 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 increased oxidant production without altering its kinetics. COSphox cells expressing p40phox also produced more superoxide when stimulated with PMA (Figure 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) (Figure 2B and 2C, gray line).

Mutation of selected amino acids (Arg57, Arg58, and Arg105) in the PX domain of p40phox abolishes its interaction with PtdIns(3)P (4,6,8,26). We prepared an R57Q mutation (Figure 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 (Figure 2A). However, cells co-transfected with p40R57Q failed to respond to fMLF with superoxide production (Figure 2B, dashed line). Likewise, expression of p40R57Q markedly reduced the PMA-stimulated oxidant production (Figure 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 Figure 2C. Mutation of Arg58 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 only seen in COS-phox cells or is applicable to leukocytes, we used nucleofection to deliver the plasmid encoding p40-phox or p40R57Q into the PLB-985 myelomonoblastic leukemia cell line (29,30). As shown in Figure 3A, a low level of endogenous p40-phox was detected in undifferentiated PLB-985 cells. An increase in p40-phox expression was evident after nucleofection, with up to 30% of the cells receiving the plasmid of interest based on flow cytometry analysis (data not shown). As expected, exogenous expression of p40-phox in the PLB-985 cells caused an increase in superoxide production (Figure 3B and 3C, solid lines), whereas expression of p40R57Q led to a decrease in superoxide production (dashed lines). These changes were observed in both fMLF- and PMA-stimulated cells (Figure 3B and 3C). Because of the low transfection efficiency, actual inhibition in the transfected cells could be greater that what was observed.

We next examined whether p40R57Q acted as a dominant negative mutant. COS-phox cells were transfected with a fixed amount of plasmid DNA coding for the wild type p40-phox, and variable amounts of plasmid DNA coding for the mutant. The resulting changes in p40-phox expression levels were determined with an anti-p40-phox antibody (Figure 4A). When the transfected cells were stimulated with fMLF (Figure 4B), the R57Q mutant of p40-phox could overcome the potentiation effect of the wild type p40-phox. At a p40R57Q: p40-phox DNA input ratio of 4:1, the mutant brought superoxide production below the level obtained without p40-phox. In cells stimulated with PMA, similar changes were observed (Figure 4C), but the magnitude of potentiation and inhibition was smaller because only a 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 b555. We sought to determine whether the R57Q mutant of p40-phox could affect this important process of NADPH oxidase activation. At resting state (Figure 5A), there was minimal membrane association of p40-phox and p47-phox in vector- or p40-phox-transfected COS-phox cells, which was consistent with the accepted notion that p40-phox is present as a cytosolic protein in unstimulated phagocytes. In COS-phox cells expressing p40-phox, enhanced membrane translocation of both p40-phox (green fluorescence) and p47-phox (red fluorescence) was observed upon PMA stimulation (Figure 5B). In cells transfected to express p40R57Q, PMA-induced membrane translocation of the mutant p40-phox was greatly impaired. Reduced membrane translocation of p47-phox was also observed in cells expressing p40R57Q. We next examined whether membrane translocation of p67-phox was affected by the R57Q mutation. As shown in Figure 5C (unstimulated cells) and 5D (PMA-stimulated cells), the PMA-induced p67-phox translocation (red fluorescence) was markedly impaired in cells expressing p40R57Q. This result is expected since p40-phox is closely associated with p67-phox and translocates to plasma membrane in a complex consisting of p67-phox and p47-phox.

To determine whether inhibition of p47-phox and p67-phox membrane translocation is a mechanism for the p40R57Q mutant to negatively regulate NADPH oxidase activation, COS-phox cells transfected with wild type p40-phox 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 p40-phox, p47-phox, p67-phox and p22-phox (a membrane marker for loading control). As shown in Figure 6A, PMA potently increased the level of membrane-associated p40-phox. In unstimulated cells, a small amount of p47-phox and p67-phox was found in the membrane fraction. PMA stimulation caused an increase in membrane-associated p47-phox and p67-phox without p40-phox. In cells expressing the wild type p40-phox, PMA stimulation resulted in a more potent increase in p47-phox and p67-phox membrane association. Densitometry analysis of the blots were shown in Figure 6B (p40-phox), 6C (p47-phox), and 6D (p67-phox). Since the PMA-induced changes only occurred in about half of the cell population that were transfected with the p40-phox expression plasmids and untransfected cells lacking p40-phox could also respond to PMA, the actual effect of p40-phox could be greater. Indeed, after normalization against the transfection efficiency, the enhancement effect of p40-phox on p47-phox

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membrane translocation became more apparent (Figure 6C, solid bar in 4th 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 (Figure 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 (Figure 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 p40 phox 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 Asp289 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 (Figure 7A). However, the immunoprecipitated, FLAG-tagged double mutant failed to associate with p67phox or p47phox in co-immunoprecipitation and Western blotting assays (Figure 7B). Interestingly, the mutation at Asp289 abolished the dominant inhibitory effect of p40R57Q in fMLF- and PMA-induced superoxide production (Figure 7C and 7D, respectively). 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 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 (Figure 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 Asp289 abolished the dominant inhibitory effect of p40R57Q (Figure7), we next examined whether the double mutant was able to retain the cytosolic factors in the Triton X-100 insoluble fraction. As shown in Figure 8, the D289A mutation caused a marked decrease in the amount of p67phox and p47phox in the Triton X-100 insoluble fraction, while having 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 Figure 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 p4057Q.

The effect of the R57Q mutation on p40phox association with the actin cytoskeleton was further examined using co-IP of a FLAG-tagged p40phox or a similarly tagged R57Q mutant and Western blotting detection of actin. As shown in Figure 9A, significantly more actin was found associated with p40R57Q than with p40phox in COS9phox cells (left panel). The same experiment was repeated in COS9p91/p22 cells, which lack p67phox and p47phox (Figure 9B). In the absence of these cytosolic factors, there was a decrease in the overall association of both p40phox and its R57Q mutant.
with actin. Still, relatively more actin association was detected with the p40R57Q mutant than with p40phox. This result corroborates with the findings in the Triton X-100 insoluble fraction. Likewise, we found in the transfected COSphox cells an increased association of p40R57Q with moesin, an actin-binding protein that serves to bridge the interaction between actin and many proteins (Figure 9C). When expressed in COS91/22 cells, the R57Q mutant still associated more strongly with moesin than the wild type p40phox did, although there was a decrease in the overall level of association (Figure 9D). Taken together, these results suggest a shift to increased actin cytoskeleton association when a PtdIns(3)P binding site in p40 phox was mutated. It is also evident that both p67 phox and p47 phox contribute to cytoskeleton association, as previously reported (20,33).

Cytochalasin B eliminates the dominant inhibitory effect of p40R57Q on superoxide production. Cytochalasin B is a fungal metabolite that binds actin filaments and prevents actin polymerization. It is widely used in phagocyte studies due to its potentiation effect on superoxide production and degranulation. How cytochalasin B enhances oxidant production remains incompletely understood. We treated the transfected COSphox cells with cytochalasin B (5 μg/ml, 10 min) or with vehicle control prior to PMA stimulation. As shown in Figure 10, cytochalasin B increased the relative level of superoxide production by approximately 3 to 4-fold. More interestingly, the dominant inhibitory effect produced by p40R57Q was completely abolished in the presence of cytochalasin B. This result further support the notion that increased association of p40R57Q with the actin cytoskeleton is responsible for its dominant inhibitory effect.

**DISCUSSION**

In this study, we attempted to characterize the PX domain in the full-length p40 phox for p40 phox 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 interaction with p67 phox through the PB1 motif in the C-terminus of p40 phox. We took advantage of the COSphox cell line, which exhibits many properties similar to those of neutrophils in terms of NADPH oxidase activation, including responsiveness to PMA with SOD-inhibitable superoxide production, membrane translocation of the cytosolic factors, and requirement for the Rac small GTPase (22,23,34). The absence of endogenous p40phox allowed us to examine the effects of the p40phox mutations on NADPH oxidase activity with relative ease. We observed an unexpected and remarkable change caused by a point mutation in the PX domain of p40phox. The R57Q mutation not only abolished the potentiation effect of the wild type p40phox on NADPH oxidase activity, but also strongly suppressed superoxide production in fMLF and PMA-stimulated cells. Our experimental data suggest that a mechanism for the observed inhibition is increased association of the cytosolic factors with the actin cytoskeleton, which is mediated by the R57Q mutant of p40phox. This study also identifies the association between p40phox and p67phox as being important for the dominant inhibitory effect of the R57Q mutation. These results, when considered together with recent observations made by other investigators, suggest a dual regulatory function of p40phox in NADPH oxidase activation.

Intracellular localization of full-length p40 phox 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 p67 phox and p47 phox (Figure 5). Upon agonist stimulation, a portion of the cytoplasmic p40phox moved to plasma membrane (Figures 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 and colleagues, published during the course of this work, provides a mechanism for the
lack of PtdIns(3)P binding with the full-length p40\textsuperscript{phox} (28). Using deletion mutagenesis, the authors found that the C-terminal PB1 domain could fold back to mask the N-terminal PX domain in p40\textsuperscript{phox} (28). Subsequently, a structural analysis of the full-length p40\textsuperscript{phox} 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 and colleagues observed differential targeting of p67\textsuperscript{phox} by p40\textsuperscript{phox} (to early endosome) and by p47\textsuperscript{phox} (to plasma membrane). During Fc\textgamma R mediated phagocytosis, transient vesicular accumulation of GFP-p40\textsuperscript{phox} and its fusion with phagosome was observed. Therefore, the early endosome-targeted p67\textsuperscript{phox}, 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 p40\textsuperscript{phox}, 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 p40\textsuperscript{phox} in the absence of p47\textsuperscript{phox}, 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 p40\textsuperscript{phox} in the absence of p47\textsuperscript{phox}, 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 p40\textsuperscript{phox} in the absence of p47\textsuperscript{phox}, allowing access of the PX domain to membrane-associated PtdIns(3)P (28).

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. Since 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 resting state (20,21,40). Of interest is the findings 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 Arg57 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 “entrap” the cytosolic complex through an enhanced interaction with the actin cytoskeleton, thereby preventing its membrane translocation and interaction with flavocytochrome b558.

Arg57 and Arg58 are highly conserved among the PX domains identified so far (1,41). In p47\textsuperscript{phox}, the analogous residue of Arg57 is Arg42 which, when mutated to a Gln, results in an autosomal recessive CGD (42). Studies of the isolated PX domain of p40\textsuperscript{phox} have shown that mutations of Arg57 (4), Arg58 (5,7,8) and Arg105 (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 p40\textsubscript{phox} 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 p40\textsubscript{phox} gene. The possibility that natural mutations at these sites lead to embryonic lethality due to defective superoxide production merits further investigation.

The interaction between p40\textsubscript{phox} and p67\textsubscript{phox} is essential for both potentiation and inhibitory effects of p40\textsubscript{phox} in superoxide production. We showed that the R57Q mutation abolished the potentiation effect of p40\textsubscript{phox} on superoxide production as well as induced membrane translocation. This mutation, however, did not affect the interaction of p40\textsubscript{phox} with p67\textsubscript{phox}, which is mediated through a C-terminal PB1 interaction with the corresponding PB1 domain in p67\textsubscript{phox}. 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 p40\textsubscript{phox} so as to weaken its interaction with p67\textsubscript{phox}. This property of p40R57Q must be considered when evaluating its functional impact on NADPH oxidase activity. Indeed, p40\textsubscript{phox} was originally identified as a cytosolic protein tightly associated with p67\textsubscript{phox} (9-11). As we have shown above (Figure 7), when this association was disrupted by a second mutation at Asp289, the resulting p40\textsubscript{phox} double mutant could no longer inhibit NADPH oxidase in superoxide production assays. Consistent with the functional change, dissociation of p67\textsubscript{phox} from p40R57Q due to the D289A mutation caused a significant reduction in the amount of cytoskeleton-associated p67\textsubscript{phox} and p47\textsubscript{phox} found in the Triton X-100 insoluble fraction. Kuribayashi \textit{et al} previously reported that the D289A mutation could abolish the potentiation effect of the wild type p40\textsubscript{phox} (13), a finding confirmed in our study of FPR-reconstituted COS\textsubscript{phox} cells (data not shown). Taken together, these experimental results support the notion that both the potentiation effect and the inhibitory effect of p40\textsubscript{phox} require its association with p67\textsubscript{phox}.

We have shown that, in resting state, p40\textsubscript{phox} as well as p40R57Q could be co-immunoprecipitated with p67\textsubscript{phox} and p47\textsubscript{phox} (Figure 7). Upon agonist stimulation, p40\textsubscript{phox} translocated to plasma membrane rather than early endosome (Figure 1), and colocalized with p47\textsubscript{phox} and p67\textsubscript{phox} (Figures 5 and 6). The absence of early endosome localization could indicate that the PX domain of p40\textsubscript{phox} remains “closed” under the experimental condition used in this study, or the p47\textsubscript{phox}-directed membrane translocation of the cytosolic complex is predominant. Interaction between p40\textsubscript{phox} and p47\textsubscript{phox} may be secondary to the p40\textsubscript{phox}–p67\textsubscript{phox} interaction, as reported previously (13,31,32) and confirmed in this study (Figure 7). Our findings are consistent with one of the original observations that p40\textsubscript{phox} remains associated with p67\textsubscript{phox} and p47\textsubscript{phox} in activated neutrophils (9), and together suggest that p40\textsubscript{phox} facilitates membrane targeting of p67\textsubscript{phox}. The tight association between p40\textsubscript{phox} and p67\textsubscript{phox} apparently helps to stabilize their structures, as CGD patients with diminished p67\textsubscript{phox} expression also display reduced p40\textsubscript{phox} 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 p67\textsubscript{phox} and even p47\textsubscript{phox}, thereby prohibiting their membrane translocation. In this regard, it is notable that published reports showed that p40\textsubscript{phox} dissociates from p67\textsubscript{phox} in activated membranes (20). Another published study suggested that p40\textsubscript{phox} stabilizes the resting state and should be dissociated from p67\textsubscript{phox} for maximal oxidase activity (15).

In summary, the current study examines a PX domain mutation in the context of a full-length p40\textsubscript{phox}, and found that alteration of the PtdIns(3)P binding site may produce drastic changes in the way p40\textsubscript{phox} 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 p40\textsubscript{phox} 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 p67\textsubscript{phox} 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.

REFERENCES

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FOOTNOTES

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4 Abbreviations used are: Phox, phagocyte oxidase; PX domain, phox homology domain; PtsIns(3)P, phosphatidylinositol 3-phosphate; CGD, chronic granulomatous disease; fMLF, fMet-Leu-Phe; FPR, formyl peptide receptor; HRP, horseradish peroxidase; SOD, superoxide dismutase; CL, Chemiluminescence; cps, counts per second; PMSF, phenylmethylsulfonyl fluoride; GFP, green fluorescence protein; EEA-1, early endosome antigen 1.

FIGURE LEGENDS

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 DAPI for nuclei (blue), anti-EEA-1 for early endosome (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, Gαi2, and PKCδ, 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 time. Superoxide production was recorded as described in Materials and Methods. Representative traces from 3-4 experiments are shown. Solid lines, p40phox; dotted lines, vector control; dashed lines, p40R57Q; and gray lines, with SOD (250U) added to the assay buffer.

Figure 3. Exogenous expression of p40phox and p40R57Q in PLB-985 cells and the effects on superoxide production. PLB-985 cells were transfected using nucleofection with expression constructs encoding FPR, together with either an empty vector or expression constructs of p40phox or p40R57Q. Twenty-four hours after nucleofection, the expression levels of p40phox and p40R57Q were determined by Western blotting with an anti-p40phox antibody (A). Cells were collected and stimulated with either 1 µM of fMLF (B) or 200 ng/ml of PMA (C) as indicated. Superoxide production was monitored for the indicated period of time, as described in Materials and Methods. Representative traces from 3 independent experiments are shown. Solid lines, p40phox; dotted lines, vector control; and dashed lines, p40R57Q.

Figure 4. A dominant inhibitory effect of p40R57Q in superoxide production. COSphox cells were transiently transfected with expression constructs encoding FPR, Gαi2, PKCδ, and either an empty vector (-) or a vector for p40phox or p40R57Q. The amount of p40phox and 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 Figure 1, and for stimulation with 1 µM of fMLF (B) or 200 ng/ml of PMA (C) as indicated. Superoxide produced in a 20-min period was recorded and integrated as described in Materials and Methods. The integrated chemiluminescence (CL) was shown as % change relative to vector-transfected cells (set as 100%). Data shown in B and C are mean ± SEM from three independent experiments. *, p < 0.05 and **, p < 0.01, compared to control.

Figure 5. Confocal imaging analysis of the effects of p40phox and p40R57Q on membrane translocation of p47phox and p67phox. COSphox cells were transiently transfected with either an empty vector or vectors encoding p40phox-GFP or p40R57Q-GFP as indicated on top of each panel. Twenty-four hours after transfection, the cells were stimulated for 5 min with 200 ng/ml of PMA (B and D) or vehicle (A and C, with same amount of DMSO). The cells were fixed and stained with an anti-p47phox antibody (A and B) or an anti-p67phox antibody (C and D), followed with a rhodamine red-X-conjugated goat anti-rabbit IgG (red). All samples were stained with DAPI for nuclei (blue). Three experiments were conducted, and the proportion of responding cells was consistent with transfection efficiency in each experiment. A representative set of confocal images is shown for the PMA- and vehicle-treated cells. Scale bar = 20 µm.

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 in Materials and Methods. The relative levels of p40phox, p47phox and p67phox in the membrane fractions were determined using antibodies against p40phox,
p47\textsuperscript{phox} and p67\textsuperscript{phox}, respectively. An anti-p22\textsuperscript{phox} 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 p40\textsuperscript{phox} (B), p47\textsuperscript{phox} (C) and p67\textsuperscript{phox} (D) was quantified against p22\textsuperscript{phox}, 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 p40\textsuperscript{phox} or p40R57Q constructs.

Figure 7. Requirement of p67\textsuperscript{phox} association for the inhibitory effect of p40R57Q. A, COS\textsuperscript{phox} cells were transfected with expression vectors coding for FPR, Gai2, PKC\textgreek{d}, the empty vector, and the wild type or mutant p40\textsuperscript{phox} constructs as indicated. Twenty-four hours after transfection, cell lysate was prepared, and equal expression of p40\textsuperscript{phox} and the indicated mutants were determined using Western blotting with an anti-p40\textsuperscript{phox} antibody. B, COS\textsuperscript{phox} cells were transfected with the above constructs of p40\textsuperscript{phox}, which were tagged with FLAG. Immunoprecipitation was carried out using an anti-FLAG monoclonal antibody, and Western blotting was conducted with the respective antibodies against p40\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox} as described above. The p40R57Q/D289A double mutant was unable to interact with p67\textsuperscript{phox} or p47\textsuperscript{phox}. The transfected cells were stimulated with either 1 \textmu M of fMLF (C) or 200 ng/ml of PMA (D). Superoxide produced in a 20-min time span was recorded as described in Materials and Methods. The integrated chemiluminescence (Int. CL) was shown as % change relative to vector-transfected cells (set as 100\%). Data shown in C and D are mean \pm SEM from three independent experiments. **, p < 0.01, compared to control.

Figure 8. Effects of the R57Q mutation and R57Q/D289A double mutation on the retention of the cytosolic factors in Triton X-100 insoluble fraction. COS\textsuperscript{phox} cells were transfected with expression vectors coding for FPR, Gai2, PKC\textgreek{d}, the empty vector, and the wild type or mutant p40\textsuperscript{phox} constructs. Cells were lysed in a buffer containing 1\% Triton X-100 at 4oC. The insoluble fraction was collected, resolved on SDS-PAGE, and blotted with antibodies against the respective cytosolic factors. An antibody against \beta-actin was used for equal loading control. The expression level of the wild type and mutant p40\textsuperscript{phox} constructs was determined by Western blotting (top panel). Three independent experiments were conducted and a representative set of blots is shown. Quantitative analysis of the relative levels of p40\textsuperscript{phox} (B), p47\textsuperscript{phox} (C) and p67\textsuperscript{phox} (D) in the Triton X-100 insoluble fraction was shown.

Figure 9. Increased interaction of p40R57Q with actin and moesin. COS\textsuperscript{phox} cells (A and C) and COS\textsuperscript{91/22} cells (B and D) were transfected with either empty vector or FLAG-tagged p40\textsuperscript{phox} and p40R57Q expression vectors. Twenty-four hours after transfection, p40\textsuperscript{phox} and p40R57Q proteins were immunoprecipitated from cell lysate with an anti-FLAG antibody. Immunoprecipitates were separated on SDS-PAGE, blotted to nitrocellulose membrane, and detected with antibodies against actin (A and B) and moesin (C and D). In all panels, an anti-p40\textsuperscript{phox} antibody was used to determine the level of p40\textsuperscript{phox} in the immunoprecipitates. Representative blots from three independent experiments are shown. The relative intensity of the Western blot bands was quantified with the Quantity One software (Bio-Rad, Version 4.3.1), and shown in the bar charts below each blot.

Figure 10. Elimination of the inhibitory effect of p40R57Q by cytochalasin B. COS\textsuperscript{phox} cells were transfected with either empty vector or expression constructs of p40\textsuperscript{phox} or p40R57Q. A, The expression level of p40\textsuperscript{phox} and p40R57Q in the transfected cells was determined by Western blot with a p40\textsuperscript{phox} antibody. Anti-GAPDH antibody was used as a control for equal loading. Twenty-four hours after transfection, cells were incubated for 10 min with vehicle (B, 0.1\% DMSO) or cytochalasin B (C, 5 \mu g/ml), and then stimulated with PMA (200 ng/ml) as indicated by the arrows. The production of superoxide was determined as described in Materials and Methods, and expressed as counts per second.
(CPS) per $1 \times 10^6$ cells. Three independent experiments were conducted, and representative traces are shown. Solid lines, $p40^{\text{phox}}$; dotted lines, vector control; and dashed lines, $p40R57Q$. 
Figure 2

A

Vector  p40phox  p40R57Q

p40phox

p47phox

B

CPS / 10^6 cells

0  10  20  30  40

Time (min)

fMLF

C

PMA

CPS / 10^6 cells

0  1000  2000  3000  4000  5000  6000  7000  8000  9000  10000

Time (min)
Figure 4

A

B

C

Chen et al.
Figure 5

A

Vector          p40phox-GFP          p40R57Q-GFP

−PMA

GFP (p40phox)

B

Vector          p40phox-GFP          p40R57Q-GFP

+PMA

GFP (p40phox)
C

Vector  p40phox-GFP  p40R57Q-GFP

Anti-p67phox

− PMA

GFP (p40phox)

+ PMA

GFP (p40phox)

D

Vector  p40phox-GFP  p40R57Q-GFP

Anti-p67phox

− PMA

GFP (p40phox)

+ PMA

GFP (p40phox)
Figure 6

A

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B

![Graph showing p40 in membrane (relative intensity)]

C

![Graph showing p47 in membrane (relative intensity)]

D

![Graph showing p67 in membrane (relative intensity)]
A

Total cell lysate

Triton X-100

Insoluble fraction

Vector

p40phox

p40R57Q

p40R57Q/D289A

IB

p40phox

p47phox

p67phox

Actin

B

C

D

Relative intensity

IB

p40phox

p47phox

p67phox