**p47phox** Phox Homology Domain Regulates Plasma Membrane but Not Phagosome Neutrophil NADPH Oxidase Activation*†‡**

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The assembly of cytosolic subunits p47phox, p67phox, and p40phox with flavocytochrome b558 at the membrane is required for activating the neutrophil NADPH oxidase that generates superoxide for microbial killing. The p47phox subunit plays a critical role in oxidase assembly. Recent studies showed that the p47phox Phox homology (PX) domain mediates phosphoinositide binding in vitro and regulates phorbol ester-induced NADPH oxidase activity in a K562 myeloid cell model. Because the importance of the p47phox PX domain in neutrophils is unclear, we investigated its role using p47phox knock-out (KO) mouse neutrophils to express human p47phox and derivatives harboring R90A mutations in the PX domain that result in loss of phosphoinositide binding. Human p47phox proteins were expressed at levels similar to endogenous murine p47phox, with the exception of a chronic granulomatous disease-associated R42Q mutant that was poorly expressed, and wild type human p47phox rescued p42phox KO mouse neutrophil NADPH oxidase activity. Plasma membrane NAPDH oxidase activity was reduced in neutrophils expressing p42phox with Arg90 substitutions, with substantial effects on responses to either phorbol ester or formyl-Met-Leu-Phe and more modest effects to particulate stimuli. In contrast, p47phox Arg90 mutants supported normal levels of intracellular NADPH oxidase activity during phagocytosis of a variety of particles and were recruited to phagosome membranes. This study defines a differential and agonist-dependent role of the p47phox PX domain for neutrophil NADPH oxidase activation.

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of phagocytic leukocytes plays a key role in innate host defense against bacterial and fungal infections (1–3). The phagocyte NADPH oxidase is composed of membrane-integrated flavocytochrome b558 (a heterodimer composed of gp91phox (NOX2) and p22phox) and four cytosolic subunits: p47phox, p67phox, p40phox, and Rac2 (2–4). Upon activation by either soluble or particulate stimuli, the cytosolic subunits translocate to flavocytochrome b558 to form the activated NADPH oxidase complex, resulting in electron transfer from cytosolic NADPH through FAD and heme groups to extracellular or phagosome-located oxygen, from which superoxide is generated (2–4). Genetic defects in any of the five phox subunits of the NADPH oxidase complex result in chronic granulomatous disease (CGD),2 which is characterized by absent or deficient NADPH oxidase activity, recurrent pyogenic infections, and granulomatous inflammation (1, 5, 6).

The assembly of the NADPH oxidase complex is essential for activation of superoxide production, and p47phox plays a central role in this assembly (2–4, 7–12). From the N terminus to the C terminus, p47phox contains a Phox homology (PX) domain, two tandemly arranged Src homology 3 (SH3) domains, an autoinhibitory region (AIR), and a proline-rich region (PRR; Fig. 1A). In the resting state, p47phox is autoinhibited via intramolecular interactions of the PX and two SH3 domains with the AIR and adjacent region (4, 10, 13–15). p47phox forms a heterotrimetric complex with p67phox and p40phox via a tail-to-tail interaction between the C-terminal SH3 domain of p67phox and proline-rich region of p47phox and a P1B-P1B association between p67phox and p40phox (4, 16). Upon cell stimulation, p47phox is phosphorylated on multiple serine residues in the AIR, which acts as a molecular switch to liberate its autoinhibited structure and release the PX and tandem SH3 domains, with the latter binding to the proline-rich region of membrane-bound p22phox (8, 14, 17, 18). The p47phox-p22phox interaction mediates the recruitment of the heterotrimetric phox complex, and neither p67phox nor p40phox undergoes membrane translocation in the absence of p47phox (7, 19).

**The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–4 and Movies 1–3.**

**The abbreviations used are: CGD, chronic granulomatous disease; ROS, reactive oxygen species; PMN, polymorphonuclear leukocyte; PX, Phox homology; SH3, Src homology 3; BM, bone marrow; PMA, phorbol 12-myrystate 13-acetate; fMLF, formyl-methionyl-leucyl-phenylalanine; SOZ, serum-opsonized zymosan; PI3P, phosphatidylinositol 3-phosphate; PI(3,4)P 2, phosphatidylinositol 3,4-bisphosphate; PI(3,4,5)P 3, phosphatidylinositol 3,4,5-trisphosphate; YFP, yellow fluorescent protein; hIgG, human IgG.**

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The PX domain is a phosphoinositide binding module that was first described as a motif in the p47\textsuperscript{phox} and p40\textsuperscript{phox} subunits of the NADPH oxidase complex (20–24). Binding of the p40\textsuperscript{phox} PX domain to its target, P13P, plays a critical role in NADPH oxidase activity in neutrophil phagosomes (6, 25–28). Unlike the p40\textsuperscript{phox} PX domain, which has a single binding pocket with high affinity for P13P, the PX domain of p47\textsuperscript{phox} has two distinct lipid binding pockets. The main pocket prefers PI(3,4)P\textsubscript{2} but also weakly binds other phosphoinositides (23, 24). The PX domain of full-length p47\textsuperscript{phox} also has a PX domain, which binds to PI3(3,4)P\textsubscript{2} via interaction with the 3- and 4-phosphates, respectively, based on crystallography (30) and mutagenesis studies (23, 32, 34). The PX domain of full-length p47\textsuperscript{phox} is masked in unstimulated cells but exposed upon activation-induced phosphorylation of the AIR (34). In a whole-cell model using K562 cells, an R90K mutation in p47\textsuperscript{phox} markedly reduced phorbol ester-induced recruitment of p47\textsuperscript{phox} to membranes and NADPH oxidase activity (34). The NOX1 (Nox-organizing protein 1) homolog of p47\textsuperscript{phox} also has a PX domain, which binds to PI3(3,4)P\textsubscript{2}, PI5P, and P14P (35). The PX domain in NOX1, which lacks an AIR, does not appear to be masked and mediates the constitutive localization of NOX1 to the plasma membrane and its activation of the NOX1 homolog of gp91\textsuperscript{phox} in an HEK293 cell model (35, 36).

The physiological function of the p47\textsuperscript{phox} PX domain in phagocytic leukocytes remains unknown. In this study, we introduced PX domain mutations that impair phosphoinositide binding into full-length p47\textsuperscript{phox} and examined the impact on NADPH oxidase activity elicited by soluble and particulate stimuli. We took advantage of the p47\textsuperscript{phox} knock-out (KO) mouse (37) to express wild type human p47\textsuperscript{phox} and derivatives. Human p47\textsuperscript{phox} is 82% identical to murine p47\textsuperscript{phox} and derivatives, can rescue phorbol ester-elicited NADPH oxidase activity in p47\textsuperscript{phox} KO mouse neutrophils (38). Here, we showed that human p47\textsuperscript{phox} or a derivative tagged at its C terminus with YFP could rescue NADPH oxidase activity in response to PMA, fMLF, and particulate stimuli, including IgG-opsonized latex beads, serum-opsonized zymosan (SOZ), serum-opsonized Staphylococcus aureus, and sterilized Aspergillus fumigatus hyphae. Mutations in the PX domain of p47\textsuperscript{phox} that impair phosphoinositide binding led to impaired neutrophil NADPH oxidase activation on the plasma membrane but had little effect on intracellular reactive oxygen species (ROS) production during phagocytosis, thus defining a differential role for the p47\textsuperscript{phox} PX domain.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Chemicals were purchased from Sigma-Aldrich unless otherwise stated. Phosphate-buffered sodium (PBS), pH 7.2, penicillin/streptomycin, neomycin, and RPMI 1640 were from Invitrogen; fetal calf serum (FCS) was from HyClone Laboratory (Logan, UT). G418 was purchased from Calbiochem. The ECL detection kit came from Pierce. Polyclonal antibody against DsRed (red fluorescent protein) was obtained from Clontech (catalog no. 632496). Latex beads (3.3 mm) were from Bangs laboratory Inc. (Fisher, IN). Polyclonal antibody against green fluorescent protein (GFP) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against p40\textsuperscript{phox} and p47\textsuperscript{phox} were from Upstate Biotechnology, Inc. (Lake Placid, NY), and monoclonal antibody against p67\textsuperscript{phox} was from BD Biosciences. Monoclonal antibodies 54.1 and NS2 against gp91\textsuperscript{phox} and p22\textsuperscript{phox}, respectively, were gifts from Dr. Jessaitis (Montana State University, Bozeman, MT). Rabbit polyclonal antibody against p47\textsuperscript{phox} was a gift from Dr. Lambeth and Dr. Uhlinger (Emory University, Atlanta, GA). 7D5 mAb (anti-gp91\textsuperscript{phox}) was collected from hybridoma cells kindly provided by M. Nakamura (Nagasaki University, Japan). The Amaxa kit V was from Amaxa Biosystems (Cologne, Germany). S. aureus was purchased from ATCC (Manassas, VA).

**Plasmid Construction**—The human p47\textsuperscript{phox} cDNA was subcloned into a pEYFP-N1 vector to generate a fluorescently tagged p47\textsuperscript{phox} probe. The cDNA encoding wild type (WT) p47\textsuperscript{phox} was amplified from pHK5-p47\textsuperscript{phox} (gift from Dr. Lambeth, Emory University Medical School, Atlanta, GA) by polymerase chain reaction (PCR) and cloned into EcoRI and KpnI sites of pEYFP-N1 (BD Biosciences Clontech) to generate p47\textsuperscript{phox}. Site-directed mutagenesis was performed in p47\textsuperscript{phox} using the QuickChange site-directed mutagenesis kit (Stratagene; La Jolla, CA). The constructs were confirmed by sequencing. The cDNA fragment of p47\textsuperscript{phox} or mutants was then excised by digesting with NotI, blunting, and then digesting with EcoRI; pMSCV (Clontech) was digested with ClaI, followed by blunting, and then digested with EcoRI. The cDNA for p47\textsuperscript{phox} or mutants was ligated to pMSCV using EcoRI to generate pMSCV-pac-p47\textsuperscript{phox}. The cDNA fragment of p67\textsuperscript{phox} from p67\textsuperscript{YFP} was subcloned into mCherry-N vector (gift from J. Swanson, University of Michigan) at the Xhol and HindIII sites to generate p67\textsuperscript{Cherry} (39, 40). Retroviral vectors were packaged as described previously (28). Other plasmids for expression of phox subunits have been described previously (28, 41).

**Cell Lines**—K562 cells were grown in RPMI 1640 with 10% FCS and 1% penicillin/streptomycin at 37 °C in 5% CO\textsubscript{2}. K562 cells stably expressing gp91\textsuperscript{phox} were generated by retroviral transduction with VSVG-pseudotyped MFG-gp91\textsuperscript{phox} (41), and gp91\textsuperscript{phox}-expressing cells were sorted using 7D5 monoclonal antibody (10). After sorting, K562-gp91\textsuperscript{phox}/p67Cherry cells were transfected with the p67\textsuperscript{Cherry} plasmid under 1.5 mg/ml G418 selection for 3 weeks and sorted using a PE-Texas Red laser (BD FACSAria). K562-gp91\textsuperscript{phox}/p67\textsuperscript{Cherry} was cultured in the presence of 0.9 mg/ml G418. Amaxa kit V (Amaxa Biosystems) was used to transiently transfect 2 x 10\textsuperscript{6} K562-gp91\textsuperscript{phox}/p67\textsuperscript{Cherry} cells with 2 μg of pRK5-p40\textsuperscript{phox} and 2 μg of pEYFP-N1-p47\textsuperscript{phox} (p47\textsuperscript{YFP}) or mutants (40). Cells were generally analyzed 24 h after transfection.

**Retroviral Transduction of p47\textsuperscript{phox} KO Mouse Bone Marrow (BM) and Neutrophil Differentiation**—Retroviral transduction of p47\textsuperscript{phox} KO mouse BM cells with MSCV-p47\textsuperscript{YFP} or mutants or with MSCV-pac-p47\textsuperscript{phox} or mutants was performed as described (41, 42). Transduced BM cells were differentiated...
into neutrophils in α-minimum essential medium with 20% heat-inactivated FCS, 1% penicillin/streptomycin, 50 ng/ml human G-CSF and 50 units/ml mIL-3. The first day of starting differentiation was considered as day 0. Cells were counted and replated to a concentration of 0.5 × 10^6/ml in fresh differentiation medium every 2 days; activity and live images were analyzed on days 6 and 7. Puromycin (1 μg/ml) was used to select the MSCV-pac-p47phox, R90K, or R42Q mutant transduced cells. After differentiation, transduction efficiency was determined by flow cytometry (FACScalibur, BD Biosciences). Except for p47-YFP-R42Q, the YFP-positive cells were ~30–50% of the total, and mean fluorescent intensity was 150–400 relative units.

Preparation of Opsonized Particles and Non-opsonized Microbes—3.3-μm latex beads were opsonized with human IgG (hIgG) as described (26). 12.5 × 10^5 hlgG-latex particles were added to 2 × 10^5 cells (cells/beads = 1:6) or 2.5 × 10^5 mouse neutrophils (cells/beads = 1:5) to initiate NADPH oxidase activity. SOZ particles (Sigma catalog no. Z-4250) or Aspergillus hyphae were also prepared as described (6, 40, 43), and then 400 μg/ml SOZ or 60 μg/ml hyphae were used to activate mouse PMNs. Serum-opsonized or heat-inactivated S. aureus (Wood 46) were prepared as previously described (25), and neutrophils were activated with bacteria at a ratio of 1:40.

Analysis of phox Subunit Expression—Cell lysates were prepared from K562 cells or mouse neutrophils using 1% Triton X-100, and 15 or 30 μg were subjected to SDS-PAGE and immunoblotting using ECL detection (40). ImageJ (available from the National Institutes of Health Web site) was used for densitometry analysis of YFP-tagged or non-YFP-tagged p47phox or mutants. In some experiments, the Triton X-100-insoluble pellet was also analyzed by SDS-PAGE and immunoblotting as described (28, 44). YFP expression was also analyzed by flow cytometry (FACScalibur, BD Biosciences) (40).

NADPH Oxidase Activation in Intact Cells—NADPH oxidase activity was assayed using chemiluminescence enhanced by luminol or isoluminol, which is membrane-impermeable; both compounds detect ROS in a peroxidase-dependent reaction (40, 45, 46). PMA (300 ng/ml) or hlgG-latex beads was used to activate 2 × 10^5 K562-gp91phox/p67Cherry cells, co-transfected with p40phox and p47YFP or mutants in the presence of 20 μM squalene and 20 units/ml horseradish peroxidase (HRP). An Lmax microplate luminometer (Molecular Devices, Sunnyvale, CA) was used to record luminescence every 1–1.5 min at 37 °C for a total of 46 readings. A similar protocol was used to measure extracellular ROS release in 2.5 × 10^5 BM differentiated neutrophils after PMA, FMLF, or particulate stimulation. Intracellular ROS production during synchronized phagocytosis of hlgG-latex or SOZ was measured in the presence of luminol and superoxide dismutase (28), and intracellular ROS generation during synchronized phagocytosis of serum-opsonized or heat-inactivated S. aureus or hyphae was measured in the presence of luminol as described by Hawkins’ group (25). Activity was normalized for p47phox expression determined by densitometry.

Live Images by Confocal Video Microscopy—SOZ-induced phagocytosis in p47phox KO neutrophils expressing p47YFP or

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RESULTS

p47phox PX Domain Is Important for Supporting Plasma Membrane NADPH Oxidase Activity in K562 Cells in Response to PMA and hlgG-Latex Beads—K562-gp91phox cells, a primitive myeloid cell line that expresses endogenous p22phox and is engineered to express flavocytochrome b by insertion of a stable transgene for gp91phox, were used to validate the function of fluorescence-tagged cytosolic phox subunit probes. K562-gp91phox cells that were transiently co-transfected for expression of p47phox tagged at its C terminus with YFP (p47YFP) (Fig. 1A) and p67phox produced similar amounts of ROS induced by PMA, compared with cells transfected with untagged p47phox and p67phox (supplemental Fig. 1, A–C), showing that p47YFP is fully functional. In contrast, p47phox tagged at its N terminus (YFP–p47phox) supported only 50% activity compared with p47phox in response to PMA (data not shown). No ROS production was observed in K562-gp91phox expressing YFP alone in the presence of p67phox (data not shown). For subsequent studies, we generated K562 cells stably expressing both gp91phox and p67Cherry, and a population expressing both transgenes was isolated by FACS. The function of p67Cherry was validated by showing that K562-gp91phox cells co-transfected for expression of p67phox or p67Cherry with p47YFP showed similar ROS release capacities upon PMA stimulation (data not shown).

To examine the role of phosphoinositide binding to the p47phox PX domain, we mutated two amino acids (Arg43 and Arg49) that are critical for interactions with the 3- and 4-phosphates, respectively, of PI(3,4)P2 (Fig. 1A) (30, 32, 47). We tested these mutants in K562-gp91/p67Cherry cells stimulated with either PMA or IgG-opsonized particles following co-transfection of plasmids for expression of p47YFP WT or derivatives, along with p40phox; co-expression of p40phox enhances oxidase activity in response to PMA (48), and we found that p40phox was required for response to hlgG-latex beads (data not shown). For comparison with p47phox PX domain mutants, we assayed in parallel a p47phox derivative with a W193R mutation in the N-terminal SH3 domain, which is unable to translocate to the membrane because it cannot bind to the flavocytochrome b via p22phox (8, 18, 49) (Fig. 1A). YFP-tagged p47phox and mutants were similarly expressed in K562-gp91/p67Cherry cells (Fig. 1B), and protein levels of p40phox, p67Cherry and gp91phox, and endogenous p22phox were also similar.
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A structural motif of p47phox and the proposed interactions of p47phox PX domain (Fig. 1B), K562-gp91/p67Cherry cells were co-transfected with p40phox and p47YFP WT or PX module or SH3A domain mutants with Amaxa V latex beads compared with p47phox WT or PX module or SH3A domain mutants with Amaxa V (Amaxa) under the T-16 program. 15 μg of cell lysate was loaded in each lane. Immunoblot analysis of p47YFP, p67Cherry, p40phox, gp91phox, and p22phox was performed using anti-GFP polyclonal antibody, which also recognizes Cherry protein, anti-p40phox polyclonal antibody, anti-gp91phox 54.1, and anti-p22phox N52, respectively. Blots are representative of three independent experiments. Shown is ROS production in K562 cells in response to PMA (C) and hlgG-latex beads (D) in the presence of isoluminol and HRP. E, ROS production in K562 cells (n = 3). Assays were performed in triplicate, and mean values ± S.D. (error bars) are shown. **, p < 0.01. PRR, proline-rich region.

Shown in Fig. 1 are representative assays and a summary of three independent experiments in which the activity of YFP-tagged p47phox mutants relative to p47YFP was normalized to protein expression level. As expected, a W193R mutation in the SH3a domain of p47phox abolished NADPH oxidase activity in response to PMA and hlgG-latex (Fig. 1, C–E). The double mutant p47phox R43A and p47phox R90A mutations resulted in a 50–70% reduction, respectively, in ROS production in response to PMA (Fig. 1, C and E), confirming a previous report on the importance of the p47phox PX domain for PMA-induced oxidase activity in the K562 model (34). We further showed that these mutations also significantly reduced ROS production in response to hlgG-latex beads compared with p47phox WT (Fig. 1, D and E). The double mutant p47phox R43A/R90A reduced ROS generation by 80–85% (Fig. 1, C–E), a modest decrease over the R90A mutation alone. The significant difference in ROS generation was also observed in R43A versus R90A and R90A versus R43A/ R90A in response to both PMA and hlgG-latex beads. Both PMA- and hlgG-latex bead-induced ROS production were fully superoxide dismutase-sensitive (not shown), indicating that the detected ROS was released at the plasma membrane, as expected, because this K562 cell line does not ingest IgG particles.3 Non-YFP-tagged p47phox R90K was also tested in K562-gp91/p67Cherry cells, with similar results (data not shown).

Thus, our results suggest that Arg43 and Arg90 are each important for supporting NADPH oxidase activity on the plasma membrane in response to either PMA or hlgG-latex beads in K562 cells. Although Arg90 is conserved in human and mouse p47phox, there is a lysine rather than an arginine at position 43 in mouse p47phox (50). Because p47phox R90A is both more conserved and showed a greater reduction in ROS production than R43A in the K562 cell model (Fig. 1), we therefore focused on this mutant in subsequent studies.

Expression and Function of Human p47phox in Murine Neutrophils—Human and murine p47phox are 82% identical, and human p47phox partially rescued NADPH oxidase activity in PMA-simulated p47phox KO mouse neutrophils (38). To further evaluate the function of human p47phox in p47phox KO mouse neutrophils, we used an MSCV-p47phox vector containing a puromycin-resistant cassette and selected for transduced cells undergoing neutrophil differentiation from myeloid progenitors. Human p47phox was expressed in puromycin-selected mouse p47phox KO mouse neutrophils at levels somewhat higher than endogenous p47phox in WT mouse neutrophils (supplemental Fig. 2A). Endogenous p47phox was absent in p47phox KO neutrophils, as expected (supplemental Fig. 2A). Other phox proteins were expressed at similar levels (supplemental Fig. 2A). Human p47phox partially rescued ROS generation (41 ± 11% of WT mouse neutrophils) in response to PMA (supplemental Fig. 2, B and J) and fully rescued NADPH oxidase activity (98 ± 30%) in response to fMLF (supplemental Fig. 2, C and J). We next characterized ROS production in response to model particles and to microbial stimuli. Human p47phox expression completely rescued NADPH oxidase activity in p47phox-deficient mouse neutrophils in response to hlgG-latex beads, SOZ, serum-opsonized S. aureus, heat-inactivated S. aureus, and sterilized A. fumigatus hyphae (supplemental Fig. 2, D–K), further extending previous findings (38) that human p47phox can replace murine p47phox in supporting NADPH oxidase activity and that the p47phox KO neutrophils are a good model for studying the function of human p47phox.

3 X. J. Li, C. C. Marchal, N. D. Stull, R. V. Stahelin, and M. C. Dinauer, unpublished observations.
dase components were expressed at similar levels in p47phox KO non-transduced or transduced cells (Fig. 2A). The p47YFP-R90A mutation resulted in a severe reduction in PMA-stimulated plasma membrane ROS production by 84% compared with p47YFP (Fig. 2B). This is consistent with results in the K562 cell model (Fig. 1, C and E) (34). The p47phox R90A mutation also produced a 72% reduction in ROS generation upon fMLF stimulation (Fig. 2C). Similar results were observed in puromycin-selected neutrophils expressing the p47phox-R90K mutant (without YFP tag) as compared with WT p47phox (Fig. 2, D–F). These results indicate that Arg90 in p47phox, a residue that plays a critical role in phosphoinositide binding to its PX domain, is a positive regulator of both phorbol ester- and chemoattractant-induced NADPH oxidase activity on neutrophil plasma membranes.

Effect of PI3K Inhibition by Wortmannin—To evaluate whether the effect of the PX domain mutation on reduced p47phox function in PMA-stimulated neutrophils is mediated by PI3K-derived phosphoinositides, we examined the effect of wortmannin. As reported for K562 cells (where wortmannin also did not affect translocation) (34), PMA-induced activity in mouse neutrophils was not inhibited by wortmannin (supplemental Fig. 3A), suggesting that phosphoinositides with a 3′-phosphate are not required for the p47phox PX domain to regulate PMA-induced ROS production. Although PI3K inhibitors abolish neutrophil NADPH oxidase activity in response to fMLF (51), PI3K plays multiple roles downstream of this agonist, including Rac activation, and it is not possible to tie its inhibition of ROS production to effects on binding targets of the p47phox PX domain.

Effect of p47phox Arg90 Mutations on Neutrophil Plasma Membrane and Intracellular NADPH Oxidase Activity Induced by Model Particles and Microbial Stimuli—We next examined the impact of mutations in the p47phox PX domain on NADPH oxidase activation by particulate stimuli. The kinetics of particle-induced extracellular ROS production in mouse p47phox KO neutrophils expressing p47YFP-R90A was similar to the kinetics of neutrophils expressing p47YFP (Fig. 3A and B). However, total integrated extracellular ROS generation was reduced by 20–40% in response to hIgG-latex beads (Fig. 3, A and B), SOZ (Fig. 3, A and B), serum-opsonized S. aureus (Fig. 3, A and B), heat-inactivated S. aureus (data not shown) (Fig. 3B), and hyphae (Fig. 3, A and B), respectively. In contrast, the time course and amount of intracellular ROS elicited during phagocytosis were comparable in neutrophils expressing either p47YFP or p47YFP-R90A (Fig. 3, A and B). Similar results were observed using the p47phox-R90K mutant (without YFP tag) compared with p47phox WT (Fig. 3C), although there was small but statistically significant reduction in serum-opsonized S. aureus-induced intracellular ROS generation in neutrophils expressing

**FIGURE 2. NADPH oxidase activity of p47phox WT and p47phox Arg90 mutants in p47phox KO mouse PMNs. A and D, Western blotting. 15 μg of cell lysate was used in immunoblotting. A vertical line was added when the sample was loaded in a non-continuous lane. Shown is PMA-stimulated (B and E) or fMLF-stimulated (C and F) ROS production in p47phox KO mouse PMNs or PMNs transduced with p47YFP, p47YFP-R90A, p47phox, or p47-R90K. Bar graphs represent four or five independent experiments. **, p < 0.01. Error bars, S.D.**
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Taken together, our data suggest that although phosphoinositide binding to the p47phox PX domain plays an important role in regulating extracellular ROS responses, particularly for chemoattractants and phorbol ester, it is not involved in regulating intracellular NADPH oxidase activity during phagocytosis.

The p47phox R90A Mutation Does Not Impair the Recruitment of p47phox during SOZ Phagocytosis—To investigate the localization of YFP-tagged p47phox and YFP-tagged p47phox R90A during serum-opsonized zymosan phagocytosis, we took advantage of time lapse confocal video microscopy. p47YFP, which was cytosolic in unstimulated cells, accumulated on the cup prior to phagosome sealing and persisted after sealing for at least 200 s during SOZ phagocytosis (Fig. 4A and supplemental Movie 1). Like p47YFP, p47YFP-R90A was cytosolic in resting cells, and during SOZ phagocytosis it was recruited to the phagosome cup and visible on the phagosome for at least 200 s (Fig. 4A and supplemental Movie 2). As previously observed for accumulation of p67YFP in PLB-985 granulocytes during hlgGzymosan phagocytosis (40), only approximately one-half of the phagocytic cups and internalized phagosomes accumulated p47YFP (Fig. 4B). Although cells expressing p47phox R90A demonstrated an ~20% reduction in extracellular ROS production (Fig. 3, A and B), there was no effect on the frequency of p47YFP-R90A-positive phagocytic cups or phagosomes compared with p47YFP in comparable studies (Fig. 4B). The kinetics and relative amount of p47YFP and p47YFP-R90A accumulating on individual phagosomes were also similar (Fig. 4C).

FIGURE 3. Extracellular and intracellular ROS production of p47phox WT and p47phox Arg90 mutants in p47phox KO mouse PMNs. A, extracellular and intracellular ROS production was induced by hlgG-latex beads (n = 4), SOZ (n = 5), serum-opsonized S. aureus (n = 4), or hyphae (n = 3). ◦, p47YFP-transduced p47phox KO neutrophils; ○, p47YFP-R90A-transduced p47phox KO neutrophils; *, p47phox KO neutrophils. The percentage of extracellular and intracellular ROS of p47phox R90A mutant (B) or p47phox R90K mutant (C) compared with p47phox WT was normalized with the protein expression level in response to different particulate stimuli from 3–5 independent experiments. *, p < 0.05; **, p < 0.01. Error bars, S.D.
vations in RAW 26.4.7 cells (52), and did not accumulate on phagosomal membranes during SOZ phagocytosis (Fig. 4C).

Analysis of an R42Q p47phox Mutant Identified in p47phox-deficient CGD—Noack et al. (53) studied two p47phox CGD patients with absent O$_2^*$ production who had point mutations in NCF1 (neutrophil cytosolic factor-1), the gene encoding p47phox, predicting an R42Q substitution in the phosphoinositide binding pocket. Both patients were compound heterozygotes, with the second allele in each patient harboring small deletions that result in frameshift and premature stop codons. The introduction of the R42Q mutation into the isolated p47phox PX domain abolished its phosphoinositide binding in vitro (23, 29). However, the above mentioned patients had no p47phox expression by immunoblot, suggesting that the R42Q renders the full-length protein unstable (54).

To explore the behavior of this CGD-related p47phox mutation, we first used K562-gp91/p67Cherry cells. As detected by flow cytometry following transient transfection with plasmid expression vectors, p47YFP-R42Q was expressed at only 7% of wild type p47YFP levels (Fig. 5A). Increasing the amount of the pEYFP-N1-p47phox-R42Q plasmid did not significantly increase the expression of the mutant protein (data not shown). Immunoblots using an anti-GFP polyclonal antibody showed that p47YFP-R42Q was trapped in the Triton X-100-insoluble fraction (Fig. 5B). Interestingly, co-expressed p47phox and p67Cherry were also partly retained in the insoluble fraction (Fig. 5B), suggesting that these three cytosolic p47phox proteins can form a trimeric complex in K562 cells, as in neutrophils (4, 16). Under confocal microscopy, we observed a punctate distribution of p47YFP-R42Q (Fig. 5C) in many cells, whereas p47YFP was distributed uniformly in the cytosol (Fig. 5C). A punctate distribution of p67Cherry was also observed in cells with this distribution of p47YFP-R42Q (Fig. 5C). PMA-stimulated cells expressing p47YFP-R42Q supported only ~1% of the ROS release seen in cells expressing p47YFP (Fig. 5, D and F). No ROS release was detected in p47YFP-R42Q in K562 cells stimulated with hIgG-latex beads (Fig. 5, E and F).

We next expressed p47YFP-R42Q in mouse p47phox KO neutrophils using retroviral transduction of myeloid progenitors, as above. Flow cytometry showed a 10-fold reduction in the level of expression of p47YFP-R42Q compared with p47YFP (Fig. 6A). We also saw markedly reduced expression of p47YFP-R42Q by Western blot of Triton-soluble neutrophil extracts (Fig. 6B). Other p47phox proteins in neutrophils expressing p47YFP or p47YFP-R42Q were present at comparable levels (Fig. 6B), suggesting that bone marrow progenitors differentiated similarly into neutrophils. To try to enhance expression of p47phox-R42Q, we used a retroviral vector with a marker for puromycin selection, but protein expression of p47YFP-R42Q was still much lower than that of p47YFP WT (Fig. 6C). Similar to K562 cells (Fig. 5C), neutrophil p47YFP-R42Q showed a punctate distribution (Fig. 6D). For reasons that are uncertain, we did not detect p47YFP-R42Q (supplemental Fig. 4) or p47phox-R42Q (not shown) in the Triton X-100-insoluble fraction from neutrophil extracts, in contrast to K562 cells expressing p47YFP-R42Q.

We used confocal video microscopy to examine whether p47YFP-R42Q could accumulate on SOZ phagosomes. We did not detect any membrane translocation (Fig. 6E and supplemental Movie 3), although the interpretation of this observation is difficult given the low level of protein expression and abnormal distribution (Fig. 6, A–D). We also examined NADPH oxidase activity in neutrophils expressing p47phox-R42Q. Extracellular ROS in response to PMA, fMLF, or different particles was not detectable (data not shown). However, low but detectable intracellular ROS were observed in response to hIgG-latex beads, serum-opsonized S. aureus, and sterile hyphae (Fig. 6, F–H).

**DISCUSSION**

PX domains are important motifs for binding membrane phosphoinositides that regulate the localization and activity of proteins and are contained in two regulatory subunits of the NADPH oxidase. Unlike the p40phox PX domain, which is specific for PI3P (23, 33), the p47phox PX domain binds preferentially to PI(3,4)P$_2$, and can also bind with lower affinity to other phosphoinositides (23, 29, 30, 32, 47). The current study identifies a differential and agonist-dependent role of the p47phox PX domain during neutrophil NADPH oxidase activation in response to soluble and particulate stimuli, showing that the p47phox PX domain regulates ROS production on the plasma membrane, particularly in response to fMLF and phorbol ester, but not intracellular ROS production during phagocytosis.

An arginine residue at amino acid 90 in the p47phox PX domain plays a critical role in ligating the 4-position phosphate in target phosphoinositides (30). In initial studies, we confirmed the importance of p47phox Arg$^{90}$ for PMA-activated...
ROS release by K562 cells on the plasma membrane (34) and extended findings in this model to show that p47<sub>Phox</sub> Arg<sup>90</sup> regulates IgG particle-induced NADPH oxidase activity on the plasma membrane. We also studied the effect of mutating Arg<sup>43</sup>, which normally interacts with the 3-phosphate of PI(3,4)P<sub>2</sub> in the phosphoinositide-binding pocket of human p47<sub>Phox</sub>. A R43Q mutation in the isolated p47<sub>Phox</sub> PX domain resulted in a more severe impairment of phosphoinositide binding in vitro compared with R90A (30). However, in the K562 model, the p47<sub>Phox</sub> R43A mutant had less effect on PMA-induced ROS generation than the p47<sub>Phox</sub> R90A mutant. This disparity could result from the complexity of the in vivo microenvironment compared with the simplicity of in vitro lipid binding studies.

In mouse p47<sub>Phox</sub> KO neutrophils, p47<sub>Phox</sub> R90A or R90K mutants showed substantial defects in both PMA- and fMLF-induced release of ROS at the plasma membrane. Upon fMLF stimulation, PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> increase and PI(4,5)P<sub>2</sub> and PI3P decrease in human PMNs (55), as a result of activation of Class I PI3Ks (51, 56). Thus, fMLF-induced increases in plasma membrane PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> could stimulate NADPH oxidase activity via the p47<sub>Phox</sub> PX domain. However, PMA-induced plasma membrane NADPH oxidase activity is insensitive to wortmannin while still dependent on p47<sub>Phox</sub> Arg<sup>90</sup>, which ligates the 4-position phosphate of target phosphoinositides. This suggests that the p47<sub>Phox</sub> PX domain can regulate ROS production via phosphoinositides lacking a 3′/H11032 phosphate, such as PI(4,5)P<sub>2</sub> which is ~25-fold more abundant in the plasma membrane compared with PI(3,4)P<sub>2</sub> (57), or other targets. Interaction with PI(4,5)P<sub>2</sub> may also account for the observation that a fluorescently tagged probe derived from the p47<sub>Phox</sub> PX domain was reported to accumulate on the plasma membrane of resting neutrophils (58), which only have low levels of PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>.

To examine the impact of p47<sub>Phox</sub> Arg<sup>90</sup> mutants on neutrophil NADPH oxidase activity during phagocytosis, we used IgG-latex beads, serum-opsonized zymosan, and sterilized Aspergillus hyphae as model particles as well as complement-opsonized S. aureus and heat-killed S. aureus. These particular stimuli bind to receptors that activate phagocytosis and ROS production through overlapping pathways that include activation of Class I III PI3Ks (51). Results from all partic-

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**FIGURE 5.** CGD-associated p47<sub>Phox</sub> R42Q mutant expressed in K562 cell model. A, YFP fusion protein expression in K562 cells was measured by flow cytometry. B, Western blot analysis of Triton X-100-soluble and -insoluble fractions. A vertical line was added when the sample was loaded in a non-continuous lane. C, distribution of p47YFP WT and R42Q in K562 cells stably expressing gp91<sub>Phox</sub> and p67Cherry. PMA-stimulated (D) or hlgG-latex-stimulated (E) ROS production in K562-gp91/p67Cherry cells co-transfected with p40<sub>Phox</sub>. Insets show the ROS production measured in the R42Q mutant. F, percentage of ROS production in K562 cells (n = 3). Error bars, S.D.
ulate stimuli tested were similar. There was a modest but statistically significant decrease in the release of oxidants at the plasma membrane when p47\textsuperscript{phox} Arg\textsuperscript{90} was replaced with either alanine or lysine, with the greatest effect seen with stimulation of IgG-opsonized particles. However, p47\textsuperscript{phox} Arg\textsuperscript{90} substitutions had little effect on intracellular ROS production, indicating that the p47\textsuperscript{phox} PX domain regulates oxidase activity only before phagosome sealing. We were also unable to detect reduced translocation of p47\textsuperscript{phox} R90A during ingestion of serum-opsonized particles, including to the phagocytic cup formed prior to internalization.

The differential influence of p47\textsuperscript{phox} R90A on extracellular and intracellular NADPH oxidase activity and the more marked sensitivity of the former to soluble ligands is likely to reflect differences in the composition of the plasma and phagosome membranes. In activated neutrophils, the spatiotemporal dynamics of membrane phosphoinositides during phagocytosis reflect the sequential activity of Class I and III PI3Ks in concert with lipid phosphatases, such as SHIP-1 (Src homology 2-containing inositol phosphatase) and PTEN (phosphatase and tensin homolog) (59, 60). Although the p47\textsuperscript{phox} PX domain has a variety of targets, the lipid with the highest affinity is PI(3,4)P\textsubscript{2}, largely derived from Class I PI3K-generated PI(3,4,5)P\textsubscript{3}. The absence of a fluorescently tagged p47\textsuperscript{phox} PX domain probe from phagosomes (58) and the lack of dependence of phagosome NADPH oxidase activity on p47\textsuperscript{phox} Arg\textsuperscript{90} are consistent with prior studies showing that although PI(3,4,5)P\textsubscript{3} or PI(3,4)P\textsubscript{2} accumulates in the phagocytic cup during ingestion of SOZ- or IgG-opsonized particles by macrophage or in neutrophilic HL60 cells, these disappear after phagosome sealing (59, 61).

The secondary binding pocket for phosphatidic acid or phosphatidylserine in the p47\textsuperscript{phox} PX domain synergizes with ligand binding to the primary pocket to increase overall affinity and penetration of the isolated p47\textsuperscript{phox} PX domain into the membrane (30, 32). As a result, the otherwise poor binding affinity of p47\textsuperscript{phox} PX R90A to PI(3,4)P\textsubscript{2} is enhanced by the presence of phosphoserine or phosphatidic acid (30, 32). Phosphatidylserine is present on plasma membranes and phagosomes (62). In the current study, we did not specifically study mutations in this secondary pocket (amino acids 55 and 70) as an independent factor; however, the p47\textsuperscript{phox} PX R90A mutation alone was sufficient to profoundly impair neutrophil plasma membrane NADPH oxidase activity in response to PMA or fMLF.

FIGURE 6. CGD-associated p47\textsuperscript{phox} R42Q mutant expressed in p47\textsuperscript{phox} KO mouse PMNs. A, YFP fusion protein expression in p47\textsuperscript{phox} KO mouse PMNs was measured by flow cytometry. B, phox protein expression in p47\textsuperscript{phox} KO mouse PMNs transduced with p47YFP WT or R42Q mutant. 15 μg of protein was loaded in each lane, except 30 μg of protein from R42Q mutant-transduced PMNs was loaded for probing p47\textsuperscript{phox}. C, phox protein expression in p47\textsuperscript{phox} KO mouse PMNs transduced with p47\textsuperscript{phox} WT or R42Q mutant under puromycin selection. A vertical line was added when the sample was loaded in a non-continuous lane. D, distribution of p47YFP and p47\textsuperscript{phox}-R42Q in resting p47\textsuperscript{phox} KO mouse PMNs. N, nucleus. E, live image of p47\textsuperscript{phox} KO mouse PMNs expressing p47\textsuperscript{phox}-R42Q during SOZ phagocytosis. The arrow indicates the cup of phagosomes, and asterisks indicate the internalized phagosomes. Bar, 5 μm. Intracellular ROS production induced by hIgG-latex beads (F), serum-opsonized S. aureus (G), or hyphae (H), was measured in p47\textsuperscript{phox} KO mouse PMNs transduced with p47YFP or p47\textsuperscript{phox}-R42Q. The inset shows the intracellular phagosome ROS production measured in p47\textsuperscript{phox}-R42Q mutant induced by serum-opsonized S. aureus. F, p47\textsuperscript{phox}-transduced p47\textsuperscript{phox} KO neutrophils; ◇, p47\textsuperscript{phox}-R42Q-transduced p47\textsuperscript{phox} KO neutrophils; *, p47\textsuperscript{phox}-KO neutrophils (n = 3).
p47\textsuperscript{phox} PX Domain in NADPH Oxidase Activation

p47\textsuperscript{phox} is also reported to bind to the cytoskeletal protein moesin via its PX domain as assayed in vitro (63), although the molecular details of this interaction are not further characterized. Because of insensitivity to p47\textsuperscript{phox} PX domain mutations affecting phosphoinositide binding, interaction with moesin was proposed to mediate p47\textsuperscript{phox} plasma membrane translocation after insulin-like growth factor-1 stimulation in COS cells (29), although there was no direct evidence for this conclusion. The PX domain of p47\textsuperscript{phox} also binds to cytosolic phospholipase A\textsubscript{2}\textalpha, which is required for maximal response to fMLF or PMA stimulation in neutrophils or PLB-985 granulocytes (64). However, the interaction of p47\textsuperscript{phox} and cytosolic phospholipase A\textsubscript{2}\textalpha involves the face of the p47\textsuperscript{phox} PX domain opposite the phosphoinositide binding pocket (64).

A G125A mutation in NCF1, predicting a point substitution R42Q in p47\textsuperscript{phox}, was identified in two p47\textsuperscript{phox} CGD patients who lacked p47\textsuperscript{phox} protein and neutrophil NADPH oxidase activity (53, 54). Phosphoinositides do not bind to the isolated p47\textsuperscript{phox} PX domain harboring a R42Q mutation, which has been taken as evidence for phosphoinositide-mediated regulation of NADPH oxidase activity by the p47\textsuperscript{phox} PX domain (23, 29). However, we observed very low levels of full-length p47\textsuperscript{phox} R42Q and an abnormal subcellular distribution when expressed in either K562 cells or neutrophils. Despite this abnormal expression, small amounts of intracellular but not extracellular ROS release during phagocytosis were detected in murine neutrophils expressing p47YFP-R42Q, consistent with the concept that phosphoinositide binding to the p47\textsuperscript{phox} PX domain does not regulate intracellular NADPH oxidase activity. Taken together with prior studies of CGD patients with p47\textsuperscript{phox}-R42Q mutations (53, 54), these results indicate that this NCF1 mutant behaves as a null allele due to deleterious effects on neutrophil expression of p47\textsuperscript{phox} rather than deficient oxidant production from loss of p47\textsuperscript{phox} function.

The differential effect of the p47\textsuperscript{phox} PX domain on NADPH oxidase activity on plasma membrane versus phagosomes is consistent with prior studies showing that the environment and regulation of the NADPH oxidase differ in these two compartments. For example, the tail-to-tail interaction of p47\textsuperscript{phox}/p67\textsuperscript{phox} becomes dissociated after phagosome sealing during ingestion of IgG-opsonized zymosan (40). In addition, whereas the binding of PI3P to the p40\textsuperscript{phox} PX domain is not required for NADPH oxidase activity on the plasma membrane, PI3P is a strong positive regulator of NADPH oxidase activity after phagosome closure and is also required to retain p40\textsuperscript{phox} on the phagosome (6, 28).

In conclusion, we establish for the first time that the p47\textsuperscript{phox} PX domain plays a role in positively regulating neutrophil NADPH oxidase activity but that this role is differentially dependent on the membrane compartment and the agonist. Substitutions at residues critical for the ability of p47\textsuperscript{phox} to bind phosphoinositides substantially decrease plasma membrane enzyme activity in response to fMLF and PMA and, to a lesser extent, particulate stimuli. However, intracellular ROS production during phagocytosis was not affected by p47\textsuperscript{phox} PX mutations, in contrast to the important role for PI3P, binding to the p40\textsuperscript{phox} PX domain for oxidant production in phagosomes (6, 25, 26, 28, 51, 65).

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