FcyR-stimulated activation of the NADPH oxidase: phosphoinositide-binding protein p40phox regulates NADPH oxidase activity after enzyme assembly on the phagosome

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The phagocyte NADPH oxidase generates superoxide for microbial killing, and includes a membrane-bound flavocytochrome b558 and cytosolic p67phox, p47phox, and p40phox subunits that undergo membrane translocation upon cellular activation. The function of p40phox, which binds p67phox in resting cells, is incompletely understood. Recent studies showed that phagocytosis-induced superoxide production is stimulated by p40phox and its binding to phosphatidylinositol-3-phosphate (PI3P), a phosphoinositide enriched in membranes of internalized phagosomes. To better define the role of p40phox in FcγR-induced oxidase activation, we used immunofluorescence and real-time imaging of FcγR-induced phagocytosis. YFP-tagged p67phox and p40phox translocated to granulocyte phagosomes before phagosome internalization and accumulation of a probe for PI3P. p67phox and p47phox accumulation on nascent and internalized phagosomes did not require p40phox or PI3 kinase activity, although superoxide production before and after phagosome sealing was decreased by mutation of the p40phox PI3P-binding domain or wortmannin. Translocation of p40phox to nascent phagosomes required binding to p67phox but not PI3P, although the loss of PI3P binding reduced p40phox retention after phagosome internalization. We conclude that p40phox functions primarily to regulate FcγR-induced NADPH oxidase activity rather than assembly, and stimulates superoxide production via a PI3P signal that increases after phagosome internalization. (BLOOD. 2008;112:3867-3877)

Introduction

Phagocytic leukocytes are the front-line cellular defense against microbial attack, and are mobilized rapidly to the sites of infection where they ingest and kill opsonized microorganisms. The NADPH oxidase complex plays a central role in this process, as its assembly and activation on phagosomal membranes generate superoxide, the precursor of potent microbicidal oxidants. The importance of this enzyme is demonstrated by genetic defects in the NADPH oxidase complex that cause chronic granulomatous disease (CGD), characterized by recurrent severe and potentially lethal bacterial and fungal infections.1

The NADPH oxidase includes the membrane-integrated flavocytochrome b, composed of gp91phox and p22phox, and the cytosolic components p47phox, p67phox, p40phox, and Rac, a Rho-family GTPase, which translocate to flavocytochrome b upon cellular stimulation to activate superoxide production.2,4 Segregation of regulatory components to the cytosol in resting cells facilitates the temporal and spatial regulation of NADPH oxidase activity. The p67phox subunit is a Rac-GTP effector4 containing a domain that activates electron transport through the flavocytochrome.3 In resting cells, p67phox is associated with p40phox via complementary

PB1 (phagocyte oxidase and Bem1p) motifs present in each protein.2,6-8 p67phox is also linked to p47phox via a high-affinity interaction involving an SH3 domain and a proline-rich region, respectively, in the C-termini of these subunits.2,4,6,9 The p67phox, p47phox, and p40phox subunits can be isolated as a complex from neutrophil cytosol, and upon cellular activation, are believed to translocate as such to the flavocytochrome. p47phox plays a key role as a carrier protein as the other 2 cytosolic phox proteins fail to undergo membrane translocation in p47phox-deficient CGD neutrophils.10-12 Translocation to the flavocytochrome is mediated by a pair of SH3 domains within p47phox that are unmasked by activation-induced phosphorylation, which then bind to a proline-rich target sequence in p22phox.2,4,13

The role of p40phox, the most recently discovered NADPH oxidase subunit, has been controversial.14 Mutations in p40phox are not a cause of CGD,1 and p40phox is not required for high-level superoxide production in response to soluble agonists in either cell-free assays or whole-cell models.14-16 In addition to the PB1 domain that mediates binding to p67phox, p40phox has a PX (phox homology) and an SH3 domain. The physiologic target of the


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p40(phox) SH3 domain is uncertain, whereas the PX domain specifically binds phosphatidylinositol-3-phosphate (PI3P), which is enriched in early endosomes and also appears on phagosomes by the action of class III PI3 kinase (PI3K) within minutes of phagosome internalization in macrophages.

Despite the importance of phagocytosis-induced superoxide production for host defense, the events regulating NADPH oxidase assembly and activation on the phagosome are incompletely defined. p47(phox) and p67(phox) are detected on the cup of newly forming phagosomes, and then on internalized phagosomes for many minutes after ingestion. Oxidant production can also begin on the plasma membrane and continues after phagosome internalization. Phagocytosis activates multiple signaling pathways, including PI3K’s, although their specific roles are still being defined.

Additional target is required for regulation of FcγR-induced phagocytosis. Although FLB-985 neutrophils expressing fluorescently tagged p40(phox) was substantially reduced in neutrophils and FLB-985 granulocytes following recognition that the PX domain of p40(phox) binds to p31,18-22 p40(phox) was established as an important regulator of phagocytosis-induced superoxide production. In COS(phox) cells with transgenes for flavocytochrome b, p47(phox), p67(phox), and the FcyRII receptor, NADPH oxidase activity induced by phagocytosis of IgG-opsonized targets required corexpression of p40(phox). In addition, superoxide production in response to IgG-opsonized particles and Staphylococcus aureus was substantially reduced in neutrophils and PB-985 granulocytes lacking p40(phox).

PI3P binding to p40(phox) was essential to stimulate phagosomal oxidase activity in both neutrophils and COS phenomes. In both the COS(phox) model and in permeabilized human neutrophils, mutants in the p40(phox) PHB and SH3 domains, especially the double mutation, also impaired p40(phox) function, which suggests that binding of p40(phox) to p67(phox) as well as an additional target is required for regulation of FcγR-induced superoxide production.

The underlying mechanism(s) by which p40(phox) regulates phagocytosis-activated superoxide production is not fully understood. p40(phox) has been proposed both to function as a second cascade protein that mediates recruitment of p67(phox) to PI3P-rich phagosome membranes and to regulate activity of the oxidase complex in combination with PI3P. To better define the role of p40(phox) in superoxide production during phagocytosis, we examined the dynamics of FcγR-induced p40(phox) accumulation on phagosomes and its coordination with NADPH oxidase assembly and activation, using both the COS(phox) model and videomicroscopy of PB-985 neutrophils expressing fluorescently tagged phox subunits and/or the PX domain of p40(phox), a robust probe for PI3P.
Western blotting

Triton X-100 lysates from COS7 or neutrophil-differentiated PLB-985 cells were prepared for electrophoresis and Western blots as described.36,41 In some experiments, the Triton X-100-insoluble pellet was also analyzed by electrophoresis and Western blotting as described.42

IgG-opsonized particles and assays for NADPH oxidase activity

SRBCs and 3.3-μm Latex beads were opsonized with human IgG as described.30 Zymosan A particles were opsonized with either Bioparticle Oposning Reagent (rabbit anti–zymosan IgG) according the manufacturer’s instructions or with human IgG at 20 μg/mL at 37°C for 60 minutes. Final stock solutions were prepared at 20 μg/mL in PBS and stored at −20°C until use.

NADPH oxidase activity was assayed using chemiluminescence enhanced by luminol or isoluminol, which is membrane-impermeant; both compounds detect superoxide in a peroxides-dependent reaction.43,44 PLB-985 cells (2.5 × 10^5) or COS7-derived cells (2.5 × 10^5) in PBSG (PBS plus 0.9 mM CaCl2, 0.5 mM MgCl2, 20 mM dextrose) in the presence of 50 μM luminol or 50 μM luminol, without or with superoxide dismutase (SOD; final concentration: 75 μg/mL), were preincubated at 37°C for 10 minutes. Horseradish peroxidase (HRP; final concentration: 20 μU/mL) was added to isoluminol assays and to luminol assays of COSphox lines. IgG-opsonized particles or phorbol myristate acetate (PMA, 300 ng/mL) was added to activate cells (final volume: 200 μL), and the relative light units (RLU) were monitored at 60- to 90-second intervals for up to 1 hour by the Long Kinetic module in an Lmax microplate luminometer from Molecular Devices (Sunnyvale, CA). Integrated RLU values were calculated by SOFTmax software (Molecular Devices). In some experiments, a synchronized phagocytosis protocol45 was adapted for NADPH oxidase assays. Briefly, PLB-985 neutrophils in 200 μL PBSG were incubated on ice for 5 minutes in 50 μM luminol and 20 μU/mL HRP or 20 μM luminol, 75 μg/mL SOD, and 2000 U/mL catalase, then 25 μL cold IgG-zymosan (final concentration: 400 μg/mL) or cold IgG-Latex beads (cell:beads = 1:8) were added. Cells and particles were spun at 240g for 5 minutes at 4°C, then immediately placed at 37°C in the luminometer. For some experiments, cells were preincubated for 30 minutes at 37°C in the presence or absence of 100 nM wortmannin.

Immunofluorescence microscopy

COSPF-p67YFP, COSPF40-p67YFP, and COS7-WF cells were plated into coverslip-bottomed dishes (MatTek Cultureware, Ashland, MA) and incubated at 37°C for 24 hours, washed with fresh media, loaded with IgG-SRBCs prelabeled with goat Alexa-633 anti–rabbit IgG, incubated for 15 minutes at 37°C, and washed with cold PBS. After distilled water lysis of external SRBCs, cells were fixed with 4% paraformaldehyde for 30 minutes. Samples were permeabilized with 0.2% Triton X-100 in PBS, blocked with 10% goat serum plus 2% BSA in PBS, and immunostained with anti-p47phox followed by Alexa-555 goat anti–mouse IgG. Nuclei were stained with 5 μg/mL DAPI in PBS for 5 minutes. Slides were imaged on a Zeiss LSM-510 confocal microscope (Carl Zeiss, Jena, Germany) using a 100×/1.4 NA oil-immersion objective. Samples were scanned sequentially at each excitation wavelength to minimize crosstalk between signals. Zeiss LSM software (Carl Zeiss) was used for image handling. Images shown are representative of at least 3 independent experiments.

Live cell imaging

A spinning-disk confocal system mounted on a Nikon TE-2000U inverted microscope (Nikon, Melville, NY) with an Ixon air-cooled EMCCD camera (Andor Technology, South Windsor, CT) and 100×/1.4 NA objective was used to film phagocytosis in living PLB-985 cells. Differentiated PLB-985 cells were loaded onto coverslip-bottomed dishes, which were mounted on the microscope and maintained at 37°C using a stage incubator (Warner Instruments, Hamden, CT). After 3 minutes, IgG-zymosan was loaded into the dish. Videos were made over an approximately 30-minute interval after the addition of IgG-zymosan. Fields were monitored randomly to identify cells beginning to ingest a particle, and sequential images collected with 488 nm and/or 568 nm excitation and 0.3-second exposure with a time lapse of 5 or 10 seconds for 5 to 8 minutes. Phagosomes being filmed often moved away from the focal plane, so a new cell beginning to ingest a particle was identified for filming. MetaMorph (Universal Imaging, Downingtown, PA) was used for image handling. Images shown are representative of at least 4 independent experiments except for studies on PLB-985 neutrophils transiently expressing YFP-p40phox-W207R or YFP-p40phox-D289A, which were performed 2 and 3 times, respectively.

To further assess translocation of fluorescent probes, images were analyzed using Image J (National Institutes of Health [NIH], Bethesda, MD). An area of the phagosome rim (∼ 25% of the total rim) was outlined by hand, and the mean fluorescence intensity within the area determined, and ratios were determined against the value from a corresponding area in the cytoplasm near the phagosome. At least 5 phagosomes monitored at each stage—cup, closure (time of sealing), and after internalization (200-300 seconds after closure, or 120 seconds after closure for YFP-p40phox R105A and for wortmannin-treated cells)—were analyzed by this method and the mean plus or minus SEM was determined.

Results

Recruitment of p67phox and p47phox to phagosomes is independent of p40phox in COSphox cells

We examined whether expression of p40phox is required for recruitment of p67phox and p47phox to phagosomes in the COSphox model. To facilitate imaging, COS7 cells were generated that stably expressed p67phox-YFP along with p91phox, p22phox, p47phox, and the Fcy-IIA receptor, without or with coexpression of p40phox (Figure 1A). Previous studies showed that p67phox tagged in this manner supports NADPH oxidase activity at levels similar to untagged p67phox.23 In NADPH oxidase assays using luminol-enhanced chemiluminescence, although both lines responded similarly to PMA stimulation (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article), there was very little IgG-SRBC–stimulated superoxide in the absence of p40phox (Figure 1B,C), confirming our previous study.20

Next, the fate of p67phox-YFP was monitored by confocal microscopy in COSPF-p67YFP and COSPF40-p67YFP cells incubated with IgG-SRBCs. The results showed that p67phox-YFP was present on phagosomes in both COSPF-p67YFP and COSPF40-p67YFP cells (Figure 2). Indirect immunofluorescence showed that p47phox colocalized with p67phox-YFP on phagosomes in both cell lines (Figure 2B; Figure S2), consistent with the concept that p47phox and p67phox are tightly linked via a tail-to-tail interaction and translocate as a unit.2,6,9,46 No p47phox staining was observed in control COS-WF cells (Figure S2). These data indicate that although FcγR-activated superoxide production has a strong requirement for p40phox in COSphox cells, the absence of p40phox does not prevent phagosomal translocation of p47phox and p67phox.

NADPH oxidase activation and translocation of p67phox-YFP and YFP-p40phox during phagocytosis in PLB-985 neutrophils

Assembly and activation of the NADPH oxidase complex was studied in neutrophil-differentiated PLB-985 cells expressing YFP-p40phox (PLB-YFP40) or p67phox-YFP (PLB-p67YFP). Endogenous and YFP-tagged subunits were expressed at comparable levels (Figure 3A and data not shown). IgG-zymosan–induced NADPH oxidase activity in PLB-p67YFP neutrophils was assayed where particles were bound to cells at 4°C followed by warming to 37°C to initiate phagocytosis. Superoxide production began before

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sealing of phagosomes, as detected by isoluminol-enhanced chemiluminescence (Figure 3B,C), although the magnitude of this response is small in comparison to extracellular superoxide detected following fMLP (not shown) or PMA (Figure S3A) stimulation. Peak rates of superoxide release occurred within minutes of warming, then declined to lower levels. NADPH oxidase activity in internalized phagosomes was detected using luminol in the presence of SOD and catalase (Figure 3D,E), with peak rates shifted in time compared with isoluminol. IgG-zymosan–induced NADPH oxidase activity both before and after phagosome internalization was substantially decreased by 100 nM wortmannin (Figure 3B-D). Of note, wortmannin had no effect on the ability of PLB-985 neutrophils to ingest IgG-zymosan particles (average of 1.9 phagosomes per cell in 50 to 65 cells analyzed in videos of untreated and wortmannin-treated cells). IgG-zymosan–stimulated superoxide production and wortmannin sensitivity of parental PLB-985 cells and PLB-YFPp40 were similar to PLB-p67YFP cells (not shown).

Finally, as previously reported by many groups (eg, Arcaro and Wymann47; Laudanna et al48), wortmannin had no effect on PMA-induced NADPH oxidase activity (Figure S3B).

Time-lapse confocal videomicroscopy was used to monitor recruitment of YFP-p40phox and p47phox to nascent phagosomes during ingestion of IgG-zymosan by PLB-985 neutrophils (Figure 4A,B; Videos S1,S2). Both p47phox-YFP and YFP-p40phox, which were otherwise distributed homogenously in the cytoplasm, accumulated on the phagosomal cup, visible 40 to 60 seconds before phagosome closure (sealing), and probes remained detectable on phagosomes for at least 5 minutes after closure. Accumulation of fluorescently tagged phox subunits often appeared nonuniform and/or “clumpy” (Figures 4-7 and Videos S1-S8). Although superoxide production was strongly dependent on PI3K activity (Figure 3B), wortmannin did not prevent recruitment of either p47phox-YFP or YFP-p40phox to nascent phagosomes (Figure 4C,D; Videos S3,S4). Interestingly, although p40phox-YFP accumulation persisted after internalization, we observed that p40phox-YFP accumulation disappeared in 2 of 7 phagosomes within 2 minutes after sealing, leading to a decline in average fluorescence intensity of the p40phox-YFP probe (Figure 4D).

NADPH oxidase assembly and activation on phagosomes were next analyzed in p40phox-deficient PLB-p67YFP cells generated using a p40phox-targeted shRNA. Western blotting showed a substantial decrease in p40phox and a small reduction in endogenous p67phox, similar to previous studies in p40phox-deficient mouse neutrophils,28 but expression of YFP-tagged p67phox appeared to be
unaffected (Figure 5A). Similar to previous studies,28,33,34 superoxide production in internalized phagosomes was reduced by approximately 2-fold in p40\textsubscript{phox}-deficient PLB-p67YFP cells stimulated with either IgG-zymosan (Figure 5D,E) or IgG-opsonized Latex beads (not shown). Superoxide production before phagosome sealing was also reduced by approximately 40% (Figure 5B,C). However, p67\textsubscript{phox}-YFP accumulated on the phagosomal cup in p40\textsubscript{phox}-deficient PLB-p67YFP cells and persisted for at least 5 minutes after closure (Figure 5F; Video S5), similar to PLB-p67YFP cells that express endogenous p40\textsubscript{phox} (Figure 4A). The independence of p67\textsubscript{phox}-YFP translocation on p40\textsubscript{phox} is consistent with our findings in COS\textsubscript{phox} cells (Figure 2), and supports a model in which translocation of p67\textsubscript{phox} to nascent and internalized phagosomes does not require p40\textsubscript{phox}.

**Role of PI3P and the PX, SH3, and PB1 domains of p40\textsubscript{phox} in Fc\textsubscript{R} induced p40\textsubscript{phox} translocation to phagosomes**

The appearance of YFP-p40\textsubscript{phox} in the phagosomal cup before the expected accumulation of phagosomal PI3P, which in macrophages occurs after phagosome internalization,18-20 and its insensitivity to wortmannin suggested that the initial recruitment of p40\textsubscript{phox} to phagosomes does not require binding to PI3P. To directly examine the temporal relationship between the appearance of p40\textsubscript{phox} and PI3P on neutrophil phagosomes, phagocytosis was analyzed in PLB-YFPp40 neutrophils coexpressing the p40\textsubscript{phox} PX domain (PX\textsubscript{40}), a probe for PI3P,2,3,30,49,50 tagged with mCherry. Although YFP-p40\textsubscript{phox} was detected on the phagosomal cup, Cherry-PX\textsubscript{40} did not accumulate until 40 to 60 seconds after the phagosome was sealed and internalized (Figure 6A; Videos S6,S7). Preincubation of cells in 100 nM wortmannin abolished accumulation of Cherry-PX\textsubscript{40}, consistent with a requirement for PI3K to generate PI3P,18,19 but YFP-p40\textsubscript{phox} was still present on many phagosomes (Figure 6B). These results further establish that recruitment of p40\textsubscript{phox} is not mediated by PI3P.

The role of each of the 3 p40\textsubscript{phox} modular domains in translocation to phagosomes was examined by expressing YFP-tagged p40\textsubscript{phox} mutants (Figure 7 A) in PLB-985 neutrophils. These included a PX domain mutant, R105A, that eliminates binding to PI3P, the SH3 domain mutant W207R, and a PB1 domain mutant, D289A, that prevents binding of p40\textsubscript{phox} to p67\textsubscript{phox}.2,3,30,49,50 The p40\textsubscript{phox}D289A mutant was poorly expressed from a stable transgene in PLB-985 granulocytes, most likely because binding to p67\textsubscript{phox} is important for p40\textsubscript{phox} stability in neutrophils, as inferred from studies of p67\textsubscript{phox}-deficient CGD neutrophils which have markedly reduced levels of p40\textsubscript{phox}14; p40\textsubscript{phox}W207R was also poorly expressed in PLB-985 granulocytes, for uncertain reasons (Figure S4). Thus, we used a transient expression protocol developed for neutrophils40 to study translocation of W207R, D289A, and a double W207R/D289A p40\textsubscript{phox} mutant in comparison with wild-type p40\textsubscript{phox}. Translocation of p40\textsubscript{phox}R105A was studied both as a transiently expressed protein and as expressed from a stable transgene, with similar results. Note that although an R57Q PI3P-binding mutant of p40\textsubscript{phox} was enriched in the Triton X-100–insoluble fraction,42 this was not observed for p40\textsubscript{phox}R105A (Figure S4).
Imaging by confocal videomicroscopy showed that R105A and W207R YFP-p40 phox mutants were recruited to the phagosomal cup, similar to wild-type YFP-p40 phox (Figure 7B,C). In contrast, YFP-p40 phox derivatives with a mutation that prevents binding to p67 phox, D289A, and W207R/D289A YFP-p40 phox were rarely detected on phagosomes, either before or after phagosome closure (Figure 7B and data not shown). For example, D289A YFP-p40 phox translocation was seen in only 2 of 30 phagosomes analyzed.

Wild-type YPP-p40 phox and W207R YFP-p40 phox were present on internalized phagosomes, similar to previous observations in the COS phox model. However, although the PI3P-binding mutant R105A YFP-p40 phox appeared on nascent phagosomes, it often disappeared within minutes after closure (Figure 7B–D). In phagosomes followed from time of cup formation, R105A YFP-p40 phox appeared on only approximately one third of internalized phagosomes for longer than 3 minutes after sealing, in contrast to wild-type YFP-p40 phox (P < .03; Fisher exact test; Figure 7D). In contrast, a similar analysis in p40phox-deficient PLB-p67YFP cells found that p67phox-YFP was detected on 11 of 11 phagosomes monitored for at least 3 minutes after phagosome closure (see also Figure 5F; Video S5). These data indicate that the PB1 domain–mediated interaction between p40phox and p67phox is required for recruitment of p40phox, but not p67phox, to both nascent and internalized phagosomes, even with an intact binding site for PI3P in p40phox. However, p40phox binding to PI3P appears to partially influence whether p40phox is present on phagosomal membranes after internalization. This effect is more pronounced compared with wortmannin treatment, which may reflect an influence of other wortmannin-inhibited pathways.

Role of the p40phox PX domain in FcγR-induced NADPH oxidase activity

To examine how loss of PI3P binding by p40phox affects NADPH oxidase activity, PLB-985, PLB-YFPp40, or PLB-YFPp40R105A cells were made deficient in endogenous p40phox using an shRNA targeting the 3′ untranslated region (Figure 7E). As in a previous study using this shRNA, p67phox expression in neutrophil-differentiated p40KD knockdown (p40KD) lines was similar to PLB-985 cells (Figure 7E). PMA-induced superoxide release in p40KD PLB-985 cells was robust and similar to p40KD PLB-985 cells expressing YFP-p40phox or YFP-p40phoxR105A. However, IgG-particle–activated superoxide production both before and after phagosome internalization was decreased by at least 40% in p40KD cells and p40KD cells expressing YFP-p40phoxR105A, compared with p40KD cells expressing YFP-p40phox (Figure 7F). Indeed, expression of YFP-p40phoxR105A tended to decrease activity to a greater extent than p40KD alone, suggestive of an inhibitory effect. Similar to studies in PLB-985 cells (Figure 7B–D), the R105A mutation did not prevent p40phox translocation to nascent phagosomes in p40KD cells (Video S8), consistent with the concept that although PI3P binding to p40phox stimulates enzyme activity, it is not required for initial p40phox translocation. However, as in Figure 7B–D, YFP-p40R105A...
often disappeared from internalized phagosomes in p40KD cells; of 8 phagosomes analyzed from time of cup formation, YFP-p40R105A was present on only 3 for longer than 3 minutes (see also Video S8).

**Discussion**

PI3Ks products play important roles in regulating superoxide production during phagocytosis, particularly PI3P that is enriched in membranes of internalized phagosomes and whose effects on NADPH oxidase activity are mediated via p40

phox

. The current study used real-time imaging of phagocytosis of IgG-opsonized particles to analyze assembly of the NADPH oxidase complex in neutrophil-differentiated PLB-985 cells. Particular emphasis was given to investigating the recruitment of the cytosolic NADPH oxidase subunits, p40

phox

 and p67

phox

, and the relationship between their translocation, the accumulation of PI3P, and superoxide production, to better characterize the role of p40

phox

, which has high-affinity binding domains for both p67

phox

 and PI3P.

This study confirms previous reports that p67

phox

 is recruited to nascent granulocyte phagosomes and is present after internalization, and shows for the first time that p40

phox

 translocation during phagocytosis exhibits similar behavior and fails to accumulate on phagosomes if unable to bind to p67

phox

. Conversely, p40

phox

 was not required for accumulation of p67

phox

 and p47

phox

 on IgG-zymosan phagosomes in COS

phox

 cells expressing FcγIIA, although NADPH oxidase activity was minimal unless p40

phox

 was coexpressed. Translocation of p67

phox

 on nascent and internalized phagosomes was also observed in p40

phox

- deficient PLB-985.
neutrophils. Furthermore, p67phox and p40phox accumulated on nascent phagosomes before the appearance of a PI3P probe, and in the presence of the PI3K inhibitor wortmannin, indicating that PI3P binding to p40phox is dispensable for the initial recruitment of p67phox and p40phox. Taken together, although confirming the importance of p40phox for phagocytosis-induced oxidant production, our results do not support a model in which PI3P-bound p40phox plays a significant role as a second carrier protein for p67phox to the phagosome, in addition to p47phox. Although a recent study in arachidonic acid–stimulated RAW 267.4 macrophage cells showed that PI3P-dependent binding of p40phox can mediate recruitment of p67phox to early endosomes,53 this setting is likely to have differences with phagocytic receptor–induced recruitment.

The current studies also reveal new insights into FcγR-induced p40phox recruitment to granulocyte phagosomes and the relative roles of its PI3P- and p67phox-binding domains. Simultaneous imaging of fluorescently tagged probes determined that full-length p40phox translocated to the phagosomal cup, whereas accumulation of the p40phox PX domain, a probe for PI3P, occurred 40 to 60 seconds after phagosome sealing, kinetics similar to PI3P probes in macrophages.18,19 Neither inhibition of PI3K by wortmannin nor a R105A mutation in p40phox that prevents PI3P binding eliminates recruitment of full-length p40phox to the phagosomal cup. However, a p40phox D289A mutation that disrupts its binding to p67phox almost completely abolishes IgG-zymosan–induced translocation of p40phox in PLB-985 neutrophils, similar to the COSphox model.30 The importance of the PB1 domain for p40phox translocation in response to a physiologic signal extends results in a K562 cell model stimulated with either PMA or a muscarinic receptor50 and in a PMA-stimulated permeabilized human neutrophil system.34,37 Taken together, our data indicate that translocation of p40phox to both nascent and PI3P-rich internalized phagosomes is dependent on p67phox and does not initially require binding to PI3P. However, both wortmannin, and to an even greater extent, an
R105A PI3P-binding mutant affected the persistence of p40\textsuperscript{phox} on internalized phagosomes. Our results support a model in which p40\textsuperscript{phox}, p67\textsuperscript{phox}, and p47\textsuperscript{phox}, linked by separate high-affinity binding sites in p67\textsuperscript{phox} for p47\textsuperscript{phox} and for p40\textsuperscript{phox}, translocate as a trimeric complex to the membrane-integrated flavocytochrome b after activation-induced unmasking of the p47\textsuperscript{phox}-binding site for p22\textsuperscript{phox} 2.4-37,52-55 This model is consistent with studies in CGD patients lacking either flavocytochrome b or p47\textsuperscript{phox} showing that stable translocation of the trimeric phox complex requires interactions between p47\textsuperscript{phox} and flavocytochrome b.10-12 For example, neither p67\textsuperscript{phox} nor p40\textsuperscript{phox} undergoes membrane translocation in p47\textsuperscript{phox}-deficient CGD neutrophils, whereas p47\textsuperscript{phox} translocation is unaffected in p67\textsuperscript{phox}-deficient CGD.30-12 Taken together, these data suggest that p47\textsuperscript{phox} is necessary and sufficient for stable recruitment of p67\textsuperscript{phox} to the flavocytochrome b in phagosomal membranes, which in turn mediates that of p40\textsuperscript{phox}.

A second implication from our results is that recruitment of cytosolic phox subunits of the NADPH oxidase complex to the phagosome is insufficient for high-level superoxide production, and the predominant role of PI3P binding by p40\textsuperscript{phox} is to stimulate FcγR-induced superoxide production after enzyme assembly rather than to drive p67\textsuperscript{phox} translocation. Whereas wild-type p40\textsuperscript{phox} rescued phagocytosis-induced superoxide production in p40\textsuperscript{phox}-deficient PLB-985 neutrophils phagosomes, the R105A PX domain mutant did not, confirming previous studies.30,33 and extending these findings to show that an intact PI3P-binding domain is also required before sealing. This suggests that there may be small amounts of PI3P in the plasma membrane not detectable by imaging probes, as previously postulated.28,33 and that the marked increase in PI3P on internalized phagosomes may function to up-regulate oxidative activity in this sequestered compartment. In addition, since p40\textsuperscript{phox}-R105A is present in phagosomal cup, these results further establish a role for this domain apart from one in translocation. That PI3P-bound p40\textsuperscript{phox} has a direct effect on the assembled enzyme is supported by studies in semirecombinant systems in which p40\textsuperscript{phox} stimulates superoxide production in the presence of PI3P,22,35 and where PI3P binding by the p40\textsuperscript{phox} PX domain is required for oxidative activity but not for subunit translocation in vitro.34 Finally, a comparison of FcγR-induced oxidative activity in wortmannin-treated PLB-985 cells with cells expressing p40\textsuperscript{phox}-R105A suggests that a substantial portion of the wortmannin effect both before and after internalization is mediated through inhibition of PI3P production, again confirming and extending a study in murine neutrophils expressing a PI3P-binding p40\textsuperscript{phox} mutant.33

The R105A PX domain mutant of p40\textsuperscript{phox}, which is unable to bind to PI3P, was recruited to nascent phagosomes but often disappeared within a few minutes after internalization. Why the PB1 domain–mediated interaction between p67\textsuperscript{phox} and p40\textsuperscript{phox}, which is necessary for p40\textsuperscript{phox} translocation to the phagosome, appears insufficient to maintain p40\textsuperscript{phox}-R105A after internalization is a paradox that will require further investigation. This observation also suggests that protein-protein interactions between oxidase subunits may be dynamic and are modified after assembly of the NADPH oxidase complex. In the resting state, the ability of the p40\textsuperscript{phox} PX domain to access PI3P in the membrane is masked by an intramolecular interaction with the p40\textsuperscript{phox} PB1 domain on the face opposite of the region that binds to p67\textsuperscript{phox}.56 The mechanism that disrupts the intramolecular p40\textsuperscript{phox} PX-PB1 interaction, leading to exposure of the PI3P-binding site, is unknown, although it does not appear to involve p40\textsuperscript{phox} phosphorylation.55,56 It is possible that conformational changes in p40\textsuperscript{phox} that accompany the unmasking of its PX domain or other events after phagosome internalization result in a requirement for PI3P binding in order for p40\textsuperscript{phox} to be retained on phagosomes. A role for PI3P in p40\textsuperscript{phox} retention after phagosome internalization may contribute to reduced NADPH oxidase activity in cells expressing a PI3P-binding mutant of p40\textsuperscript{phox}.

In summary, this study demonstrates that membrane translocation of p40\textsuperscript{phox} during FcγR-induced phagocyte NADPH oxidase assembly begins in the phagosomal cup and requires binding to p67\textsuperscript{phox}. The data are consistent with a model in which p47\textsuperscript{phox} functions as the key adaptor protein that is necessary and sufficient to recruit p67\textsuperscript{phox} and p40\textsuperscript{phox} to the membrane-bound flavocytochrome b. Although p40\textsuperscript{phox} is not required to mediate assembly of the other cytosolic phox subunits on the phagosome, p40\textsuperscript{phox} stimulates activity of the assembled NADPH oxidase complex via a PI3P signal that is spatially and temporally regulated to increase on internalized phagosomes. Future challenges include identifying underlying mechanisms by which the PX domain in p40\textsuperscript{phox} becomes accessible to PI3P, how PI3P influences localization of p40\textsuperscript{phox} after phagosome closure, and how p40\textsuperscript{phox} stimulates activity of the NADPH oxidase complex. For example, it is possible that PI3P-bound p40\textsuperscript{phox} induces conformational changes in other NADPH oxidase subunits, or acts to tether the oxidase complex to an optimal membrane microdomain, as recent studies in a cell-free model system suggest that the membrane phospholipid environment can have a large influence on the activity of the assembled oxidase complex.57

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Authorship

Contribution: W.T. and X.J.L. designed, performed, and analyzed experiments, prepared the figures, and helped draft the paper; W.M. and N.D.S. performed and analyzed experiments; C.-I.S. prepared critical reagents; S.A.B. and M.B.Y. provided a critical reagent; S.G. helped with interpretation of data and paper preparation; S.J.A. and M.B.Y. analyzed and interpreted data and helped with paper preparation; and M.C.D. oversaw this entire project including the experimental design, analysis, interpretation of the data, and preparation of the paper.

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FcγR-stimulated activation of the NADPH oxidase: phosphoinositide-binding protein p40phox regulates NADPH oxidase activity after enzyme assembly on the phagosome

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