MOLECULAR AND CELLULAR BIOLOGY, Jan. 2007, p. 312–323 Vol. 27, No. 1
0270-7306/07/$08.00+0 doi:10.1128/MCB.00985-06

The Rac Effector p67^{phox} Regulates Phagocyte NADPH Oxidase by Stimulating Vav1 Guanine Nucleotide Exchange Activity▽†

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Received 2 June 2006/Returned for modification 10 July 2006/Accepted 9 October 2006

The phagocyte NADPH oxidase catalyzes the reduction of molecular oxygen to superoxide and is essential for microbial defense. Electron transport through the oxidase flavocytochrome is activated by the Rac effector p67^{phox}. Previous studies suggest that Vav1 regulates NADPH oxidase activity elicited by the chemoattractant formyl-Met-Leu-Phe (fMLP). We show that Vav1 associates with p67^{phox} and Rac2, but not Rac1, in fMLP-stimulated human neutrophils, correlating with superoxide production. The interaction of p67^{phox} with Vav1 is direct and activates nucleotide exchange on Rac, which enhances the interaction between p67^{phox} and Vav1. This provides new molecular insights into regulation of the neutrophil NADPH oxidase, suggesting that chemoattractant-stimulated superoxide production can be amplified by a positive feedback loop in which p67^{phox} targets Vav1-mediated Rac activation.

The NADP (NADPH) oxidase in phagocytic leukocytes plays a crucial role in host defense by virtue of its ability to convert molecular oxygen to superoxide, the precursor to microbicidal oxidants (2). The redox center is a heterodimeric flavocytochrome b_{558} comprised of two integral membrane proteins, gp91^{phox} and p22^{phox} (where *phox* stands for *ph*agocyte *ox*idase). Activation of electron transfer from NADPH to molecular oxygen requires recruitment of the cytosolic oxidase subunits p47^{phox} and p67^{phox}, in addition to Rac-GTP. In humans, genetic deficiency of any one of these four *phox* subunits of NADPH oxidase results in a disorder of innate immunity known as chronic granulomatous disease, which is characterized by recurrent and severe bacterial and fungal infections (2).

The molecular events associated with NADPH oxidase assembly and activation are partially defined. Phagocyte activation by soluble or particulate inflammatory mediators initiates signaling cascades that lead to p47^{phox} phosphorylation and Rac activation to assemble the active oxidase complex. Phosphorylation of p47^{phox} unmasks a pair of Src homology 3 (SH3) domains that mediate its translocation to the flavocytochrome via a proline-rich sequence on p22^{phox} (18). The p47^{phox} subunit functions as an adaptor protein to recruit p67^{phox} via a high-affinity interaction between a proline-rich region and SH3 domain in the C termini of p47^{phox} and p67^{phox}, respectively (18). p67^{phox} is a target of Rac-GTP and contains an "activation" domain that regulates flavocytochrome *b* (29). This domain is just distal to the N-terminal portion of p67^{phox} that binds to the switch 1 effector domain of Rac-GTP (18, 24, 25). Current experimental evidence supports a model in which complex formation between p67^{phox} and membrane-anchored Rac-GTP is required to optimally position p67^{phox} for activation of electron transport through flavocytochrome *b* (12, 18, 36).

The Rac GTPase translocates to the plasma membrane independently of p67^{phox} and p47^{phox} (13, 15, 20) and is required for NADPH oxidase activity. In resting cells, Rac-GDP is present as a complex with Rho-GDP disassociation inhibitor (Rho-GDI), a negative regulator of Rho family GTPases, but this complex rapidly dissociates, and Rac-GTP forms in stimulated cells. This process is facilitated by activation of guanine nucleotide exchange factors (GEFs) and is accompanied by translocation of Rac to the plasma membrane with kinetics similar to p47^{phox} and p67^{phox} (13). The catalytic activity of the oxidase is dependent on interactions between Rac-GTP and p67^{phox} (12, 24, 25), as mentioned above. Phagocytic leukocytes express Rac1 and Rac2 (13), two closely related isoforms that interact with the Rac binding domain of p67^{phox} with similar affinity (25). The hematopoietic cell-restricted Rac2 is the preferred isoform to regulate neutrophil NADPH oxidase activity in response to most agonists (13, 22, 26, 34, 44), whereas Rac1 appears to play a more important role in macrophages and human monocytes (43, 46).

The specific GEFs that regulate the NADPH oxidase are not well defined. Recent studies indicate that P-Rex1, a GEF that is activated by phosphoinositol-3,4,5-phosphate and Gβγ subunits of heterotrimeric G proteins, participates in chemoattrac-

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† Supplemental material for this article is available at http://mcb.asm.org/.

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▽ Published ahead of print on 23 October 2006.

tant-mediated activation of superoxide production (14, 40, 41). Additional studies suggest that Vav1, a hematopoietic cell-specific isoform of the Vav family of Rho/Rac GEFs, which are activated by tyrosine phosphorylation (37), also plays an important role in regulating chemoattractant-induced NADPH oxidase activity. Vav1 undergoes tyrosine phosphorylation in murine neutrophils stimulated with formyl-Met-Leu-Phe (fMLP), and superoxide production by fMLP-stimulated neutrophils from Vav$^{-/-}$ mice is attenuated by approximately threefold compared to stimulated neutrophils from wild-type mice (23). However, overall levels of activated Rac1 and Rac2 are similar in fMLP-stimulated Vav1$^{-/-}$ and wild-type neutrophils (16, 23). Additional studies in COS-7 cells stably expressing transgenic NADPH oxidase subunits found that a constitutively active form of Vav1 stimulates translocation of p67^{phox} and p47^{phox} to the plasma membrane and activates superoxide production (32). Moreover, constitutively active Vav1 stimulates NADPH oxidase activity more efficiently than Vav2 or Tiam1 (32). Taken together, these data suggest that Vav1 may regulate a specific pool of Rac in proximity to the NADPH oxidase and are consistent with the concept that GEFs are not functionally redundant but act in the context of specific downstream signaling pathways (35).

Three structurally related Vav isoforms have been identified in mammalian cells (37, 38). Vav1 expression is predominantly limited to the hematopoietic lineage, whereas Vav2 and Vav3 are widely expressed. Typical of other Rho family GEFs, all three Vav family members have a Dbl homology (DH) region that mediates guanine nucleotide exchange. This is flanked by domains that regulate GEF activity and others that mediate protein-protein interactions. These include a calponin homology domain and an acidic region N-terminal to the DH domain, and at the C-terminal side are a pleckstrin homology (PH) domain, a cysteine-rich region also referred to as the zinc finger (ZF) domain, and three juxtaposed Src homology domains (SH3-SH2-SH3) (37). Vav1 facilitates guanine nucleotide exchange for Rho family GTPases with some preference for Rac (10, 38). Phosphorylation of tyrosines within the acidic domain of Vav family GEFs relieves autoinhibitory intramolecular interactions involving the N-terminal portion of the protein that block access to the DH domain, thereby activating GEF activity. The PH domain also appears to regulate GEF activity via binding of phosphatidylinositols (19, 37). Vav family GEFs are activated by ligation of a variety of receptors, including the T-cell and B-cell antigen receptors, growth factor receptors, integrins, and chemokine receptors (37, 38). Genetic deletion of Vav1 family proteins leads to a variety of defects in T- and B-cell development and function, along with other phenotypic changes including leukocyte adhesion and chemokine signaling (37, 38).

This study further examines the role of Vav1 in regulation of the NADPH oxidase. The results show that p67^{phox}, a target of Rac-GTP in the oxidase complex, interacts with Vav1, which activates nucleotide exchange on Rac and enhances superoxide production. The studies presented here suggest that Vav1 plays an important role as a NADPH oxidase-targeted GEF and provide new molecular insights into the function of p67^{phox} in the oxidase complex and the regulation of Vav1 exchange activity, which act in a positive feedback loop for amplification of superoxide production in response to chemoattractants.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Rabbit anti-Vav1 antibody (SC-132) and goat anti-p67^{phox} antibody (N-19) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies (MAbs) against Vav1 (catalog no. 05-219), Rac1 (23A8; 05-389), Myc-epitope tag (9E10; 05-419), and phosphotyrosine (4G10; 05-321) were from Upstate Biotechnology (Lake Placid, NY). His epitope tag MAb (27471001), an ECL detection kit, protein A-Sepharose-4 Fast Flow (17-5280-01), and glutathione-Sepharose-4B (17-0756-01) were purchased from Amersham Biosciences (Piscataway, NJ). p67^{phox} (610913), p47^{phox} (610354), and Rac1/2 MAb (610651) were purchased from BD Transduction Laboratories (San Diego, CA), and tissue culture medium and Lipofectamine Plus transfection reagents were from Invitrogen (Carlsbad, CA). Glutathione *S*-transferase (GST) MAb (26H1; 2624) was purchased from Cell Signaling (Beverly, MA). A rabbit anti-Rac2 antibody was a gift from G. Bokoch and U. Knaus (The Scripps Research Institute, La Jolla, CA).

Cell lines and cDNA constructs. PLB-985 myelomonocytic cells, COS-7 cells, and derivative COS^{phox} cells expressing NAPDH oxidase subunits gp91^{phox}, p22^{phox}, p67^{phox}, and p47^{phox} were maintained as previously described (32, 33, 47). Transient transfection of COS-7 and COS^{phox} cells was also as previously described (32). A total of 1 to 2 μg of plasmid DNA was used for a 100-mm plate of cells, and 0.5 μg was used for a 60-mm plate. Transfected cells were generally harvested 24 h after transfection. For experiments involving stimulation with epidermal growth factor (EGF; final concentration, 50 ng/ml), 24 h after transfection, cells were cultured in medium with 0.1% serum for an additional 18 to 22 h prior to study.

Expression constructs included wild-type and mutant murine Vav1 cDNAs (Y203F/L213Q, K404A/R422G, and C529S) in pCDNA3 (4, 5) and Myc-tagged human Rac1, Rac1(G30S), and Rac2 in pRK5 (32). Other point mutants [murine Vav1(W495) and p67^{phox}(R102E)] were generated by site-directed mutagenesis (QuikChange II; Stratagene) and truncated derivatives of p67^{phox} by using internal restriction sites. cDNAs for expression of p67^{phox}, an N-terminal fragment of p67^{phox} encoding amino acids 1 to 299, and C-terminal Myc-tagged p67^{phox} were gifts from D. Lambeth (Emory University Medical School, Atlanta, GA) and were subcloned into pcDNA3. p67^{phox} was also subcloned into pGEX-2T-1 (Amersham Biosciences, NJ), and a fragment of Vav encompassing the DH, PH, and ZF domains (amino acids 186 to 545) was subcloned into pQE30 (QIAGEN, Valenica, CA). Plasmids for expression of murine Vav1 with an N-terminal enhanced green fluorescent protein (EFGP) tag and human p67^{phox} with a C-terminal enhanced yellow fluorescent protein (EYFP) tag were constructed using pcDNA3-Vav1 and pEGFP-C3 (BD Biosciences Clontech, San Diego, CA) or pcDNA3-hp67^{phox} (D. Lambeth, Emory University Medical School, Atlanta, GA) and pEYFP-N1 (BD Biosciences Clontech), respectively. Details of all plasmid constructs are available upon request. The expression vector for a 6.4-kDa fragment from p21-activated kinase 3 containing the p21-binding domain (PBD) for Cdc42/Rac and tagged with GST was from R. Cerione (Cornell University, Ithaca, NY).

Preparation of human neutrophils and PLB-985 granulocytes. Blood was collected from healthy donors by venipuncture, and neutrophils were isolated as described previously (20). To differentiate PLB-985 into granulocyte-like cells, 5×10^5 cells were taken from a log-phase culture and plated at 0.5×10^5 cells/ml in the presence of 0.65% *N*, *N*-dimethyl-formamide or 1.25% dimethyl sulfoxide for 6 days. Neutrophils and PLB-985 granulocytes were washed with cold endotoxin-free phosphate-buffered saline (PBS), and treated with the protease inhibitor diisofluorophosphate on ice for 10 min prior to use in the assays below.

NADPH oxidase activity. Superoxide production was measured in a quantitative kinetic assay based on the superoxide dismutase-inhibitable reduction of cytochrome *c* (32). In human neutrophils and PLB-985 granulocytes stimulated with 10 μM fMLP, superoxide release was measured by an isoluminol chemiluminescence assay in 96-well plates using an Lmax microplate luminometer (Molecular Devices, Sunnyvale, CA) (26).

Rac activation assays. Affinity precipitation and pull-down assays for Rac were performed essentially as described previously (3, 26, 45). Diisofluorophosphate-treated human neutrophils or differentiated PLB-985 granulocytes were suspended in 0.5 ml of PBS and stimulated at 37°C with fMLP (final concentration, 10 μM) or dimethyl sulfoxide as a vehicle control. Cell lysates were stored, if needed, at −80°C or analyzed immediately.

In vitro interaction of Vav1 with p67^{phox}. In vitro binding assays were performed as described previously (11). A GST-tagged full-length p67^{phox} and His-tagged Vav1 fragment encompassing the DH, PH, and ZF domains [His-Vav1 (DH-PH-ZF)] were each expressed in bacteria and purified by glutathioine-Sepharose-4B (Amersham Bioscience) or Ni-nitrilotriacetic acid

(NTA) resin (QIAGEN), respectively, according to the manufacturer's protocol. For in vitro GST pull-down assays, 50 pmol of immobilized GST-p67^{phox} was incubated in binding buffer [50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.5% NP-40, 0.5 mM dithiothreitol, and 0.1% bovine serum albumin (BSA), 20 μg/ml chymostatin, 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride] with different amounts of His-Vav1 (DH-PH-ZF) for 1 h at room temperature with shaking. After incubation, the glutathione (GSH)-Sepharose beads were extensively washed with binding buffer and then the bound His-Vav1 (DH-PH-ZF) protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting with anti-His antibody.

For in vitro Ni-NTA pull-down assays, purified His-Vav (DH-PH-ZF) and GST-p67^{phox} were incubated in binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM KCl, 1 mM dithiothreitol, 5 mM imidazole, and 500 μg/ml BSA) with nickel agarose beads for 1 h at room temperature with shaking. After incubation, the nickel agarose beads were extensively washed with buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, and 50 mM imidazole), and bound GST-p67^{phox} was subjected to SDS-PAGE and detected by immunoblotting with anti-p67^{phox} antibody.

Immunoprecipitation and immunoblotting. Immunoprecipitations were carried out as previously described (42). Briefly, cells were lysed in ice-cold immunoprecipitation lysis buffer [20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1% Triton X-100, 0.1% BSA, 20 μg/ml chymostatin, 2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 1 mM 4-(2-aminoethyl benzenesulfonyl fluoride] for 10 min with gentle mixing. Cleared cell lysates were incubated for 2 h at 4°C with the indicated antibodies and for an additional 2 h with protein A-Sepharose beads. Pellets were washed three times with ice-cold immunoprecipitation wash buffer and once with ice-cold PBS and then resuspended in 2× SDS sample buffer. Eluted immunoprecipitates and an aliquot of whole-cell lysate were separated by SDS-PAGE and analyzed by immunoblotting, with signals detected with enhanced chemiluminescence (ECL; Amersham Bioscience) (33).

Confocal microscopy. Twenty-four hours after transfection with indicated plasmids, COS^{phox} cells were split and replated on glass slides and incubated overnight. Cells were then fixed with 4% paraformaldehyde (pH 7.6) and permeabilized with 0.1% Triton X-100 in PBS. After incubation for 30 min in 3% BSA, the cells were incubated for 1 h at room temperature with rabbit anti-Vav1 antibody and Myc MAb (for Rac1 or p67^{phox}), followed by appropriate fluorescence-labeled secondary antibodies or rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) for 30 min. Slides were washed three times with PBS and mounted with 50% glycerol and 1% DABCO in PBS or DAPI (4′,6′-diamidino-2-phenylindole) II counterstain (32-804831; Vysis, Downers Grove, IL). Images were captured with a ZEISS LSM 510 fluorescence microscope at a magnification if ×100. The extent of ruffling, as defined by the existence of irregular membrane folds around a part or all of the margin of the cells, was scored using the following scale: −, no ruffling present; +, ruffling confined to one or several noncontiguous areas of the cell surface; or ++, diffuse ruffling over at least 75% of the cell surface. These membrane folds were outlined by F-actin staining in the submembrane cytoskeleton, although ruffling was readily visible even in the absence of F-actin staining due to staining or fluorescence of Vav and/or p67^{phox}. Ruffling was scored following transfection of cDNAs for EGFP-Vav1 alone or with those for Myc-tagged p67^{phox} or Myc-tagged Rac1, and following transfection of EFYP-p67^{phox} alone, with Myc-tagged Rac1, or with Myc-tagged Rac1 and Vav1. For each combination studied, experiments were performed three to five times, and at least 20 randomly chosen cells expressing the indicated protein(s) from transfected cDNAs, as visualized by fluorescent tag or indirect immunofluorescence staining, were scored in each experiment.

Guanine nucleotide exchange analysis. Guanine nucleotide exchange assays were conducted as described previously (28). Briefly, fluorescence spectroscopic analysis of *N*-methylanthraniloyl (mant)-GTP incorporation into GST-Rac1 purified from bacteria was carried out at 25°C using a Perkin-Elmer Life Sciences LS 50B spectrometer. Flag-tagged Vav1 was immunoprecipitated from COS-7 cells 24 h following transfection with plasmids for expression of Flag-tagged Vav1 alone or in combination with p67^{phox} and Rac. Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and incubated with Anti-FLAG M2 Affinity Gel (Sigma, St. Louis, MO) for 2 h with gentle agitation. The resin-protein complexes were washed three times, and the proteins were eluted with FLAG peptide through gentle shaking for 30 min at 4°C. Exchange reaction assay mixtures contained 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 50 mg/ml BSA, 1% glycerol, 400 nM mant-GTP (Molecular Probes), and 2 μM Rac1 or Rac2. Assays were allowed to equilibrate with continuous stirring prior to addition of immunoprecipitation complexes as agonist. Relative fluorescence (excitation λ, 360 nm; emission λ, 440 nm) was monitored, and experiments were performed in duplicate.

Statistical analysis. Statistical analysis was performed with Instat Vision 2.0 and Excel (Microsoft, Redmond, WA) software. Data are expressed as mean ± standard deviation (SD), and a P value of <0.05 was considered significant. The two-tailed Student's t test or one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test was used to determine the difference between groups.

RESULTS

Vav1 interacts with p67^{phox}. Previous studies demonstrated that Vav1 plays a role in regulating NADPH oxidase in fMLP-stimulated neutrophils (23). As an underlying mechanism that might account for the importance of this GEF to the NADPH oxidase, we explored whether Vav1 interacted with the cytosolic oxidase subunits p67^{phox} or p47^{phox}. To address this hypothesis, cDNAs encoding Vav1 and either p67^{phox} or p47^{phox} were transiently expressed in COS-7 cells, and the interaction between Vav1 and each of these two proteins was examined by coimmunoprecipitation. Figure 1A show that p67^{phox} and Vav1 were coimmunoprecipitated from cell extracts using antibodies to epitope tags on either protein. Vav1 and p47^{phox} did not coimmunoprecipitate, and coexpression of Vav1, p67^{phox}, and p47^{phox} did not alter the coimmunoprecipitation of p67^{phox} with Vav1 in cotransfected cells (not shown). In resting neutrophils, p67^{phox} is associated with p40^{phox}, an oxidase subunit whose function is not well understood via an interaction mediated by PB1 motifs in the C terminus of each protein (18). Coexpression of Vav1, p67^{phox}, and p40^{phox} also had no effect on the coimmunoprecipitation of Vav1 and p67^{phox} (not shown).

We next examined whether Vav1 and p67^{phox} interacted in human neutrophils treated with or without fMLP, and, in parallel, how this correlated with Rac activation and superoxide production. fMLP transiently stimulated an association between Vav1 and p67^{phox}, as seen in immunoprecipitates isolated using a Vav-specific antibody (Fig. 1B). The kinetics of this association, which peaked at 1 min after stimulation, were similar to the kinetics of superoxide formation elicited by this agonist (data not shown) (3, 23). Rac2, which accounts for the majority of Rac in human neutrophils (26), also coimmunoprecipitated with Vav1, with a time course similar to p67^{phox} (Fig. 1B). In contrast, Rac1 did not coimmunoprecipitate with Vav1 from fMLP-stimulated or unstimulated neutrophils (Fig. 1B). These results were confirmed in PLB-985 granulocytes (Fig. 1C), where Rac1 and Rac2 are expressed in similar amounts. The activation of Rac1 and Rac2 in fMLP-stimulated cells was also examined using an affinity precipitation assay with GST-PBD, which showed that Rac2-GTP increases rapidly and transiently in response to fMLP in human neutrophils and myeloid PLB-985 granulocytes (Fig. 1D and E), as previously reported (3), whereas levels of activated Rac1 increased more slowly (Fig. 1D and E). The transient increase in Rac2-GTP corresponded temporally with the increase in coimmunoprecipitation of Vav1, p67^{phox}, and Rac2 (Fig. 1B and C). These results correlate the activation of Rac2 and superoxide production, both rapid and transient responses to fMLP, with a physical interaction between Vav1, p67^{phox}, and Rac2.

Recombinant purified p67^{phox} and Vav1 interact directly in vitro. To examine whether p67^{phox} and Vav1 interacted directly, we performed binding assays using purified recombinant

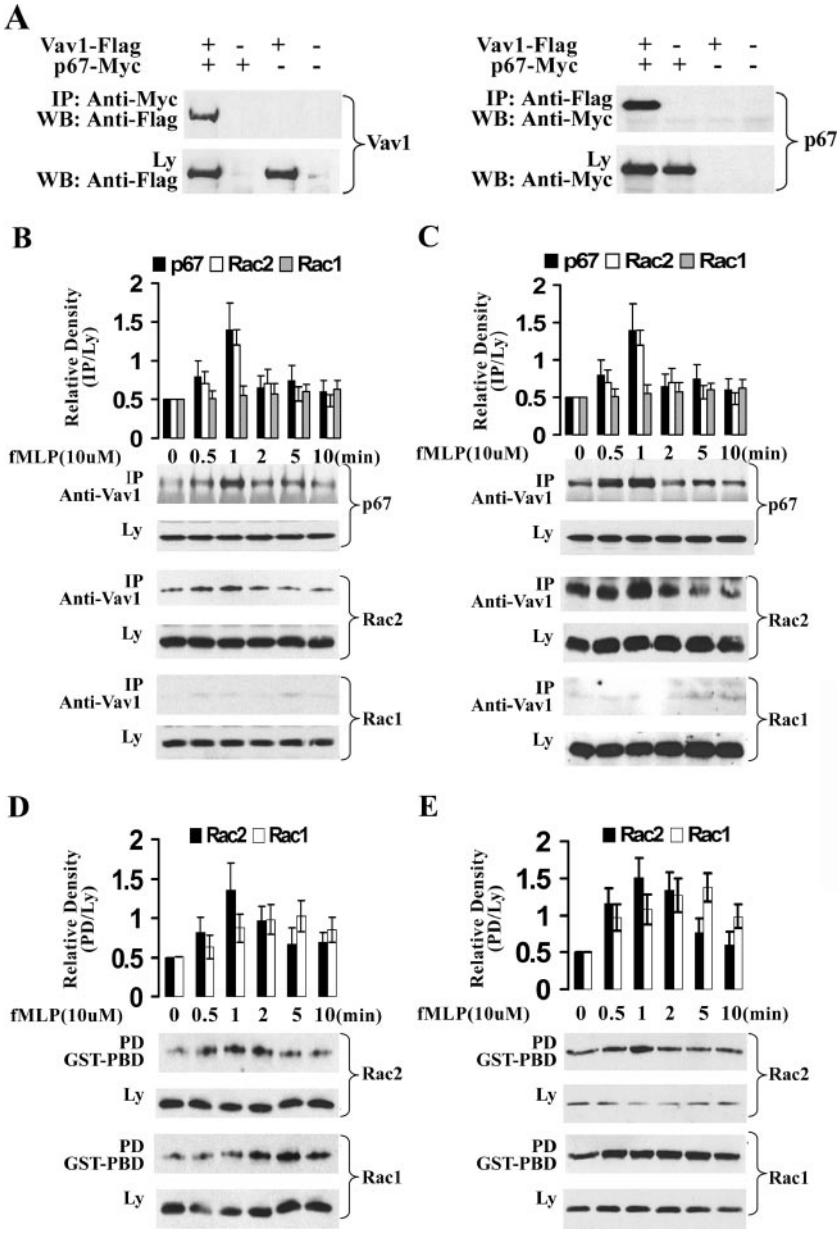

FIG. 1. p67^{phox} interacts with Vav1. (A) COS-7 cells were transiently cotransfected with cDNAs encoding Flag-tagged Vav1 and Myc-tagged p67^{phox}. Immunoprecipitation assays were carried out using Myc (for p67^{phox}) or Flag (for Vav1) MAb and Western blots probed with Flag or Myc MAb as indicated. Each blot is representative of three independent experiments. Human neutrophils (B and D) or myeloid PLB-985 granulocytes (C and E) were treated with 10 μM fMLP for the indicated amounts of time. (B and C) Cell lysates (30×10^6 cells) were immunoprecipitated with a rabbit anti-Vav1 antibody and analyzed by Western blotting with p67^{phox} MAb, rabbit anti-Rac2 antibody, or Rac1 MAb. Enhanced chemiluminescence signal was quantified by densitometry. Bar graphs show mean immunoprecipitation signal normalized to the lysate signal. Assays were carried out in triplicate and the mean ± SD was calculated. (D and E) A GST-PBD pull-down assay was used for quantifying Rac-GTP. Cell lysates were incubated with GST-PBD protein and glutathione-Sepharose beads, and eluted samples were analyzed by immunoblotting using Rabbit anti-Rac2 antibody or Rac1 MAb, as indicated. Assays were carried out in triplicate and the mean ± SD was calculated. Bar graphs showed mean pull-down signal normalized to lysate signal. WB, Western blotting; IP, immunoprecipitation; Ly, whole-cell lysates (1% of the cell lysate used for immunoprecipitation); PD, pull-down assay.

proteins. Initial studies using cotransfected COS-7 cells in which portions of Vav1 were expressed in combination with full-length p67^{phox} showed that a fragment of Vav1 comprised of the DH, PH, and the ZF domains was sufficient to coimmunoprecipitate p67^{phox} (data not shown). We next examined interactions between a His-tagged DH-PH-ZF Vav1 fragment and GST-tagged full-length p67^{phox}. His-Vav1 DH-PH-ZF was coincubated in vitro with GST-tagged p67^{phox}, and the mixtures were precipitated with glutathione-Sepharose (Fig. 2A and B) or Ni-NTA agarose (Fig. 2C). The results show that the His-tagged Vav1 fragment and GST-p67^{phox} coprecipitated regardless of whether the protein mixtures were affinity-purified

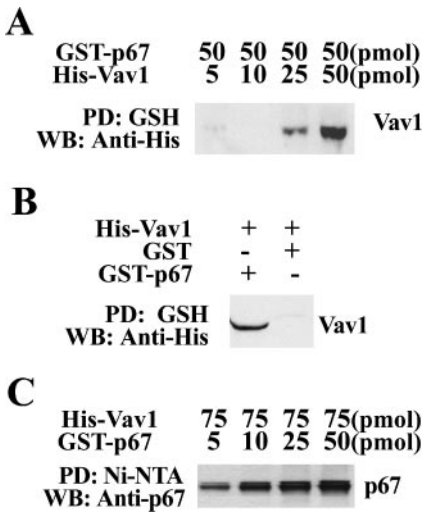

FIG. 2. The interaction of p67^{phox} with Vav1 is direct. Pull-down (PD) samples were analyzed by Western blotting (WB), as indicated. (A) Purified recombinant His-tagged Vav1 (DH-PH-ZF) and GST-p67^{phox} were preincubated in binding buffer prior to addition of glutathione-Sepharose 4B (GSH). GSH pull-down samples were probed with His MAb. (B) Recombinant His-Vav1 (DH-PH-ZF) and GST-p67^{phox} or GST were coincubated prior to the addition of GSH. Pull-down samples were detected with anti-His antibody. (C) His-tagged Vav1 (DH-PH-ZF) and GST-p67^{phox} were preincubated in binding buffer prior to the addition of Ni-NTA agarose beads. Pull-down samples were analyzed with p67^{phox} MAb. These assays were performed in triplicate with similar results.

using glutathione or Ni-NTA. Control experiments showed that the interaction was specific, because His-Vav1 was not precipitated if GST was used instead of GSTp67^{phox} (Fig. 2B). These results indicate that Vav1 can bind directly to p67^{phox} via the DH-PH-ZF domains of Vav1.

p67^{phox} enhances interaction between Vav1 and Rac. The interaction identified between Vav1 and p67^{phox}, a downstream target of Rac-GTP, led us to ask whether p67^{phox} stimulated interactions between Vav and Rac. This question was prompted by recent studies showing that downstream targets of activated Rho GTPases can, in some cases, bind to the corresponding GEF to promote activation of the Rho family member in the vicinity of its target (21, 27, 39), a model referred to as effector-tethered modulation (35).

To investigate whether p67^{phox} could regulate an interaction between Vav1 and Rac, we coexpressed Myc-Rac1 and Flag-Vav1 with or without Myc-p67^{phox} in COS-7 cells. The amount of exogenous (Myc-tagged) and endogenous (nontagged) Rac1 and Vav1 was examined in immunoprecipitates isolated with an anti-Vav1 antibody. The results show that approximately threefold more Rac1 coimmunoprecipitated when Vav1 and Rac1 were coexpressed with exogenous p67^{phox} (Fig. 3A) and that Vav1 forms a complex with both exogenous and endogenous Rac under these conditions (Fig. 3D). Similar results in this assay were seen upon coexpression of Vav1, p67^{phox}, and Rac2 (Fig. 3B). Vav1 and other Rho family GEFs bind the nucleotide-free transition state of their GTPase targets (35), whereas p67^{phox} binds Rac-GTP (25). As an approach to determine the nucleotide status of Rac in the immunoprecipitation complex, we repeated the immunoprecipitations of Myc-Rac1 and Flag-Vav1, with or without Myc-p67^{phox}, in the presence of Mg^{2+}, which stabilizes guanine nucleotide binding to Rac. The results showed that in the presence of Mg^{2+}, Rac1 was no longer recovered in the immunoprecipitation complex (Fig. 3C versus A), suggesting that it is nucleotide-free Rac that otherwise coimmunoprecipitates with Vav1 in the presence of p67^{phox}. Given that the nucleotide-free transition state of Rac should exist only fleetingly in vivo, it is likely that binding of apo-Rac to Vav is an indication that the exchange factor is in an activated or open conformation when bound to p67^{phox}, which enables it to complex more efficiently in vitro with the nucleotide-free Rac generated by $Mg2^{+}$ chelation upon cell lysis. Since nucleotide-free Rac does not interact directly with p67^{phox} (25), these results indicate that p67^{phox} enhances interactions between Vav1 and the nucleotide-free form of Rac.

Association between p67^{phox} and Vav1 involves both the C terminus of p67^{phox} and, indirectly, the N-terminal Rac-binding domain of p67^{phox}. Domains in p67^{phox} important for its association with Vav1 were mapped using a combination of deletion and site-directed mutagenesis. When expressed in cotransfected cells, Vav1 coimmunoprecipitated a C-terminal fragment of p67^{phox} (amino acids 350 to 526) but not an N-terminal fragment (amino acids 1 to 299), which contains the binding site for Rac-GTP (Fig. 4A and B). We also examined the effect of mutations at sites of contact between p67^{phox} and Rac-GTP. R102 of p67^{phox} and G30 of Rac participate in key binding interactions at the interface of these two proteins (25). For example, p67^{phox}(R102E) neither binds to Rac-GTP nor activates NADPH oxidase in vitro or in vivo (24), and G30 is required for Rac binding to p67^{phox} (25). Figure 4C shows that substitution of R102 with glutamic acid in p67^{phox} diminishes its coimmunoprecipitation with Vav1, while substitution of V204 with alanine, a mutation that abolishes the ability of p67^{phox} to promote electron transfer in the NADPH oxidase flavocytochrome (29), does not. Overexpression of Rac1(G30S), which is unable to interact with p67^{phox}, also decreases the coimmunoprecipitation of p67^{phox} and Vav1 (Fig. 4D), as does inhibiting activation of endogenous Rac upon overexpression of Rho-GDI (Fig. 4E). Taken together, these results suggest that the interaction between full-length p67^{phox} and Vav1 involves the C terminus of p67^{phox} but is enhanced upon binding of Rac-GTP to the N terminus of p67^{phox}.

We also examined whether the association of p67^{phox} with Vav depended on the level of exogenous Rac expression by comparing the amount of p67^{phox} recovered in Vav1 immunoprecipitates from cells transiently expressing increasing amounts of exogenous Rac (Fig. 4F and G). The results show that the relative amount of p67^{phox} associated with Vav increases as the level of Rac is increased. This is consistent with the ability of Rho-GDI to dose-dependently suppress endogenous Rac-GTP levels and decrease the association of p67^{phox} with Vav1 (Fig. 4E).

Coexpression of p67^{phox} with Vav1 leads to Vav-mediated Rac activation. To investigate whether interactions between p67^{phox} and Vav1 can stimulate nucleotide exchange on Rac, we conducted GST-PBD pull-down assays to quantify Rac-GTP in COS^{phox} cells transiently transfected with plasmids encoding Vav1, p67^{phox}, Vav1 plus p67^{phox} or Rac, or Vav1

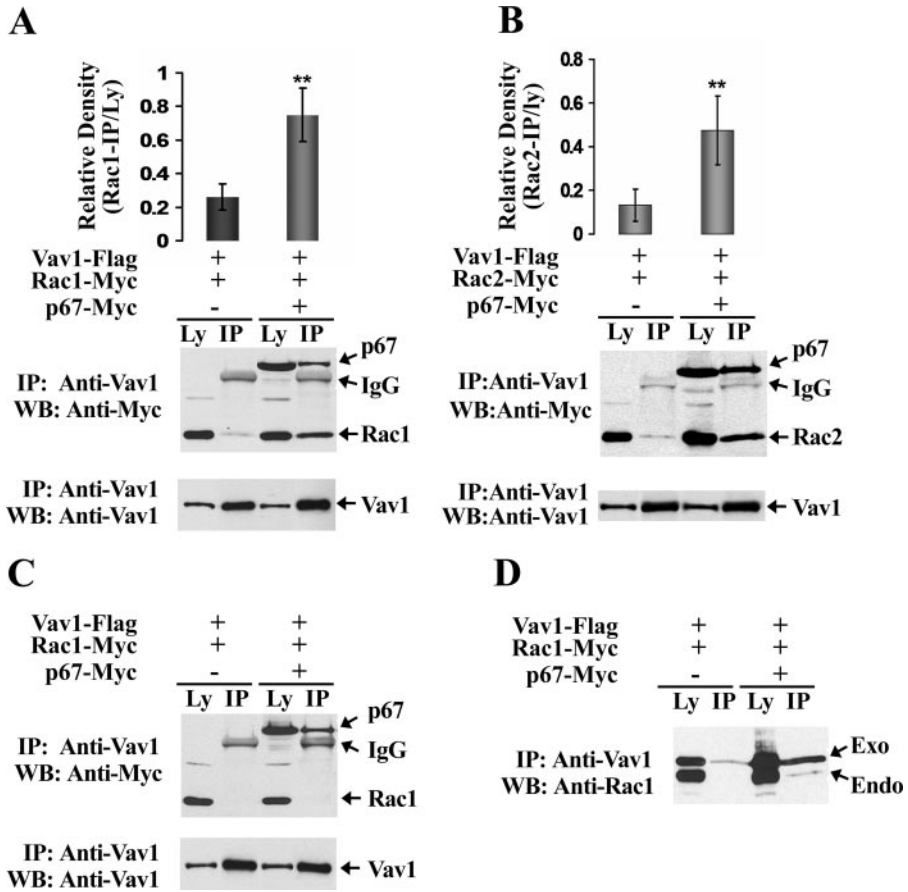

FIG. 3. p67^{phox} stimulates Vav1 binding to Rac. (A and B) COS-7 cells were transiently cotransfected with cDNAs encoding Vav1 and Myc-tagged Rac1 (A) or Myc-tagged Rac2 (B), with or without Myc-tagged p67^{phox}. Immunoprecipitation samples were prepared with rabbit anti-Vav1 antibody and analyzed by Western blotting, probed with Myc MAb (for p67^{phox} and Rac) or Vav1 MAb. Bar graphs show mean immunoprecipitation Rac signals normalized to lysate Rac signals; a two-tailed Student's *t* test was used to determine the difference between groups (**, $P < 0.0001$; $n = 5$). (C) Immunoprecipitation assays were performed as described for panel A except that cells were lysed in buffer containing 10 mM $MgCl_2$. (D) The immunoblot shown in panel A was stripped and reprobed with Rac1 MAb, showing that endogenous (Endo) and exogenous (Exo) Rac1 were included in Vav1 immunoprecipitation complexes. Results are representative of three independent experiments. IP, immunoprecipitation; Ly, whole-cell lysates prior to immunoprecipitation; WB, Western blotting.

plus Rac and p67^{phox}. COS^{phox} cells express gp91^{phox}, p22^{phox}, p67^{phox}, and p47^{phox} as stable transgenes (33) and were used in these experiments in order to examine NADPH oxidase activity in parallel (see below). Each set of transient expression combinations was tested using Rac1 or Rac2, with similar results (Fig. 5A and B). A small but detectable amount of endogenous Rac-GTP was recovered in cells transfected with empty vector or with a plasmid encoding p67^{phox}, and the level of GTP-bound endogenous Rac increased slightly upon cotransfection of Vav1 without or with p67^{phox} or Rac plasmid (Fig. 5A and B). Similarly, a small amount of exogenous Rac-GTP was recovered from cells transfected with a Rac1 or Rac2 expression plasmid, which increased modestly when either GTPase was cotransfected with a plasmid encoding Vav 1 (see Fig. S1 in the supplemental material). However, upon transient cotransfection with cDNAs encoding p67^{phox}, Vav1, and Rac, a three- to fourfold increase in activation of both exogenous and endogenous Rac was observed (Fig. 5A and B). Similar results were seen using COS-7 cells instead of COS^{phox} cells (Fig. 5C and 6A). In addition, the p67^{phox}(R102E) mutant, which has impaired binding to Rac-GTP, did not enhance formation of activated Rac when coexpressed with Vav1 and Rac in COS-7 cells (Fig. 5C). Thus, interactions between p67^{phox}, Vav1, and Rac appear to promote formation of Rac-GTP. In combination with the enhanced association of p67^{phox} and Vav1 upon the interaction between Rac-GTP and p67^{phox} (Fig. 4C to G), these interactions can create a positive feedback loop for upregulation of Vav1 activity.

To provide additional evidence that p67^{phox} can regulate Vav1 exchange on Rac, we utilized an in vitro guanine nucleotide exchange assay based on a change in mant-GTP fluorescence upon Rac binding. This assay showed that the addition of anti-Flag immunoprecipitates from cells expressing Flag-Vav1, p67^{phox}, and Rac1 to purified GST-Rac1 stimulated exchange for mant-GTP, compared to immunoprecipitates isolated from cells expressing Flag-Vav1 alone or Flag-Vav1 and p67^{phox}(Fig. 5D and E). The change in mant-GTP fluorescence reflected binding to GST-Rac1 rather than coimmunoprecipitated Rac1, since fluorescence did not increase when GST-Rac1 was omitted (Fig. 5E).

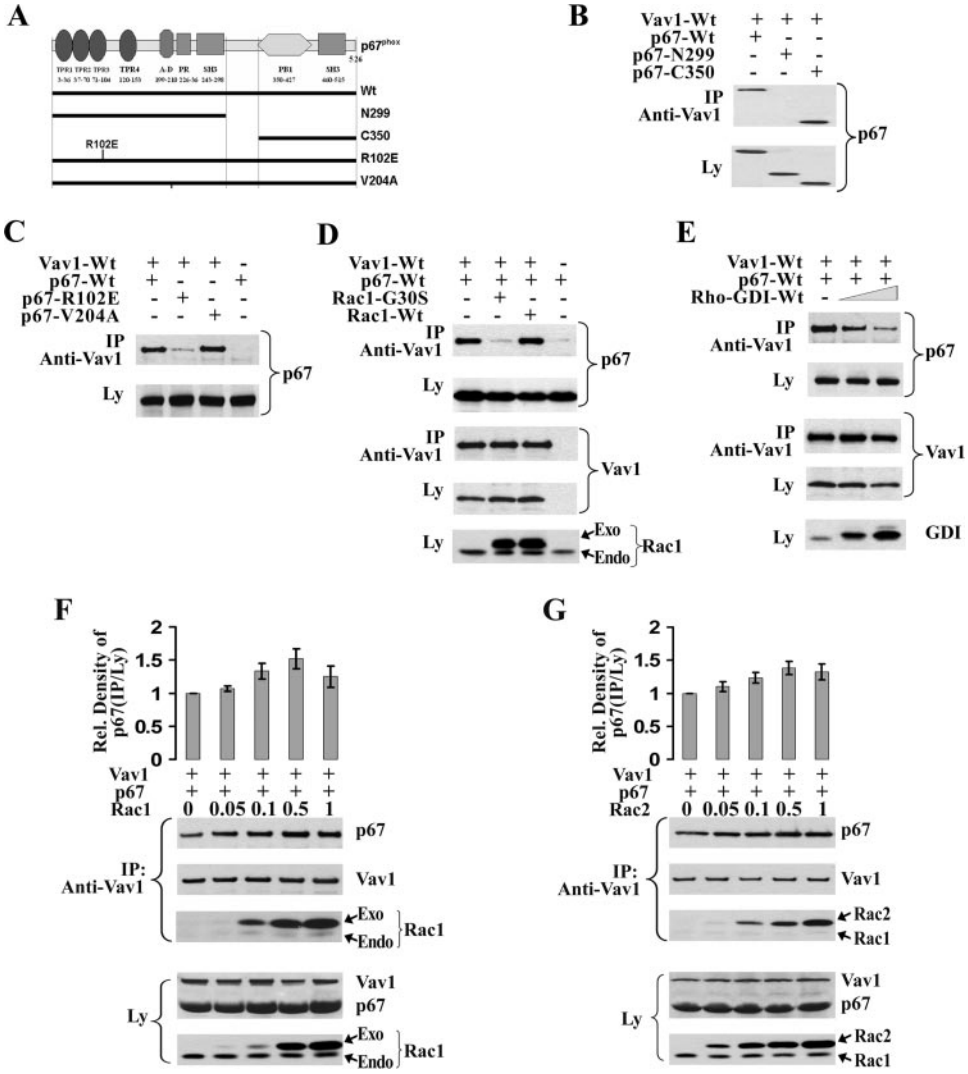

FIG. 4. Rac-GTP stimulates the interaction between p67*phox* and Vav1. (A) p67*phox* domains and mutants are shown schematically. TPR, tetracopeptide repeat; AD, active domain; PR, proline-rich region; PB1, *Phox* and Bem1 domain; N299, fragment comprised of amino acids 1 to 299; C350, truncated N-terminal 350 amino acids of p67*phox*; R102E and V204A, point mutations. (B to G) COS-7 cells were cotransfected with constructs expressing Vav1 in combination with wild-type or mutant p67*phox*, wild-type or mutant Rac1, and/or Rho-DGI, as indicated. Immunoprecipitation (IP) assays were performed with rabbit anti-Vav1 polyclonal antibody and Western blots were probed with a Vav1 MAb, p67*phox* MAb, rabbit anti-Rho-GDI antibody, Rac MAb, or Rac1 MAb, as indicated. Ly, whole-cell lysate.

p67*phox*-enhanced Rac activation requires Vav1 GEF activity but is not associated with increased tyrosine phosphorylation of Vav1. The introduction of point mutations in Vav1 DH, PH, or ZF domains, previously shown to disrupt Vav1 GEF activity (30, 48), resulted in decreased formation of endogenous and exogenous Rac1-GTP in COS-7 cells in the presence of p67*phox* (Fig. 6A). Similar results were seen when Rac2 was coexpressed with p67*phox* and the Vav1 mutants (not shown). These results demonstrate that the GEF activity of Vav1 itself is required for p67*phox*-enhanced formation of Rac-GTP rather than being mediated by another GEF or by regulation of a GTPase activating protein.

We also examined whether p67*phox*-stimulated activation of Rac in the presence of Vav1 was associated with increased tyrosine phosphorylation of Vav1. Vav1 activation is regulated by tyrosine phosphorylation in its acidic domain, which relieves autoinhibition by the N terminus of Vav1 (38). We therefore evaluated tyrosine phosphorylation of Vav1 expressed in COS*phox* cells with or without coexpression of transfected p67*phox* and Rac. These levels of tyrosine phosphorylation of Vav1 were compared to the level elicited by EGF, previously established to activate Vav1 (8). Cotransfection of Vav with p67*phox* and Rac did not result in an increase in phosphotyrosine staining of immunoprecipitated Vav1 in cells not stimulated with EGF (Fig. 6B), although their cotransfection is associated with an increase in activated Rac (Fig. 5A and B and 6A). The addition of EGF stimulated tyrosine phosphorylation on Vav1 by approximately twofold, an increase that was not affected by the presence of transfected p67*phox* and Rac (Fig. 6B). These data indicate that the interactions between p67*phox*, Vav1, and Rac and concomitant Rac activation are not associated with increased tyrosine phosphorylation on Vav. How-

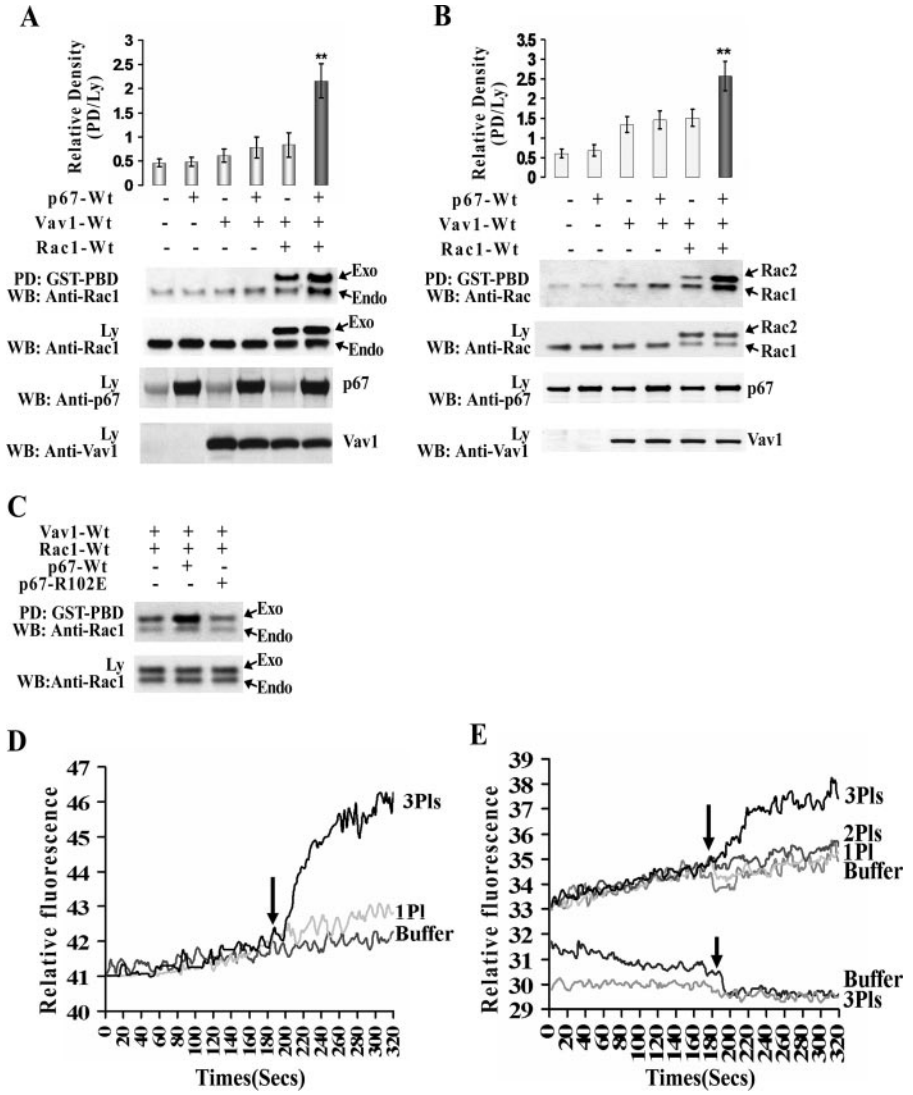

FIG. 5. Coexpression of Vav1, p67^{phox} and Rac activates endogenous and exogenous Rac. (A and B) GST-PBD assays for Rac activation were carried out as described in the legend of Fig. 1 except that COS^{phox} cells were transfected for expression of wild-type Vav1 and wild-type p67^{phox} or with Rac1 or Rac2 in different combinations; for detection of Rac, immunoblots were probed with either a Rac1 MAb (A) or a Rac1/2 MAb (B). The bar graphs show the relative level of total (endogenous [Endo] plus exogenous [Exo]) activated Rac normalized to total Rac. Assays were performed in triplicate and mean values ± SD are shown. ANOVA followed by a Tukey-Kramer multiple comparison test was used to determine the difference between groups. **, $P < 0.001$ versus all other groups. (C) GST-PBD assays for Rac1 activation were carried out for wild-type or p67^{phox}(R102E), coexpressed with Vav1 and Rac1 in COS-7 cells. (D and E) In vitro guanine exchange assays were carried out to examine GEF activity of Flag-tagged Vav1 expressed in cotransfected COS-7 cells with or without cotransfection of constructs for expression of p67^{phox} and Rac, as indicated by 3Pl (plasmids for expression of Vav1, p67^{phox}, and Rac), 2Pl (Vav1 and p67^{phox}), or 1Pl (Vav1). Cell lysates were immunoprecipitated by anti-Flag antibody-conjugated beads, and bound proteins were eluted with Flag peptide. The eluted proteins (or elution buffer only) were added to a mixture containing mant-GTP and bacterially expressed GST-Rac at the time indicated by the arrow, and the relative fluorescence of mant-GTP was monitored by a Perkin-Elmer Life Sciences LS 50B spectrophotometer. Labels indicate plasmids used to transfect COS-7 cells prior to immunoprecipitation or addition of buffer alone. The lower group curves in panel E were generated for reaction mixtures lacking GST-Rac1. Assays were performed in triplicate, and each graph represents one experiment. WB, Western blotting; PD, pull-down; Ly, whole-cell lysates.

ever, we cannot rule out the possibility that the stimulatory effect of p67^{phox} on Vav1 activity is totally independent of Vav1 acidic domain phosphorylation due to the inability to fully eliminate Vav1 phosphorylation in serum-starved cells (Fig. 6B).

NADPH oxidase is activated in COS^{phox} cells upon cotransfection of p67^{phox}, Vav1, and Rac. Since previous studies showed that constitutively activated Rac1-V12 is sufficient to activate NADPH oxidase in COS^{phox} cells (32), we examined whether cotransfection of plasmids for expression of p67^{phox}, Vav1, and Rac-WT (where WT is wild type) also led to activation of superoxide production in COS^{phox} cells. Compared to COS^{phox} cells transfected with either empty vector or vector containing the p67^{phox} cDNA, expression of Vav1 or Vav1 plus additional p67^{phox} or Rac1 consistently resulted in small but detectable increases in superoxide production by COS^{phox} cells (Fig. 7A and B). Although not statistically significant, this

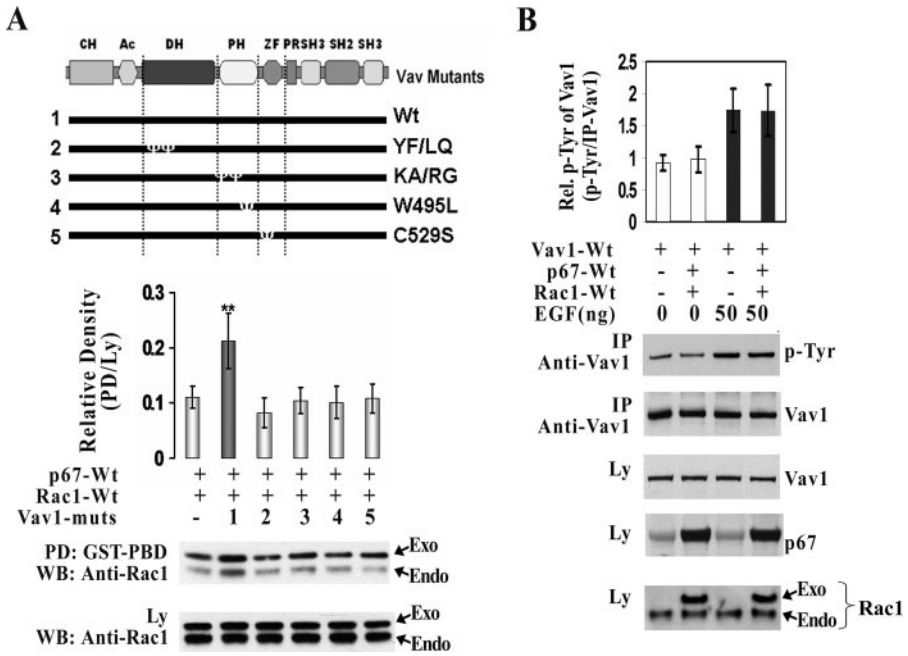

FIG. 6. Activation of Rac by coexpression of Vav1 and p67^{phox} requires Vav1 GEF activity without an increase in tyrosine phosphorylation of Vav1. (A) GST-PBD pull-down assays to assess Rac activation were carried out as described in the legend of Fig. 1 except that cell lysates were cotransfected COS-7 cells with cDNAs encoding p67^{phox}, Rac1, and Vav1 mutants as indicated. At the top is a schematic of Vav1 mutants that lack GEF activity. CH, calponin homology domain; Ac, acidic motif; PR, proline rich region; Wt, wild type Vav1; YF/LQ, Y203F and L213Q double mutant in DH domain; KA/RG, K404A and R422G double mutant in PH domain; W495L, mutant in PH domain; C529S, mutant in ZF domain. The bar graph shows the relative level of total (endogenous plus exogenous) activated Rac normalized to total Rac. Assays were performed in triplicate and mean values ± SD are shown; ANOVA followed by a Tukey-Kramer multiple comparison test was used to determine the difference between groups. **, $P < 0.05$ versus all other groups. (B) Analysis of Vav1 phosphorylation in COS^{phox} cells transfected with indicated constructs. Lysates were prepared from serum-starved cells with or without stimulation with 50 ng/ml EGF for 5 min. Immunoprecipitates isolated using rabbit anti-Vav1 antibody were analyzed by Western blotting, probed with a phosphotyrosine MAb, and reprobed with a Vav1 MAb. Phosphorylated and total Vav1 detected in the immunoprecipitation complex were quantified by densitometry. Bar graph shows the fraction of phosphorylated Vav1 normalized to total Vav1 in the immunoprecipitation complex. Whole-cell lysates were also analyzed by Western blotting for expression of Vav1, p67^{phox}, and Rac1, as indicated. Assays were performed in triplicate, and mean values ± SD are shown. IP, immunoprecipitation; Ly, whole-cell lysates prior to immunoprecipitation; WB, Western blotting; Endo, endogenous; Exo, exogenous; PD, pull-down.

stimulation of NADPH oxidase activity is consistent with the small increases in exogenous Rac1-GTP detected using pull-down assays under these conditions (Fig. 5A). In the absence of overexpressed Rac, the synergistic effect seen on superoxide production by COS^{phox} cells following coexpression of Vav1 plus additional p67^{phox} compared to Vav1 alone (Fig. 7B) is more evident compared to the effect on Rac-GTP levels (Fig. 5A and B) or ruffling (Fig. 7D) (see below). It may be that the effect of the feed-forward loop utilizing endogenous Rac and transfected p67^{phox} and Vav is more readily detectable for superoxide production, since the Rac/p67^{phox}/Vav complex is linked to the NADPH oxidase via p67$^{phox.}$ Cotransfection of COS^{phox} cells with plasmids for increased expression of p67^{phox} and Rac1, along with Vav1, produced a two- to threefold increase in NADPH oxidase activity in the absence of any additional agonist (Fig. 7A and B). Similar results were obtained using Rac2 instead of Rac1 (not shown). Thus, Rac activation upon cotransfection of p67^{phox}, Vav1, and Rac stimulates NADPH oxidase activity, which correlates with the level of Rac-GTP (Fig. 5A).

Membrane ruffling is activated in COS^{phox} cells upon cotransfection of p67^{phox}, Vav1, and Rac. Rac activation is also associated with actin remodeling events leading to ruffling at the cell periphery. We used confocal microscopy to examine whether Rac activation induced by the interaction of Vav1, p67^{phox}, and Rac could also stimulate ruffling in COS^{phox} cells. To facilitate detection of expressed proteins, an N-terminally tagged EGFP-Vav1 and/or C-terminally tagged EYFP-p67^{phox} were used in some experiments. Control studies showed that the degree of ruffling stimulated by different combinations of Vav1, p67^{phox}, and Rac was similar whether or not Vav1 and/or p67^{phox} carried a fluorescent tag (not shown). Vav1 and p67^{phox} colocalize in the cytoplasm and, in cells with extensive ruffling, also at the cell periphery (Fig. 7C). The degree of ruffling elicited by the different plasmid combinations, Vav1-EGFP alone or with p67^{phox} or Rac1 (Fig. 7C), p67^{phox}-EFYP alone or with Rac1, Vav1, or both (Fig. 7C) are summarized in Fig. 7D. Similar results were seen in COS-7 cells and when Rac2 was used instead of Rac1 (not shown). Cells coexpressing recombinant Vav1, p67^{phox}, and Rac displayed the most extensive ruffling (Fig. 7D), correlating with the levels of activated Rac (Fig. 5A).

DISCUSSION

The Rac GTPase is an essential switch for superoxide production by phagocytes. In this report, we show that p67^{phox}, a

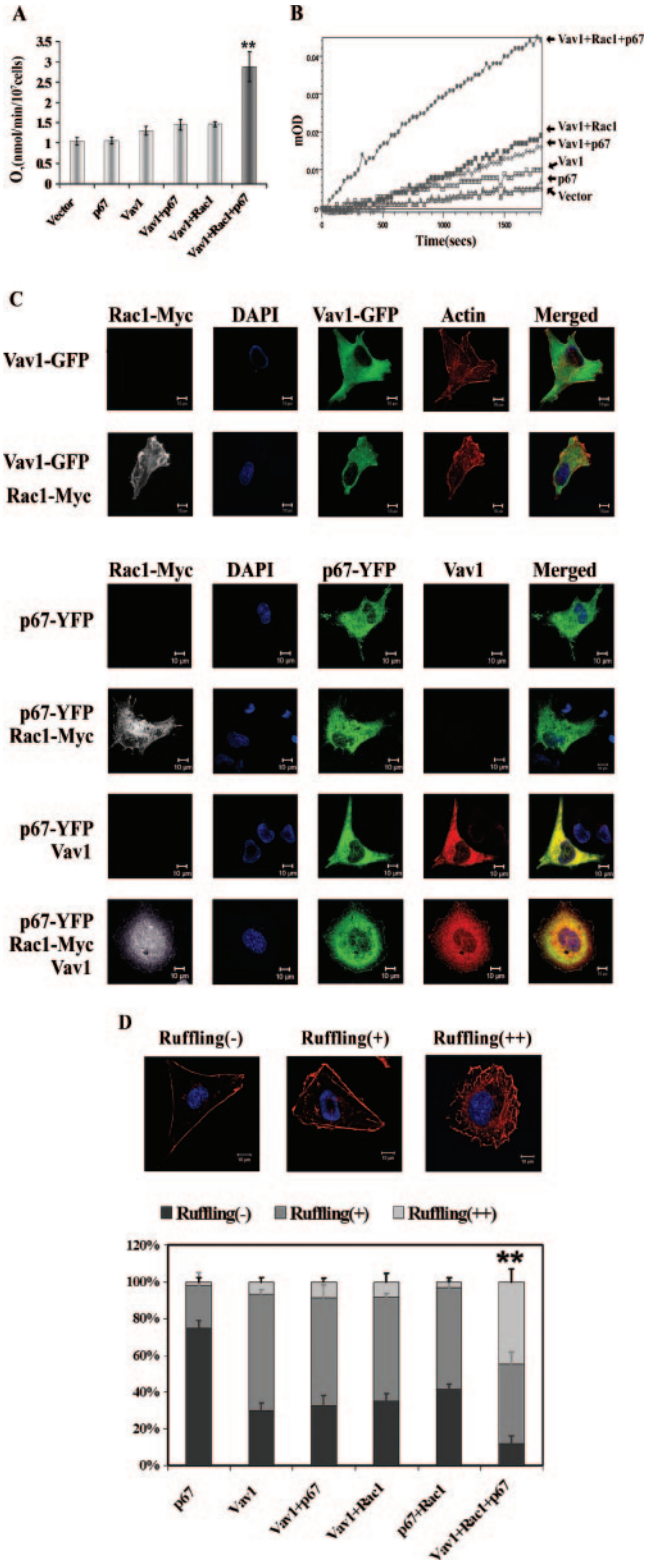

FIG. 7. Coexpression of p67^{phox}, Vav1, and Rac stimulates superoxide production in COS^{phox} cells and activates actin remodeling. (A) Maximal rates of superoxide production in COS^{phox} cells transiently transfected for expression of the indicated proteins was quantified with the cytochrome *c* assay. Values are means ± SD; ANOVA followed by a Tukey-Kramer multiple comparison test was used to determine the difference between groups. **, $P < 0.001$ versus all other groups (column 2, $n = 2$; column 3, $n = 3$; for all others, $n \geq 4$). (B) Kinetics of NADPH oxidase activity in COS^{phox} cells transiently transfected for expression of indicated proteins or empty vector, as measured by reduction of cytochrome *c*. (C and D) Confocal microscopy analysis of COS^{phox} cells transiently cotransfected for expression of p67^{phox}-EYFP or Vav1-EGFP, along with additional cDNAs, as indicated. Cells were fixed and stained with rhodamine-labeled phalloidin or indicated antibodies. DAPI staining used to visualize the nucleus. Scale bar, 10 μm. (C) Vav1-EGFP was coexpressed with or without Myc-Rac1. Cells were stained with Myc MAb (for exogenous Rac1), followed by Alexa Fluor-633 goat anti-mouse antibody. p67^{phox}-EYFP was coexpressed with Vav1 and/or Myc-tagged Rac1; cells were stained with rabbit anti-Vav1 antibody and Alexa Fluor-555 goat anti-rabbit antibody and with Myc MAb and Alexa Fluor-633 goat anti-mouse antibody. Merged images were generated from the red and green signals, and regions where these overlap appear orange or yellow. Images shown are representative fields from three to five independent experiments. Scale bar, 10 μm. (D) The bar graph shows the relative frequency of COS^{phox} cells with various ruffling scores (−, +, and ++; see Materials and Methods) for cells expressing the indicated proteins following transient transfection. For each ruffling score, representative images of rhodamine-labeled phalloidin-stained cells are shown in the panel above the bar graph. Data are the means ± SD of three to five separate experiments; to determine the difference between groups for the frequency of cells with ++ ruffling, data were analyzed using ANOVA followed by a Tukey-Kramer multiple comparison test. **, $P < 0.001$ versus all other groups.

cytosolic regulatory subunit of the NADPH oxidase and a target of Rac-GTP, can regulate Rac activation via interactions with the guanine nucleotide exchange factor, Vav1. Studies in human neutrophils showed that p67^{phox} coimmunoprecipitates with Vav1 upon fMLP stimulation, and in vitro binding assays indicate that this interaction is direct. Additional findings in transfection assays showed that interactions between Vav1 and p67^{phox} stimulate Vav1 GEF activity, and, in turn, the binding of Rac-GTP to p67^{phox} enhances interactions between p67^{phox} and Vav. This creates a positive feedback loop that can target and amplify Rac activation in the vicinity of the NADPH oxidase complex. These findings provide a molecular rationale for the observation that neutrophils isolated from Vav$^{-/-}$ mice have substantially attenuated superoxide production following fMLP stimulation (23). That chemotaxis is normal and overall levels of Rac-GTP are unaffected in fMLP-activated Vav$^{-/-}$ neutrophils (16, 23) are also consistent with an effect of Vav1 on a specific pool of Rac.

In human neutrophils and PLB-985 granulocytes, complex formation between p67^{phox}, Vav1, and Rac2, but not Rac1, is stimulated by fMLP (Fig. 1B and C). These data establish that Vav1 is an oxidase target GEF and is linked to p67^{phox} in neutrophils and provide further evidence that fMLP-initiated NADPH oxidase activation in neutrophils is mediated by a process that preferentially utilizes Rac2. The presence of Rac2 but not Rac1 in this complex is consistent with the marked defects in superoxide production in Rac2-deficient murine neutrophils, despite increased activation of Rac1 (22, 26, 34, 44), whereas NADPH oxidase activity is normal in the genetic absence of Rac1 (17). In transfection assays in COS-7 cells, the interaction of p67^{phox} with Vav1 promotes nucleotide exchange on both Rac1 and Rac2. However, it is likely that in fMLP-stimulated neutrophils, preference for the Rac2 isoform is conferred by constraints in the activation or localization of

Rac2 that promote efficient interaction between p67^{phox}, Rac2, and Vav1.

As an essential regulator of the NADPH oxidase, p67^{phox} has a well-characterized role in activating electron transport through flavocytochrome *b*, an activity that is dependent upon Rac-GTP (12, 18, 36). This study identifies two unexpected additional functions for p67^{phox}, showing that it both interacts with a Rac GEF and enhances its nucleotide exchange activity. It also maps the Vav-interacting region of p67^{phox} to the C-terminal region of p67^{phox}, a site distinct from the Rac-GTP binding domain in the N-terminal portion of p67^{phox}. However, the binding of Rac-GTP potentiates the interaction between p67^{phox} and Vav1, as this interaction is inhibited by overexpression of Rho-GDI or by mutations of Rac and p67^{phox} that specifically disrupt their association (Fig. 4C to E and 5B). Conversely, wild-type p67^{phox} enhances the coimmunoprecipitation of Rac and Vav1 (Fig. 3A and B) and stimulates Vav-mediated activation of Rac (Fig. 5A and B). Thus, we propose that in fMLP-stimulated neutrophils, Rac-GTP binds to p67^{phox}, which in turn promotes the interaction between p67^{phox} and Vav1, followed by further activation of Rac and NADPH oxidase activity. In this model (Fig. 8), p67^{phox} acts as a crucial link in a positive feedback loop for activation of Rac.

These studies also identify a new mechanism for regulation of Vav1 via protein-protein interactions that enhance its GEF activity in the context of a specific functional response. Activation of Vav1 exchange activity requires tyrosine phosphorylation in its acidic domain or N-terminal truncation, either of which relieves autoinhibition by the N terminus of Vav1 that otherwise blocks access of substrates to the catalytic DH domain (1, 10, 37, 38). It is also known that Vav GEF activity can be additionally modulated by binding of phospholipids to the PH domain or by residues in the ZF domain that may interact directly with GTPases (37, 38). In this report, we show that p67^{phox} stimulates Vav1-catalyzed nucleotide exchange on Rac (Fig. 5A and B) without increasing the tyrosine phosphorylation of Vav1 (Fig. 6B). p67^{phox} is capable of interacting directly with a Vav1 fragment comprised of the DH, PH, and ZF domains (Fig. 2). However, the underlying mechanism by which p67^{phox} binding influences Vav1 GEF activity remains to be clarified.

The modulation of Rac exchange by p67^{phox}, an effector of Rac-GTP, adds to the emerging evidence that Rho family GTPases can form signaling complexes with their upstream GEFs and immediate downstream effectors through direct or indirect scaffolding interactions (35). These complexes can promote efficient effector activation and contribute to signaling specificity. In most cases, the GEF acts as a scaffold to localize Rac effectors (6, 7, 9, 31). However, amplification of GEF activity by assembly of a GEF-GTPase-effector complex, such as described here, has been shown in only a few reports; WASP, an effector of Cdc42, recruits intersectin, a Cdc42-specific GEF, to a signaling complex and activates Cdc42 in vitro (21). Similarly, Gβγ binds to PAK1 via PIXα, a Cdc42 GEF, and activates Cdc42, which in turn activates PAK1 to regulate neutrophil polarization (27).

In summary, we report here that p67^{phox}, the NADPH oxidase subunit that is a target of activated Rac, both associates with and enhances the activity of a guanine nucleotide exchange factor, Vav1, thus creating a positive feedback loop for local amplification of Rac activation and subsequently NADPH oxidase activity. The results provide new mechanistic insights into the regulation of superoxide production by neutrophils. Additional studies are required to elucidate the molecular details by which the binding of p67^{phox} to Vav1 activates Vav1 exchange activity and to identify other GEFs that activate Rac in context of the oxidase and to examine their association with p67^{phox} or other NADPH oxidase subunits.

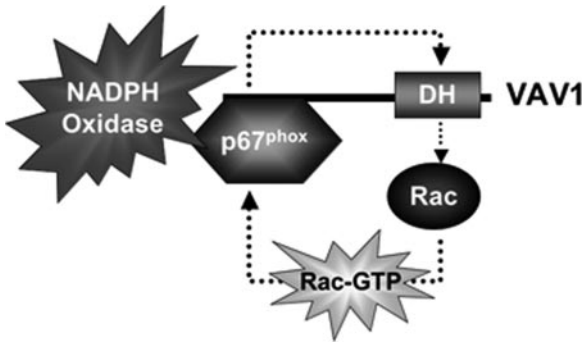

FIG. 8. Model for a p67^{phox}-Vav-Rac feedback loop for activation of the NADPH oxidase. In this model of NADPH oxidase activation in fMLP-stimulated neutrophils, interactions between Vav1 and p67^{phox} stimulate Vav1 GEF activity, and, in turn, the binding of Rac-GTP to p67^{phox} enhances interactions between p67^{phox} and Vav. This leads to further activation of Rac and NADPH oxidase activity in a positive feedback loop that targets and amplifies Rac activation in the vicinity of the NADPH oxidase complex.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants P01 HL069974 (M.C.D.), R01 HL45635 (M.C.D.), CA108647 (L.A.Q.), R01 AI065474 (D.D.B.), the Indiana University Cancer Center Flow Cytometry and Imaging Cores P30 CA082709 (M.C.D. and L.A.Q.), and the Riley Children's Foundation.

We thank Xiaohong Li and Shari Upchurch for assistance with manuscript preparation.

Wenyu Ming performed the majority of experiments, wrote the first draft of the manuscript, prepared all figures, and participated in all additional aspects of manuscript preparation. Shijun Li performed experiments and reviewed the manuscript. Daniel D. Billadeau provided key reagents along with reviewing and editing the manuscript. Lawrence A. Quilliam provided input into experimental design, manuscript preparation, and editing of the manuscript. Mary C. Dinauer oversaw all aspects of this project and manuscript preparation.

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