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RhoG Regulates the Neutrophil NADPH Oxidase[1]

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RhoG is a Rho family small GTPase implicated in cytoskeletal regulation, acting either upstream of or in parallel to Rac1. The precise function(s) of RhoG in vivo has not yet been defined. We have identified a novel role for RhoG in signaling the neutrophil respiratory burst stimulated by G protein-coupled receptor agonists. Bone marrow-derived neutrophils from RhoG knockout (RhoG$^{-/-}$) mice exhibited a marked impairment of oxidant generation in response to C5a or fMLP, but normal responses to PMA or opsonized zymosan and normal bacterial killing. Activation of Rac1 and Rac2 by fMLP was diminished in RhoG$^{-/-}$ neutrophils only at very early (5 s) time points (by 25 and 32%, respectively), whereas chemotaxis in response to soluble agonists was unaffected by lack of RhoG. Additionally, fMLP-stimulated phosphorylation of protein kinase B and p38MAPK, activation of phospholipase D, and calcium fluxes were equivalent in wild-type and RhoG$^{-/-}$ neutrophils. Our results define RhoG as a critical component of G protein-coupled receptor-stimulated signaling cascades in murine neutrophils, acting either via a subset of total cellular Rac relevant to oxidase activation and/or by a novel and as yet undefined interaction with the neutrophil NADPH oxidase. ***The Journal of Immunology*****, 2006, 176: 5314–5320.**

Neutrophils constitute the major cellular component of the innate immune response. They are recruited rapidly and in large numbers to sites of infection, where they ingest pathogens and produce reactive oxygen species (ROS)[4] required for microbial killing. Defects in neutrophil function or neutrophil depletion result in increased susceptibility to life-threatening bacterial and fungal infections. Inappropriate, excessive, or inadequately curtailed neutrophil activation contributes to many pathological processes (reviewed in Ref. 1). Generation of ROS occurs intracellularly at the phagosomal membrane after pathogen ingestion (2), minimizing the likelihood of tissue injury; involvement of ROS in the killing process may be direct, via halogenated derivatives, and/or indirect, via activation of an array of proteases (3). Extracellular ROS are generated in response to soluble agonists such as formylated bacterial peptides (e.g., fMLP) or activated complement (C5a), a response that can be massively up-regulated by priming with proinflammatory cytokines (4–6) with the potential for consequent tissue injury (7).

Generation of ROS requires the assembly of the multicomponent phagocyte NADPH oxidase. On stimulation, the cytosolic p47^{phox} and p67^{phox} proteins translocate to associate with the membrane-localized gp91^{phox} and p22^{phox}. The hemopoietic-specific GTPase Rac2 is also an essential component of the oxidase machinery. The requirement for Rac2 in the formation of ROS and for chemotaxis has been demonstrated in neutrophils from Rac2-null mice (8) and from a patient with a dominant negative mutation of Rac2 (9, 10). Although Rac2 binds oxidase components and may participate in the translocation process, the precise molecular mechanisms by which it regulates the membrane-associated enzyme are unclear (11). Rac2 shares 92% amino acid sequence identity with the ubiquitously expressed Rac1, yet each GTPase has unique physiological roles. Rac1$^{-/-}$ neutrophils show no defects in ROS production; however, deficiency of both Rac1 and Rac2 resulted in a more severe defect in ROS production than deletion of Rac2 alone (12). This suggests that there is also some functional redundancy between the two GTPases.

Rac1 shares 72% amino acid sequence homology with the Rho family small GTPase, RhoG. Despite this homology, RhoG interacts with few of the known Rac1 effectors (13); instead, it has been shown to activate Rac1 itself via an interaction with Elmo-DOCK180, with consequent regulation of the actin cytoskeleton (14). RhoG has also been shown to impact on the microtubule system via a specific interaction with the protein kinectin (15). Reported functional consequences of RhoG activation include formation of dorsal ruffles (16), neurite outgrowth from PC12 cells (17), stimulation of macropinocytosis (16), and engulfment of apoptotic cells (18). RhoG has additionally been implicated in lymphocyte signaling, transcriptional regulation, and cytoskeletal rearrangements (19), but lymphocytes derived from a RhoG$^{-/-}$ mouse demonstrated only minor functional and signaling abnormalities (20).

Because RhoG is thought to function upstream of Rac and play a role in cytoskeletal rearrangements (upon which neutrophils are exquisitely dependent to fulfill their roles in chemotaxis, phagocytosis, and ROS generation), we investigated the function of RhoG$^{-/-}$ neutrophils. Surprisingly, although chemotaxis and the

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[4] Abbreviations used in this paper: ROS, reactive oxygen species; DPBS, Dulbecco's PBS; PLD, phospholipase D; wt, wild type; CRIB, CDC42/Rac interacting and binding domain; PAK, p21-activated kinase.

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respiratory burst in response to IgG-opsonized particles were fully preserved, the generation of ROS in response to soluble agonists was severely compromised in association with a modest defect in the activation of both Rac1 and Rac2.

Materials and Methods

Materials

Unless otherwise stated, chemicals and media were purchased from Sigma-Aldrich. Recombinant murine GM-CSF was obtained from R&D Systems. Phospho-AKT (Ser^{473}), AKT, phospho-p38MAPK, p38MAPK, and phospho-p44/42 MAP Abs were purchased from New England Biolabs, and p42/44 and Rac-1 Abs were obtained from BD Transduction Laboratories. Rabbit polyclonal anti-RhoG and anti-$p22^{phox}$ were obtained from Santa Cruz Biotechnology, and rabbit polyclonal Abs to $gp91^{phox}$, murine $p47^{phox}$, and $p67^{phox}$ were obtained from Upstate Biotechnology. [^{3}H]Palmitic acid was purchased from ICN Biomedicals. Rac2 Ab was a gift from Dr. G. Bokoch (The Scripps Research Institute, La Jolla, CA), and the GST-p21-activated kinase (PAK)-CDC42/Rac interacting and binding domain (CRIB) construct was obtained from Dr. J. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (21). Millicell-PC filter inserts were purchased from Millipore. Percoll was purchased from Amersham Biosciences. Zymosan was opsonized with rabbit IgG anti-zymosan (Molecular Probes) according to the manufacturer's instructions.

Animals

$RhoG^{-/-}$ mice on a mixed C57BL/6, 129/Sv background were obtained by targeted disruption of the *RhoG* gene as previously described (20). Wild-type (wt) controls were mixed C57BL/6, 129/Sv derived from wt littermates bred in parallel. Animals were housed under specific pathogen-free conditions in the small animal breeding unit at the Babraham Institute and were used at 8–12 wk of age.

Preparation of murine neutrophils

Murine bone marrow was dispersed in 137 mM NaCl, 5.3 mM KCl, 4 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5.6 mM glucose, 30 mM HEPES, and 0.25% fatty acid-free BSA (HBSS/BSA) and centrifuged ($1256 \times g$, 30 min, room temperature) over discontinuous gradients comprised of 81, 62, and 55% Percoll in HBSS. Mature neutrophils (75–85% pure by cytospin) were obtained from the 55/62% interface, and RBC were removed by ammonium chloride lysis. Cells were washed twice in HBSS/BSA and resuspended in HBSS.

Western blotting

Neutrophil lysates (2×10^6 cells/lane) from wt and $RhoG^{-/-}$ mice and human donors (prepared by centrifugation over discontinuous plasma/Percoll gradients) were prepared by sonication into Laemmli sample buffer and analyzed by Western blotting for expression of RhoG. Lysates from wt and $RhoG^{-/-}$ neutrophils were additionally blotted for components of NADPH oxidase.

Measurement of neutrophil oxidative burst activity

Neutrophils (2.5×10^6/ml in HBSS at 37°C) were incubated with Luminol (1 μM final concentration) and HRP (62.5 U/ml final concentration) for 3 min, 150-μl aliquots were transferred to prewarmed luminometer plates, and fMLP (final concentration, 10 μM) was added via the injection port of a Berthold MicroLumat Plus luminometer. Other agonists were added via a multichannel pipette. Data were acquired and analyzed using WinGlow software.

Measurement of bacterial killing

Bacteria (*Staphylococcus aureus* Wood 46) were subcultured at 37°C to logarithmic growth from an overnight culture. Bacteria (4×10^7) were washed in Dulbecco's PBS (DPBS), resuspended in 1 ml of DPBS and 50% mouse serum, and incubated at 37°C for 15 min with end-over-end mixing, followed by washing in DPBS. Opsonized *S. aureus* (1.5×10^6) were added to 6.2×10^6 primed (mouse rGM-CSF/mouse rTNF-α) bone marrow-derived neutrophils (2.5×10^7/ml) at 37°C with mixing. After 15 min, 50-μl aliquots were removed to 950 μl of ice-cold Luria Bertoni broth containing 0.05% saponin. Samples were sonicated (Sonicator 3000; Misonix; output, 1.5, 10 s) to liberate intracellular bacteria and were returned to ice. Suspensions were serially diluted and plated on Luria-Bertoni broth-agar to enumerate surviving bacteria. A parallel bacterial incubation was run in the absence of neutrophils. Neutrophils were further incubated for 5 min with 3 μM diphenylene iodonium chloride before the addition of bacteria (oxidant-dependent killing control).

Measurement of chemotactic activity

In vitro chemotaxis was performed using 3.0 μM culture plate inserts placed within individual wells of a 24-well low adhesion plate (Corning Costar) to form an upper and a lower chamber, respectively. Three hundred microliters of fMLP (1 μM), C5a (2 nM) or medium alone (HBSS/BSA) was placed into the bottom compartment, and 200 μl of unfractionated bone marrow (5×10^6/ml in HBSS/BSA) was added to the top compartment. After 60 min at 37°C (no CO_2), migrated cells were aspirated and counted, and the percentage of input cells migrated was determined by FACS analysis as previously described (22).

For in vivo chemotaxis, peritonitis was induced by i.p. injection of 0.6 ml of sterile 3% thioglycolate in PBS. At 4 or 18 h, the peritoneum was washed twice with 5 ml of PBS/3 mM EDTA/0.5% BSA. Lavage fluid (0.25 ml) was stained with conjugated Abs (anti-Gr1 (RBG-8C5), anti-CD11b (RM815-3), and anti-F4/80 (BM8); eBioscience and BD Harlingen), washed twice, resuspended in 100 μl of PBS/0.5% BSA with 10 μl of Flow-Count fluorospheres (Beckman Coulter; for direct determination of absolute neutrophil counts), and analyzed using a FACSCalibur flow cytometer (BD Biosciences) with CellQuest (BD Biosciences) software for acquisition and FlowJo (Tree Star) software for analysis. Annexin V-positive Gr-1-positive cells were quantitated using the Annexin V^{FITC} apoptosis detection kit according to the manufacturer's (BD Pharmingen) instructions.

Measurement of macropinocytosis

Murine neutrophils were incubated (4 or 37°C) in 1 mg/ml FITC-dextran (m.w., 70,000) in HBSS and stimulated with 10 μM fMLP, 200 nM PMA, or vehicle. Reactions were stopped at 15 min by addition of 0.1% sodium azide and were quenched by resuspension in 0.4% trypan blue. Cells were washed three times in ice-cold HBSS and analyzed using a FACSCalibur flow cytometer (BD Biosciences) as described above.

Rac activation assays

Activation of Rac1 and Rac2 was quantitated as previously described (23) with minor modifications. Murine neutrophils (5×10^6 in 100 μl of HBSS) at 37°C were stimulated with fMLP (10 μM) or vehicle for 5 or 15 s, lysed with ice-cold 2× lysis buffer (20% glycerol; 100 mM Tris; 200 mM NaCl; 2% Nonidet P-40; 4 mM $MgCl_2$; 20 μg/ml leupeptin, pepstatin A, antipain, and aprotinin; and 0.4 mM PMSF), and clarified ($14,000 \times g$, 3 min, 4°C), and the supernatant was added to 10 μg of GST-PAK-CRIB coupled to glutathione-Sepharose beads. Samples were mixed end-on-end at 4°C for 20 min, washed three times, and boiled in 45 μl of Laemmli sample buffer. Samples were separated by SDS-PAGE in parallel with total cell lysates of wt and $RhoG^{-/-}$ cells, transferred to nitrocellulose, and probed with Ab to Rac2 (1/7500) and subsequently Rac1 (1/1000). Parallel samples were probed with Ab to CDC42 (Santa Cruz Biotechnology).

PKB, p38MAPK, and ERK1/2 activation

Activation of PKB, p38MAPK, and ERK1/2 were quantitated by Western blotting with phospho-specific Abs. Paired samples of wt and $RhoG^{-/-}$ neutrophils ($2–5 \times 10^6$) in HBSS were stimulated (0–10 μM fMLP or vehicle, 0–10 min), pelleted (10 s in a microfuge at 4°C), and resuspended in 60 μl of ice-cold lysis buffer (0.2% cholate; 1% Triton X-100; 3 mM β-glycerophosphate; 30 mM sodium fluoride; 150 mM NaCl; 40 mM HEPES; 5 mM EGTA; 5 mM EDTA; 10 μg/ml leupeptin, pepstatin A, antipain, and aprotinin; and 0.2 mM PMSF). Samples were clarified, and the supernatants were combined with 4× Laemmli sample buffer, boiled, and separated by SDS-PAGE. Western blotting was conducted according to the Ab supplier's instructions.

Measurement of calcium flux

Bone marrow leukocytes were flushed from hind limb bones in PBS. Residual RBC were removed by ammonium chloride lysis, and leukocytes were washed in DPBS. Bone marrow leukocytes (1×10^7/ml) were loaded with 1 μM indo-1 (Molecular Probes) for 15 min at 37°C, then resuspended at 5×10^6/ml in HBSS and 1 mM Ca^{2+}. Increases in intracellular Ca^{2+} were measured using a FACStar Plus machine with CellQuest software (BD Biosciences). Neutrophils were identified by their forward and side scatter profile. Data were analyzed with FlowJo software (Tree Star).

Phospholipase D (PLD) activation

Paired wt and $RhoG^{-/-}$ neutrophils were labeled with [^{3}H]palmitic acid in the presence of TNF-α (500 U/ml) for 115 min before the addition of 1%

butanol. After an additional 5 min, samples (7.5×10^6 cells in 500 μl, 100 μCi) were stimulated (10 μM fMLP, 200 nM PMA, or vehicle) for 0–10 min, placed on ice, and washed three times with ice-cold PBS. Cellular lipids were extracted and resolved by TLC as previously described (24).

Results

Functional defects of soluble agonist-induced responses in RhoG$^{-/-}$ neutrophils

Western blotting confirmed abundant expression of RhoG in both human and murine wt, but not RhoG$^{-/-}$, neutrophils (Fig. 1*A*). Surprisingly, ROS production in response to fMLP was substantially reduced in RhoG$^{-/-}$ vs wt bone marrow-derived neutrophils (Fig. 2*A*; $p = 0.002$, by unpaired *t* test) regardless of whether the cells had been primed with GM-CSF (Fig. 2*B*; $p = 0.0004$, by unpaired *t* test). The C5a-stimulated oxidative burst was also compromised by deletion of RhoG (Fig. 2*C*; $p = 0.011$, by unpaired *t* test). These results were confirmed using a fundamentally different FACS-based assay (dichlorodihydrofluorescein diacetate staining) and also using neutrophils from mice backcrossed onto a B10/Br background (data not shown). This reduction in ROS production was not the consequence of a basic defect in expression or assembly of the oxidase machinery, because the responses to PMA and IgG-opsonized zymosan were fully preserved in the same RhoG$^{-/-}$ neutrophils (Fig. 2, *D* and *E*), and expression of p47^{phox}, p67^{phox}, p22^{phox}, gp91^{phox}, and Rac2, assessed by Western blotting, was identical in RhoG$^{-/-}$ vs wt neutrophils (Fig. 1, *B–F*). Despite the impairment of oxidant generation in response to soluble inflammatory mediators, killing of opsonized *S. aureus* was completely unaffected in the absence of RhoG (Fig. 2*F*).

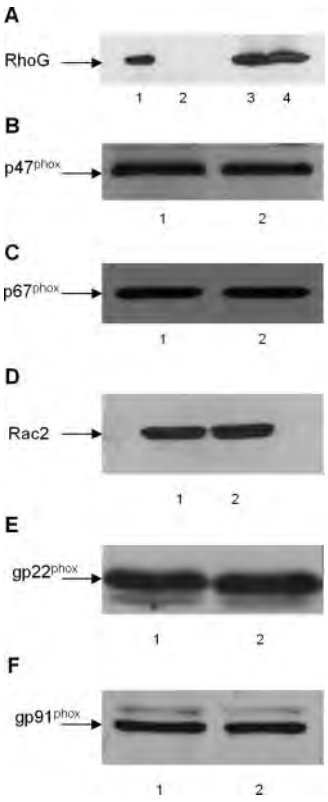

FIGURE 1. Expression of RhoG and oxidase components in wt vs RhoG$^{-/-}$ neutrophils. *A*, Neutrophil lysates (2×10^6 cells/lane) from wt mice (*lane 1*), RhoG$^{-/-}$ mice (*lane 2*), and two separate human donors (*lanes 3* and *4*) were analyzed by Western blotting for expression of RhoG. *B–F*, Neutrophil lysates (2×10^6 cells/lane) from wt (*lane 1*) and RhoG$^{-/-}$ (*lane 2*) mice were analyzed by Western blotting for expression of p47^{phox} (*B*), p67^{phox} (*C*), rac2 (*D*), p22^{phox} (*E*), and gp91^{phox} (*F*).

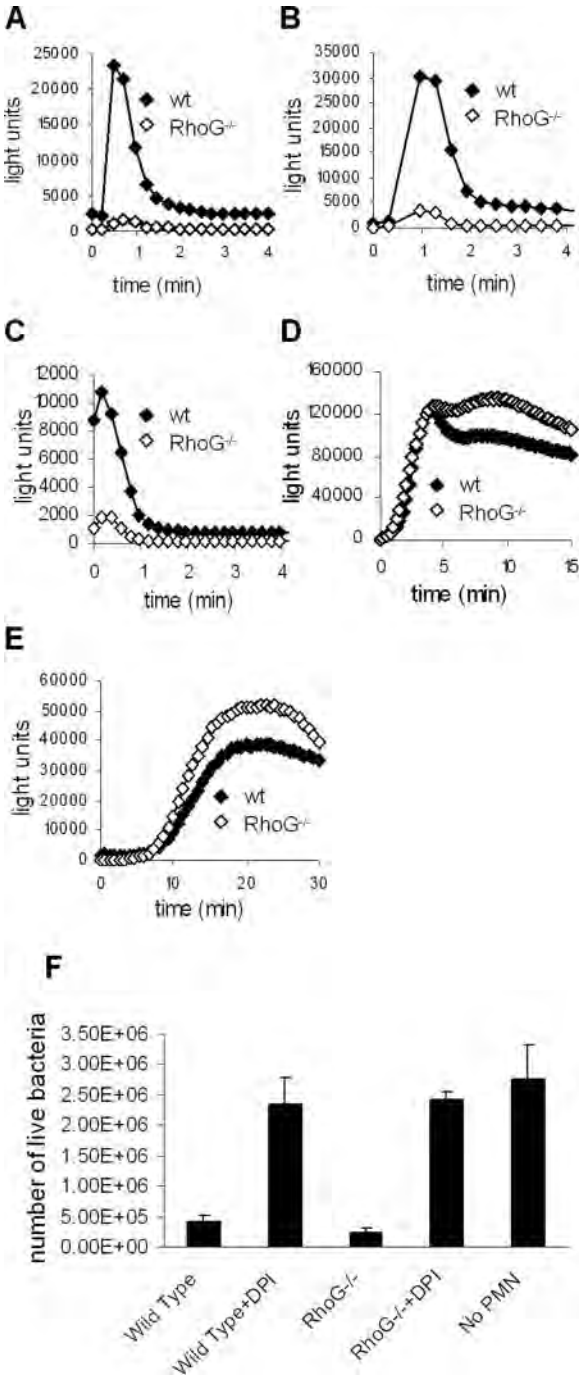

FIGURE 2. NADPH oxidase activation in wt vs RhoG$^{-/-}$ neutrophils. *A–E*, Wt and RhoG$^{-/-}$ neutrophils were incubated in the presence of HRP/Luminol and stimulated with 10 μM fMLP (*A*), 100 ng/ml GM-CSF for 1 h, then 10 μM fMLP (*B*), 25 nM C5a (*C*), 200 nM PMA (*D*), or IgG-opsonized zymosan (*E*). Chemiluminescence was monitored for the indicated times; units are arbitrary light units. A single representative trace of three or four is shown. *F*, Primed neutrophils from wt and RhoG $^{-/-}$ animals were incubated for 15 min with serum-opsonized *S. aureus* (approximate ratio of one bacterium to four neutrophils) with rapid mixing. Samples were added to ice-cold saponin and sonicated to liberate ingested bacteria. Surviving bacteria were enumerated by plating and subsequent counting of colonies. No neutrophil controls and controls with 3 μM diphenylene iodorium chloride were also run. Data are the mean ± SEM of three experiments, using three or four mice per preparation.

Interestingly, despite the relative unresponsiveness of RhoG$^{-/-}$ neutrophils to G protein-coupled receptor agonists in terms of oxidase activity, their ability to respond chemotactically to C5a and

fMLP was preserved (Fig. 3*A*). The fMLP-induced neutrophil polarization, and baseline and stimulated (with PMA; fMLP did not increase this response) macropinocytosis (Fig. 3*C*) were also unaffected, confirming the tightly restricted functional consequences of RhoG deletion on murine neutrophils. To extend these findings, we used the in vivo model of thioglycolate-induced peritonitis. Previous work (25) has shown that mice deficient in $p47^{phox}$ generated a more pronounced thioglycolate-elicited peritoneal leukocytosis than wt mice due to their inability to produce ROS. Likewise, we found that accumulation of neutrophils in the inflamed peritoneum at 18 h was significantly higher in $RhoG^{-/-}$ animals (Fig. 3*B*; $p < 0.001$, by Student's *t* test), and this was associated with a decreased percentage of annexin V-positive cells in the $RhoG^{-/-}$ peritoneum (Fig. 3*C*). Recruitment of neutrophils to the peritoneum at 4 h was not significantly different for wt and $RhoG^{-/-}$ animals (data not shown).

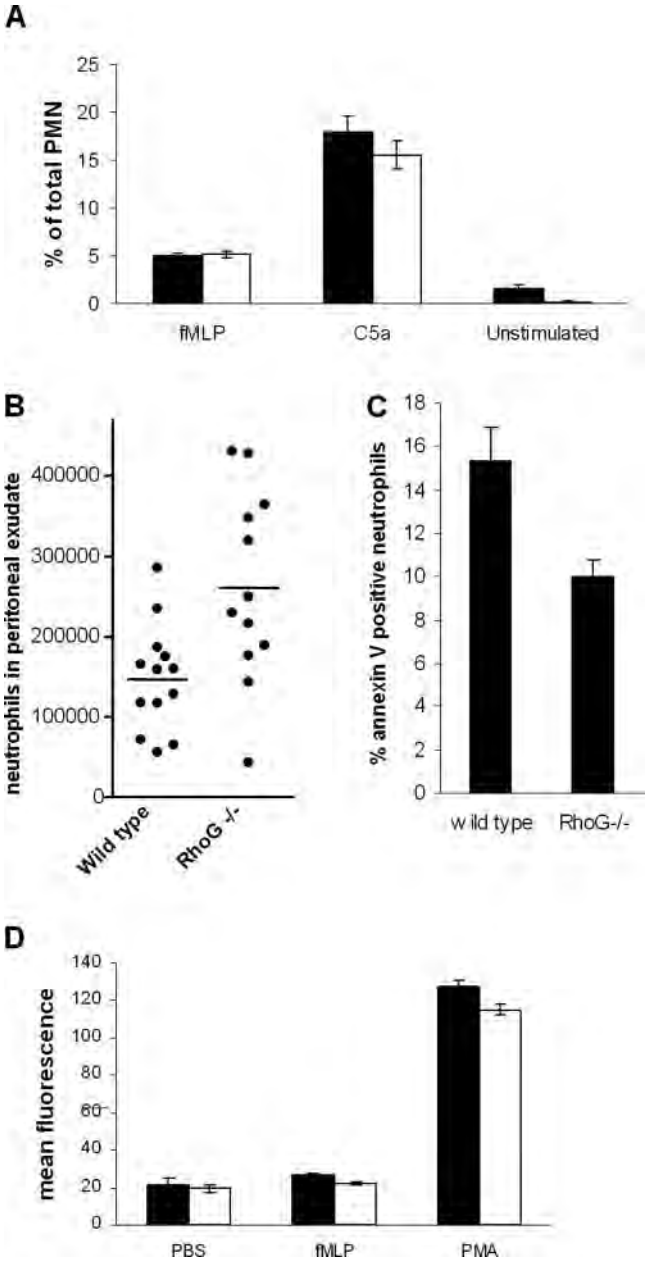

FIGURE 3. Chemotaxis and macropinocytosis in wt vs $RhoG^{-/-}$ neutrophils. *A*, In vitro chemotaxis of neutrophils to 3 μM fMLP and 30 nM C5a was performed as described. Data represent the mean ± SEM of two experiments, each performed in quadruplicate. ■, Wt neutrophils; □, $RhoG^{-/-}$ neutrophils. *B*, Thioglycolate-induced peritonitis was induced as described above. Absolute numbers of migrated neutrophils were determined by staining peritoneal exudate cells with anti-Gr1, anti-CD11b, and anti-F4/80 and running samples on a FACSCalibur flow cytometer with FlowCount fluorospheres. ●, Number of neutrophils present in the peritoneal exudates for individual animals. Bars represent the average for each group of animals. *C*, Peritoneal exudate cells were stained with anti-Gr1, anti-CD11b, and annexin V, and staining was assessed on cells positive for Gr-1 and CD11b. Data represent the mean ± SEM of two experiments with five animals of each genotype studied in each experiment. *D*, Macropinocytosis was quantitated by internalization of FITC-dextran 70 as described (*Materials and Methods*). Cells maintained at 4°C did not take up the label (data not shown). Data represent the mean ± SEM for three experiments, each performed in duplicate.

FMLP-induced Rac activation in $RhoG^{-/-}$ neutrophils

Because RhoG has been shown to act upstream of Rac1 activation in some cell systems, and because Rac1 and Rac 2 share 92% amino acid sequence identity, we investigated whether the absence of RhoG affected fMLP-stimulated activation of Rac1 or Rac2. Using a standard pull-down assay, we found that in wt murine neutrophils, peak activation of Rac proteins by fMLP occurred at 5 s (data not shown). At 5 s, the activation of both Rac1 and Rac2 was significantly less in the $RhoG^{-/-}$ than in wt neutrophils (by 25 and 32%, respectively; Fig. 4, *A–D*). There was no significant difference in the percentage of cellular Rac activation at 15 s (Fig. 4, *A–D*), and by 45 s, the response had returned almost to baseline (data not shown). Activation of CDC42 was slightly reduced in $RhoG^{-/-}$ neutrophil at 5 s, but this reduction did not achieve statistical significance (Fig. 4, *E* and *F*).

FMLP-induced activation of signaling pathways in $RhoG^{-/-}$ neutrophils

Activation of class 1 PI3Ks is required for generation of ROS in response to G protein-coupled receptor agonists (26, 27). We therefore determined the effect of RhoG deletion on PKB phosphorylation, a downstream effect of PI3K activation. Stimulation with 10 μM fMLP led to the detection of substantial phospho-PKB, peaking at 1 min, an effect that was equivalent in wt and $RhoG^{-/-}$ neutrophils (Fig. 5*A*). Concentration responses for PKB phosphorylation were also similar in wt and $RhoG^{-/-}$ neutrophils (data not shown).

Activation of p38MAPK has been implicated in regulating the neutrophil respiratory burst, and p38MAPK has been reported to phosphorylate components of NADPH oxidase (28). However, no differences in p38MAPK phosphorylation were observed between $RhoG^{-/-}$ and wt neutrophils stimulated with fMLP (Fig. 5*B*). Calcium transients in response to a range of concentrations of fMLP and C5a were similarly unaffected by deletion of RhoG (Fig. 6*A* and data not shown). Unexpectedly, phosphorylation of ERK1/2 was consistently ($n = 3$) slightly reduced in $RhoG^{-/-}$ neutrophils in response to fMLP (Fig. 5*C*). However, the detection of GTP-Ras from fMLP-stimulated neutrophils did not differ significantly between wt and $RhoG^{-/-}$ neutrophils (data not shown).

Activation of PLD correlates with respiratory burst activity in human neutrophils (29), and PLD1 has been found to bind constitutively active RhoG in NIH-3T3 cells (13). We therefore quantitated the activation of PLD in murine neutrophils treated with fMLP, but found no reduction in cells derived from $RhoG^{-/-}$ animals (Fig. 6*B*).

Agonist-stimulated translocation of the cytosolic oxidase components $p47^{phox}$ and $p67^{phox}$ to the plasma membrane has been correlated with oxidase activation in human neutrophils (30). However, at times relevant to activation of the oxidase by fMLP (30 and 60 s), we could detect no significant translocation of either

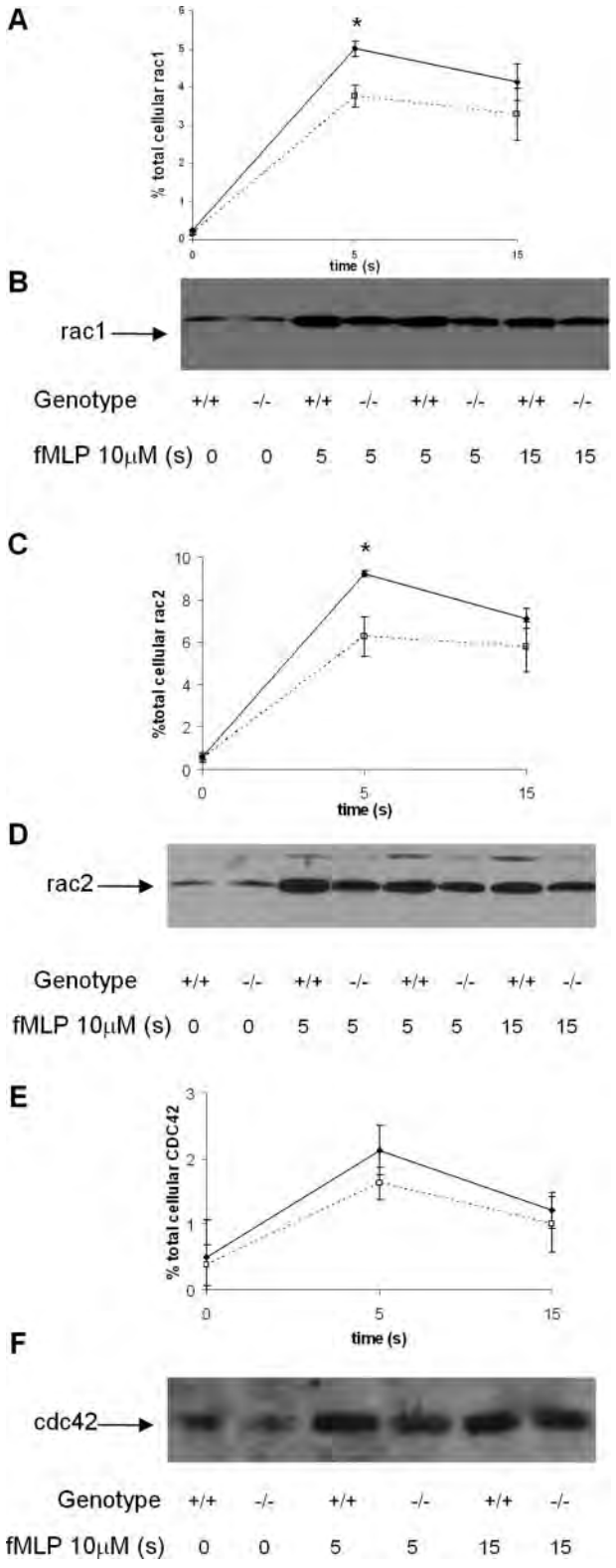

FIGURE 4. Activation of Rac and CDC42 in wt vs RhoG$^{-/-}$ neutrophils. Neutrophils (5×10^6/point) from wt and RhoG$^{-/-}$ mice were stimulated (10 μM fMLP; 0, 5, or 15 s) and lysed, and GTP-bound Rac/CDC42 was pulled down using GST-PAK-CRIB as described. Proteins were separated by SDS-PAGE. *A*, *C*, and *E*, Rac1, Rac2, and CDC42 activation was quantitated by densitometry (Image J software). Data (percentage of total cellular GTPase) represent the mean ± SEM of four experiments. *B*, *D*, and *F*, Western blotting of GST-PAK-CRIB immunoprecipitates from wt (+/+) or RhoG$^{-/-}$ (−/−) neutrophils for Rac1 (*B*), Rac2 (*D*), or CDC42 (*F*) was conducted as described; a single representative experiment of four is shown.

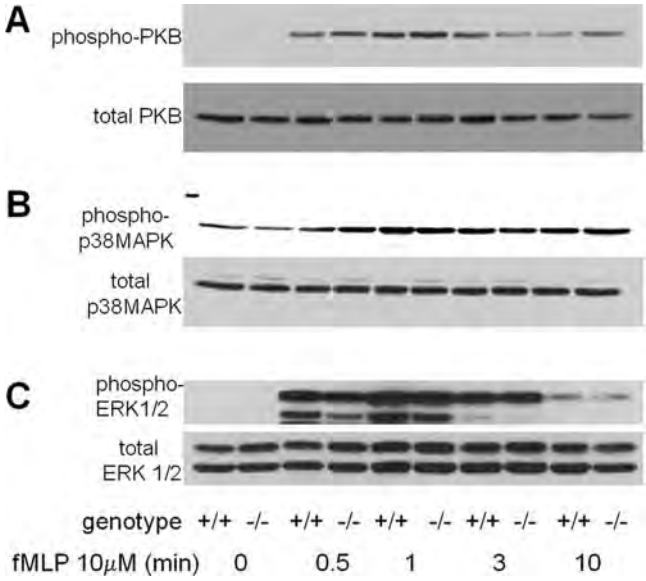

FIGURE 5. Signaling pathways in wt vs RhoG$^{-/-}$ neutrophils. *A–C*, Neutrophils from wt (+/+) and RhoG$^{-/-}$ (−/−) mice were stimulated with 10 μM fMLP for the indicated times, lysed, and Western blotted for phospho-PKB (*A*), phospho-p38MAPK (*B*), or phosphor-ERK1/2 (*C*) before stripping and reprobing for total protein.

p47^{phox} or p67^{phox} to the plasma membrane of either wt or of RhoG$^{-/-}$ murine neutrophils (data not shown).

Discussion

Previous work using overexpression systems has implicated RhoG in the regulation of both the actin cytoskeleton and the microtubule network, but the normal health status of RhoG$^{-/-}$ mice (although maintained in a specific pathogen-free environment) suggests functional redundancy or compensation by other regulatory molecules. In this report we present data demonstrating a novel, noncompensated role for RhoG linking G protein receptor stimulation with ROS generation. The functional effect of RhoG deletion seems to be narrowly restricted to NADPH oxidase activation, with chemotaxis and macropinocytosis unaffected. The fact that neutrophil accumulation was, in fact, enhanced in the inflamed peritoneum of RhoG$^{-/-}$ mice is consistent with data reported by Hampton et al. (25) demonstrating elevated neutrophil numbers in the inflammatory exudate of p47^{phox}-null mice who had received an i.p. injection of heat-killed *S. aureus*. This effect was attributed to a requirement for ROS to enable phosphatidylserine externalization and subsequent ingestion of apoptotic neutrophils.

Deletion of RhoG resulted in a partial reduction of peak Rac1 and Rac2 activation at 5 s in response to uniform stimulation with 10 μM fMLP**.** Although the rapid kinetics of Rac2 activation/deactivation that we observed differed from the more prolonged response seen by Li et al. (23), they are fully consistent with the time courses reported by Dong et al. (31) and Welch et al. (32); we do not fully understand these inconsistencies, but they may reflect differing murine genetic backgrounds and/or methodological differences in the preparation of murine neutrophils. The swift onset of the fMLP-stimulated respiratory burst (already detectable at 10 s and peaking at ~30 s) is also consistent with the rapid activation of Rac2 detected; although the respiratory burst activity was somewhat prolonged with respect to Rac2 activation, the factors responsible for switching off the former response are not well understood.

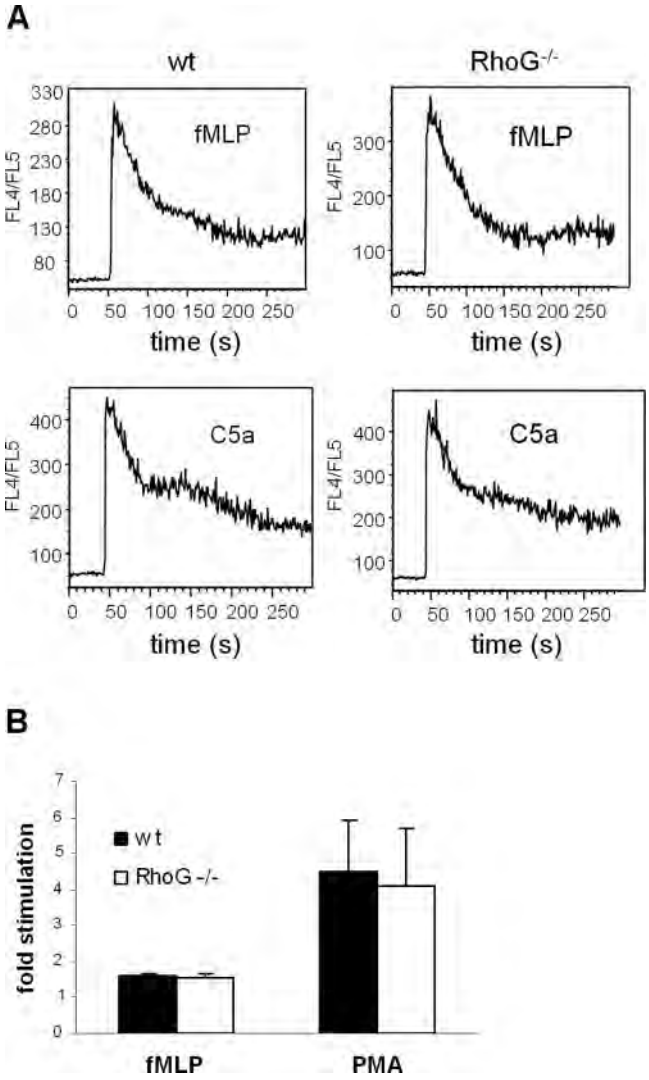

FIGURE 6. Calcium flux and PLD activation in wt vs RhoG$^{-/-}$ neutrophils. *A*, Bone marrow leukocytes were loaded with indo-1, and neutrophils were identified by the forward and side scatter profile. Intracellular calcium was monitored by flow cytometry and expressed as the ratio of indo-1 fluorescence at 424 and 530 nm (FL4/FL5). The median neutrophil: indo-1 ratio of ~500–700 events is plotted each second. Responses to 10 μM fMLP and 100 nM C5a for wt and RhoG$^{-/-}$ neutrophils are shown. *B*, Neutrophils from wt or RhoG$^{-/-}$ mice were labeled with [^{3}H]palmitic acid and stimulated with 10 μM fMLP, 200 nM PMA, or vehicle as described. Lipids were extracted and separated by TLC. Autoradiographs were analyzed using ImageJ software. Data represent the mean ± SD for two separate experiments.

The observed reduction of Rac activation was less complete than the reduction of oxidase activation in response to the corresponding stimulus; hence, it is possible that RhoG may impact on the generation of ROS by some other (as yet unidentified) mechanism. However, pull-down assays only measure overall activation of the total cellular complement of Rac; hence, the results obtained do not take into account the fact that Rac, in common with many signaling moieties, may be compartmentalized within the cell. Thus, RhoG may be acting on a subset of Rac2 required for targeted oxidase assembly or determining its precise intracellular localization (33). Neutrophils lacking Rac-GEF pRex1 (32) exhibited a partial reduction in fMLP-stimulated Rac1/2 activation (comparable to that in RhoG-deficient cells) with only a minor defect in in vitro chemotaxis assays, but a more pronounced defect in oxidase activation in LPS-primed fMLP-stimulated cells. Thus, both RhoG and pRex1 may contribute to Rac activation, with some functional overlap and, hence, partial compensation when either protein is knocked out. Elucidation of these questions may require generation of RhoG$^{-/-}$/pRex$^{-/-}$ double-knockout mice.

The complete preservation of RhoG$^{-/-}$ chemotactic responses despite a 25% reduction in Rac1 activation is surprising, but may relate to the fact that GTPase activity was measured in response to a high stimulus concentration delivered in a uniform concentration, rather than in a gradient, and that global activity measurements do not assess targeted effector localization. Although RhoG has been shown to activate Rac1 via an interaction with Dock180/Elmo, Rac1 activation is only reduced to a minor extent in the absence of RhoG, consistent with a major role for other signaling pathways in the activation of this ubiquitous small GTPase. In this context, Santy et al. (34) recently reported that ADP-ribosylation factor nucleotide site opener (ARNO) and ADP-ribosylation factor 6 (Arf6) coordinate with DOCK180/Elmo to promote Rac activation at the leading edge of migrating cells, a pathway that would remain entirely intact in RhoG$^{-/-}$ neutrophils.

In an attempt to elucidate other possible mechanisms by which RhoG could be coupled to oxidase activation, we have studied the signaling pathways implicated in this response. Both the time course and concentration dependence of PKB and of p38MAPK phosphorylation were similar in bone-marrow derived neutrophils from RhoG$^{-/-}$ and wt mice, demonstrating that activation of PI3Ks and p38MAPK alone is not sufficient for full oxidative burst activity in response to fMLP. The decrease in ERK1/2 phosphorylation in RhoG$^{-/-}$ neutrophils is surprising, but is unlikely to be mechanistically linked to the oxidase defect. Indeed, Kim and Dinauer (8) reported a more dramatic defect of ERK1/2 phosphorylation in neutrophils derived from Rac2 knockout mice, but found that the MAPK kinase-1 inhibitor, PD098059, at concentrations sufficient to abolish ERK1/2 phosphorylation had little impact on fMLP-induced superoxide generation in wt murine neutrophils. Activation of PLD correlates with respiratory burst activity in human neutrophils, an effect that may be mediated by phosphorylation of p22^{phox} (35) or direct interaction of phosphatidic acid or 1,2-diacylglycerol with oxidase components (36). Despite the fact that PLD has been identified as a possible downstream target of RhoG (13), we found no defect of PLD activation in RhoG$^{-/-}$ neutrophils. The activation of PLD activity induced by fMLP in murine neutrophils was comparatively small compared with that in human cells; this may reflect the substantially lower oxidative capacity of murine relative to human neutrophils, and the fact that such activity is further compromised by prolonged incubation at 37°C.

In summary, we have demonstrated a novel and unexpected role for the small GTPase RhoG in activation of the neutrophil NADPH oxidase in response to G protein-coupled agonists. This defect is associated with a reduction in the activation of Rac1 and Rac2, but complete preservation of chemotactic responsiveness.

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Disclosures

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