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Rac2 Is an Essential Regulator of Neutrophil Nicotinamide Adenine Dinucleotide Phosphate Oxidase Activation in Response to Specific Signaling Pathways1

Chaeyyun Kim and Mary C. Dinauer2

Rac2 is a hematopoietic-specific Rho family GTPase implicated as an important constituent of the NADPH oxidase complex and shares 92% amino acid identity with the ubiquitously expressed Rac1. In bone marrow (BM) neutrophils isolated from rac2−/− mice generated by gene targeting, we previously reported that PMA-induced superoxide production was reduced by about 4-fold, which was partially corrected in TNF-α-primed BM neutrophils and in peritoneal exudate neutrophils. We investigated receptor-mediated activation of the NADPH oxidase in the current study, finding that superoxide production in rac2−/− BM and peritoneal exudate neutrophils was normal in response to opsonized zymosan, reduced to 22% of wild type in response to IgG-coated SRBC, and almost absent in response to fMLP. In wild-type murine BM neutrophils, phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, and Akt was induced by PMA or fMLP, which was decreased in rac2−/− neutrophils for ERK1/2 and p38. Activation of p38 by either opsonized zymosan or IgG-coated SRBC was similar in wild-type and rac2−/− cells. Inhibition of ERK1/2 or p38 activation using either PD98059 or SB203580, respectively, had only a modest effect on fMLP-elicited superoxide production and no effect on the PMA-induced response. These data provide genetic evidence supporting an important role for Rac2 in regulating neutrophil NADPH oxidase activation downstream of chemoattractant and Fcγ receptors. The effect of Rac2 deficiency on superoxide production is probably exerted through multiple pathways, including those independent of mitogen-activated protein kinase activation. The Journal of Immunology, 2001, 166: 1223–1232.

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vitro (4, 5), has also been found to be essential for high level superoxide production in cell-free NADPH oxidase assays (6, 7), although its role in the NADPH oxidase complex is not fully defined.

The Rho family GTPases regulate a wide spectrum of cellular functions, including cytoskeletal organization, transcription, cell growth, development, and superoxide production (8–13). Alternating between GDP- and GTP-bound states, Rho GTPases function as intermediary switches, mediating the transfer of signals from receptors and their associated kinases to downstream effectors (12). Three Rho GTPase subclasses based on homology to the three prototypic members, RhoA, Rac1, and Cdc42Hs, are currently recognized. These encompass seven distinct proteins, including Rho (A, B, and C isoforms), Rac (1, 2, and 3 isoforms), Cdc42 (Cdc42Hs and G25K isoforms), RhoD, RhoG, RhoE, and Tc10. Rac1 and Rac2 share 92% identity and differ primarily in the C-terminal 10 residues, where Rac1, but not Rac2, contains a polybasic sequence. Rac1 and Rac3 share 77% identity, and Rac2 and Rac3 share 83% identity (14–18). Murine Rac1 has 100% amino acid identity and 92% nucleotide homology with human Rac1, and murine Rac2 differs by only two amino acids from human Rac2 (14, 15). Rac1 and Rac3 are expressed in a wide variety of tissues, whereas Rac2 expression is highly restricted to hematopoietic cells (14–18).

One of the first cellular functions ascribed to Rac was the activation of the phagocyte NADPH oxidase (6, 7). The GTP-bound form of Rac binds to both p67phox and probably also to flavocytochrome b in the assembled oxidase complex (4, 5, 19–22). Superoxide generation in cell-free assays is substantially increased by the addition of Rac (6, 7), and studies using either Rac2 transgenes or antisense oligonucleotides to manipulate Rac levels suggest that Rac regulates NADPH oxidase activity in intact cells (23, 24). Neutrophil activation with either fMLP or phorbol ester stimulates...
the formation of Rac-GTP (25, 26). Rac2 is the predominant form in human neutrophils (22, 27). In the yeast two-hybrid system, p67phox has higher affinity for Rac2 than for Rac1 (19). Recombinant isoprenylated Rac1 and Rac2 were equipotent in a cell-free NADPH oxidase assay using purified flavocytochrome b and recombinant cytosolic phox proteins, but Rac2 was more active than Rac1 when neutrophil cytosol was added (28).

To examine whether Rac2 has a specialized function in the NADPH oxidase and other neutrophil functions, mice with a targeted disruption of the hematopoietic-specific rac2 gene were generated (11). Rac2-null mice exhibited decreased exudate formation in vivo and an increased susceptibility to invasive Aspergillus. Phorbol ester-activated NADPH oxidase activity in bone marrow (BM) rac2−/− neutrophils was reduced by about 4-fold. However, the requirement for Rac2 was not absolute, in that deficient superoxide production by rac2−/− BM neutrophils could be partially overcome by first priming with TNF, and phorbol ester-elicited superoxide production in rac2−/− exudate neutrophils was almost normal. Other functional defects in rac2−/− neutrophils include diminished L-selection-mediated adhesion to glycan-1, impaired actin polymerization, and chemotaxis in response to fMLP and other chemoattractants signaling through G protein-coupled receptors, and decreased IMLP-induced activation of p42/p44 and p38 mitogen-activated protein (MAP) kinase (11). Taken together, these findings suggested that Rac2 regulates multiple cellular functions in neutrophils. Additional studies in mast cells (29) and T cells (30) have demonstrated a variety of functional abnormalities associated with intracellular signaling defects. These include reduced chemotaxis and growth factor-induced survival in mast cells, with impaired activation of Akt (29).

In the present study, we used rac2−/− mice as a genetic approach to investigate the role of Rac2 in receptor-mediated activation of the neutrophil NADPH oxidase. The results support the concept that Rac2 plays an important, but stimulus-specific, role in regulating NADPH oxidase activation and other functional responses of neutrophils, particularly those downstream of chemoattractant receptors and Fcy receptors.

### Materials and Methods

**Abs, reagents, and buffers**

Rac2 and p47phox Abs were gifts from G. Bokoch and U. Knaus (The Scripps Research Institute, La Jolla, CA) and D. Lambeth and D. Uhlinger (Emory University, Atlanta, GA), respectively. Rabbit polyclonal Abs against p42/44, Thr202/Tyr204-phosphorylated p42/44, Thr183/Tyr185-phosphorylated c-Jun N-terminal kinase (JNK), SARK, p38 MAP kinase, Thr180/Tyr182-phosphorylated p38 MAP kinase, Akt, and Ser473-phosphorylated Akt were purchased from New England Biolabs (Beverly, MA). Mouse mAbs against Akt1 and phospho-Akt (4G10) were purchased from Upstate Biotechnology (Lake Placid, NY), and those for JNK1/2 and CD16/CD32 were purchased from PharMingen (San Diego, CA). Highly purified recombinant human Rac1 and Rac2 were provided by D. Lambeth. Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. PBS (pH 7.2), HBSS (without Ca2+ and Mg2+), and phenol red, and HBSS with 1.26 mM Ca2+ were purchased from Life Technologies (Grand Island, NY). Other buffers used in this study include HBSS with 0.1% BSA and 1% glucose (pH 7.25–7.4) (HBSS-BG); PBS (pH 7.2), HBSS (without Ca2+ and Mg2+) until further processing. For some studies BM neutrophils were cultured for 24 h at 37°C in DMEM containing 20% FCS and 1 ng/ml IL-3 before measuring NADPH oxidase activity. To elicit peritoneal exudate neutrophils, mice were injected with 1 ml of 3% thioglycolate. Peritoneal cells were harvested after 18 h as previously described (31). All reagents used were endotoxin tested (<0.1 ng endotoxin/ml by the Limulus lysate assay) to minimize inadvertent priming during the isolation procedure.

Human neutrophils were separated from peripheral venous blood obtained from healthy volunteers as approved by the institutional review board of the Indiana University School of Medicine. Heparinized whole blood was sedimented using 6% dextran 70 (McGaw, Irvine, CA), and the granulocytes were purified from the leukocyte-rich rich serum by Ficoll-Hypaque. Remaining erythrocytes were removed by hypotonic lysis.

**FcγRII/III expression**

Flow cytometry was used to quantitate the relative amount of cell surface FcγRII/III expression per neutrophils. BM neutrophils (2 × 10^6/ml) in PBS containing 0.1% BSA were incubated with 2 μg/ml FITC-conjugated anti-CD16/CD32 or FITC-conjugated anti-rabbit mouse IgG2b isotype (PharMingen) for 30 min on ice. After washing cells twice with PBS containing 0.1% BSA, flow cytometric analysis was performed using a FACScan (Becton Dickinson). A minimum of 10,000 neutrophil events was recorded and analyzed using CellQuest (Becton Dickinson).

**Opsonization of zymosan and preparation of IgG-sensitized SRBC**

Zymosan particles were prepared as described previously (32), opsonized with normal human serum for 1 h at 37°C, washed with PBS, and then diluted in PBS. Ab-coated SRBC (IgG-SRBC) were freshly prepared in magnesium- and calcium-free PBS by incubating 10^6/ml SRBC (Cappel; ICN Biomedicals, Costa Mesa, CA) with an equal volume of 2 μg/ml IgG-rabbit-anti-SRBC Ab (Cappel) as previously described (33).

**Immunoblot analysis of Rac1 and Rac2 expression**

Lysates of mouse BM neutrophils and human peripheral blood neutrophils were prepared, and protein was measured with the bicinchoninic acid protein kit from Pierce (Rockford, IL) using BSA as a standard. Lysates were subjected to SDS-PAGE and immunoblotting as previously described (11, 34). Blots were probed with either mouse mAb for Rac1 or a rabbit polyclonal Ab for Rac2 and developed with the ECL method (Amersham, Arlington Heights, IL) as previously described (35). The Ab specificity of the Rac1 and Rac2 Abs was confirmed by immunoblotting Exserichia coli-purified recombinant Rac1 and Rac2, supplied by D. Lambeth (Emory University). Integrated densitometry was employed to measure the relative intensities of signals using an Eagle Eye II Still Video System and associated software (Stratagene, La Jolla, CA).

Animals

Rac2 knockout (rac2−/−) mice had previously been generated by targeted disruption of the rac2 gene (11). Rac2 knockout mice used in this study had been backcrossed into C57BL/6J mice for ≥11 generations. C57BL/6J mice were used for wild-type controls. Mice were housed in microisolator cages under specific pathogen-free conditions and were fed autoclaved food and acidified water ad libitum. Mice used in these experiments were 8–16 wk of age.
Superoxide production was measured in a quantitative kinetic assay based on the reduction of cytochrome c. The assay was performed at 37°C using a Thermomax microplate reader and associated SOFTMAX version 2.02 software (Molecular Devices, Menlo Park, CA) as reported previously (34, 35). Briefly, for the soluble stimuli, cells were suspended (2.5 × 10^5 cells/ml) in 250 µl of PBSG containing 75 µM ferricytochrome c and activated by the addition of 200 ng/ml PMA or 10 µg/ml IgG-SRBC (2.5 × 10^5 potential RBCs) before adding PBSG containing ferricytochrome c. FcγR-activated oxidant production in BM neutrophils was also measured using the Fc Oxby Burr reagent (Molecular Probes, Eugene, OR), according to the manufacturer’s instructions. Briefly, BM neutrophils were suspended at 2 × 10^6/ml in PBSG and then stimulated with Fc Oxby Burr at 37°C for a final concentration of 140 µg/ml. Flow cytometric analysis was performed using a FACSsort (Becton Dickinson) and data were analyzed using CellQuest. For measurement of nitroblue tetrazolium (NBT) reduction, neutrophils suspended in IMDM were placed on a glass chamber slide (Nunc, Naperville, IL) for 1 h before activation with different stimuli. After an additional incubation for 20 min at 37°C, slides were fixed and counterstained with safranin, and the percentage of NBT-positive neutrophils and the intensity of staining were determined by evaluating 100 randomly selected cells (34).

**MAP kinase activation**

To examine the phosphorylation of MAP kinases, paired samples of wild-type and rac2−/− BM neutrophils were suspended in HBSS (1 × 10^6 cells/ml) and stimulated for varying periods of time with 200 ng/ml PMA, 10 µM fMLP, 100 µg/ml OpZ, or a 10-fold excess of IgG-SRBC at 37°C. The activation was terminated by addition of cold PBS and placement on ice. Thereafter, cells (1 × 10^6 cells/ml) were pelleted and lysed in 200 µl of lysis buffer at 4°C for 30 min. Cell lysates were clarified by centrifuging for 2 min at 18,000 × g at 4°C. Samples were then heated at 95°C for 5 min with 0.2 vol of 5X Laemmli sample buffer and 10 µg of cell lysate (10 µl for IRG-SRBC samples) resolved on 10% SDS-PAGE. Immunoblot analysis was conducted as described above. Expression of total p42/44, p38, JNK, and Akt was detected using rabbit polyclonal Abs against p42/44, p38, JNK, and Akt and tyrosine phosphorylation were determined using phospho-specific p42/44, p38, JNK, Akt, and tyrosine Abs. Integrated densitometry was employed to measure the relative intensity of the protein signal using an Eagle Eye II Still Video System.

**MAP kinase inhibitors**

To investigate whether MAP kinase inhibitors affect superoxide production in murine BM neutrophils, the p38 MAP kinase inhibitor, SB203580 (Calbiochem, La Jolla, CA) and the MAP kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitor, PD98059 (Calbiochem), were used. BM neutrophils were incubated with SB203580 (1, 5, and 10 µM) or PD98059 (5, 25, and 50 µM) for 30 min at 37°C. Following incubation, cells were analyzed for cytochrome c superoxide production using the reduction assay and for MAP kinase activation in response to various stimuli as described above.

**Results**

**Expression of Rac1 and Rac2 in mouse and human neutrophils**

It has previously been shown that Rac2 is the predominant Rac isoform in human neutrophils (22). We compared the expression of Rac1 and Rac2 in mouse BM neutrophils to those in human neutrophils by immunoblotting with specific Abs for Rac1 and Rac2. At least three to five times more Rac1 was expressed in mouse neutrophils on a milligram protein basis compared with human neutrophils, whereas similar levels of Rac2 were detected in mouse and human neutrophils (Fig. 1). As previously reported (11), the expression of Rac1 in murine rac2−/− neutrophils was similar to that in wild-type neutrophils.

**Receptor-mediated activation of neutrophil NADPH oxidase in rac2−/− mice**

To examine the activation of the NADPH oxidase by physiological agonists, three different receptor-mediated stimuli were tested. Superoxide production in response to fMLP was ~10-fold higher in freshly isolated wild-type BM neutrophils (2.5 ± 2.3 nmol/min/10^7 cells) than in those isolated from rac2−/− mice (0.2 ± 0.7 nmol/min/10^7 cells; Fig. 2A). BM neutrophils were also cultured with 1 ng/ml IL-3 for 24 h at 37°C to enhance responsiveness to fMLP stimulation (36, 37) (Fig. 2B). fMLP-elicited superoxide production by IL-3-treated wild-type BM neutrophils increased ~2-fold compared with that by freshly isolated BM neutrophils (p < 0.05), but was still virtually undetectable in rac2−/− cells. Consistent with previous observations (11), PMA-elicited NADPH oxidase activity was reduced by about 4-fold in freshly isolated rac2−/− BM neutrophils (3.6 ± 1.2 nmol/min/10^7 cells) compared with wild-type neutrophils (17.3 ± 3.8 nmol/min/10^7 cells) (Fig. 2A). Culture in IL-3 resulted in a modest increase in PMA-elicited superoxide production by BM neutrophils (22.5 ± 6.5 nmol/min/10^7 cells), although this was still significantly reduced in cells isolated from rac2−/− mice (9.6 ± 3.2 nmol/min/10^7 cells). Activation of NADPH oxidase in response to opsonized particles was also evaluated in wild-type and rac2−/− BM neutrophils (Fig. 3). Superoxide production elicited by IgG-SRBC was reduced by ~5-fold in rac2−/− BM neutrophils compared with wild-type neutrophils (wild-type, 6.9 ± 2.5 nmol/min/10^7 cells; rac2−/−, 1.5 ± 2.4 nmol/min/10^7 cells). The expression of FcγR, as measured by flow cytometry using an Ab against Fcγ III/II receptor (CD16/CD32), was similar for both wild-type and rac2−/− genotypes (data not shown). This suggests that the significant impairment in activation of the NADPH oxidase by IgG-SRBC in rac2−/− BM neutrophils does not reflect reduced expression of Fcγ receptors. In contrast, activation of the respiratory burst by serum OpZ, which can also interact with the β2 integrin CR3 receptor for activated complement protein C3bi, was preserved in rac2−/− BM neutrophils. There was no difference in OpZ-induced superoxide release between genotypes (wild-type, 7.3 ± 3.0 nmol/min/10^7 cells; rac2−/−, 6.0 ± 2.2 nmol/min/10^7 cells; Fig. 3).

We next examined receptor-mediated activation of superoxide production in peritoneal exudate neutrophils from wild-type and rac2−/− mice. NADPH oxidase activity in response to IgG-SRBC was substantially reduced in rac2−/− peritoneal exudate neutrophils (wild-type, 5.04 ± 1.52 nmol/min/10^7 cells; rac2−/−, 0.08 ± 0.08 nmol/min/10^7 cells), while it was normal in response to OpZ (wild type, 5.0 ± 1.8 nmol/min/10^7 cells; rac2−/−, 5.0 ± 2.1 nmol/min/10^7 cells; Fig. 4). Stimulation with fMLP did not induce
sufficient superoxide production that could be detected in the cytochrome c assay in either wild-type or rac2²⁻/² peritoneal exudate cells (data not shown). In contrast to our initial observation that PMA-activated superoxide production was not significantly reduced in rac2²⁻/² peritoneal exudate neutrophils (11), we found a 2-fold reduction in rac2²⁻/² peritoneal exudate cells compared with wild-type (wild type, 14.9 ± 2.4 nmol/min/10⁷ cells; rac2²⁻/², 7.7 ± 2.8 nmol/min/10⁷ cells; p < 0.01). This may reflect differences in the mouse genetic background. In the original study, we used C57BL/6J x 129Sv F₂, F₃, and F₄ littermate mice, whereas in the current study, mice were in the C57BL/6J background.

As another approach to evaluating NADPH oxidase activity in BM and peritoneal exudate neutrophils, we used the NBT test, a sensitive semiquantitative assay for monitoring superoxide production in individual cells. In this assay NADPH oxidase is activated in the presence of NBT, which forms an insoluble purple precipitate when reduced by superoxide. The relative numbers of NBT-positive cells in response to PMA and OpZ were similar between wild-type and rac2²⁻/² BM and peritoneal exudate neutrophils (Table I). However, the relative numbers of NBT-positive cells detected upon activation with either fMLP or IgG-SRBC were significantly decreased in rac2²⁻/² cells (Table I). Activation

**FIGURE 2.** Activation of NADPH oxidase by fMLP receptor in BM neutrophils. ■ Wild type; □ rac2⁻²/². Data are expressed as the mean ± SD. *, p < 0.001, wild type vs rac2⁻²/² (unpaired t test). A. Superoxide production by freshly isolated BM neutrophils following stimulation with 200 ng/ml PMA or 10 μM fMLP was monitored by reduction of ferricytochrome c (n = 8). B. Superoxide production by BM neutrophils cultured with 1 ng/ml IL-3 for 24 h at 37°C before stimulation with either PMA or fMLP (n = 11).

**FIGURE 3.** Activation of BM neutrophil NADPH oxidase by opsonized particles. A. BM neutrophils were preincubated with 100 μg/ml OpZ (n = 9) or 10× IgG-SRBC (n = 7) for 30 min at 37°C before adding ferricytochrome c to monitor superoxide production. ■ Wild type; □ rac2⁻²/². Data are expressed as the mean ± SD. *, p < 0.001, wild type vs rac2⁻²/² (unpaired t test). B. Representative FACSscan of BM neutrophils incubated with Fc OxyBurst at 37°C. The shaded area shows fluorescence obtained immediately after mixing, which was the same for wild-type and rac2⁻²/² mice. The open curves show fluorescence after 30 min of incubation, with the dark line indicating fluorescence of wild-type neutrophils, and the thinner line indicating fluorescence for rac2⁻²/² neutrophils. C. The mean cellular fluorescence for the highly fluorescent subpopulation of Fc OxyBurst-activated neutrophils (see B) is shown after either 20 (n = 4–5) or 30 (n = 3–5) min of incubation, as indicated. ■ Wild type; □ rac2⁻²/². *, p = 0.022; **, p = 0.011.
with either PMA or OpZ produced intense cell-associated formazan deposits in both wild-type and rac2−/− neutrophils, which were concentrated in the phagosome in the case of OpZ-activated neutrophils, whereas fMLP or IgG-SRBC stimulation produced less intense formazan staining of cells (data not shown). Phagocytosis of serum-opsonized zymosan particles was equivalent in wild-type and rac2−/− neutrophils. The number of particles ingested per 100 neutrophils was 131 ± 19 for wild-type and 137 ± 26 for rac2−/− (n = 5 for both genotypes). Ingestion of IgG-SRBC was rarely seen for either wild-type or rac2−/− neutrophils, perhaps because the rabbit IgG used for opsonization is a poor stimulus inducing phagocytosis in murine neutrophils under our conditions.

The differences seen between wild-type and rac2−/− neutrophils in the NBT assay for superoxide production are overall consistent with the results from the cytochrome c assay, except for the response to PMA. However, we have previously observed that intense NBT reduction can be observed when NADPH oxidase activity is only 20–30% of wild-type levels (34, 38). This could account for the similar percentage of NBT-positive cells for rac2−/− and wild-type PMA-activated neutrophils despite a reduced amount of superoxide release by rac2−/− cells.

The decreased fraction of NBT-positive neutrophils in rac2−/− mice in response to fMLP or IgG-SRBC also indicates that reduced superoxide release measured in the cytochrome c assay could reflect either fewer numbers of activated rac2−/− neutrophils and/or decreased neutrophil NADPH oxidase activity. To examine this question directly for FcγR-activated NADPH oxidase activity, we used the Fc OxyBurst reagent to monitor oxidant production by flow cytometry. As shown in Fig. 3B, incubation with Fc Oxyburst produced a shift in fluorescence, corresponding to oxidant production, in a subpopulation of BM neutrophils. The shift in fluorescence was significantly decreased in rac2−/− neutrophils compared with wild-type cells (666 ± 76 vs 1753 ± 501 U after 30 min; n = 3–5; p = 0.011; Fig. 3C). The relative number of responding neutrophils was also decreased in rac2−/− mice, with 27 ± 9% of rac2−/− cells exhibiting a shift in fluorescence by 30 min compared with 48 ± 25% for wild-type cells, although this difference was not statistically significant. These results suggest that decreased superoxide production by FcγR-activated rac2−/− neutrophils is due to both a decrease in cellular oxidant production as well as a smaller proportion of responding cells.

**Activation of intracellular kinases in activated murine neutrophils**

We previously found that phosphorylation of ERK1/2 and p38 MAP kinases induced by fMLP was diminished in rac2−/− BM neutrophils (11). To expand on these initial observations, we compared chemoattractant-activated MAP kinase activation in mouse BM neutrophils to stimulation by PMA, OpZ, or IgG-SRBC, using Abs to detect the corresponding phosphorylated form of the protein (Fig. 5). Preliminary studies (see also Fig. 6A) determined that PMA-induced phosphorylation of ERK1/2 and p38 was initiated within 2 min, which increased further by 10 min. fMLP-induced phosphorylation of ERK1/2 and p38 peaked at 2 min, with phosphorylated ERK1/2 declining by 5 min (data not shown). Phosphorylated p38 was detected 5 min after the addition of IgG-SRBC and persisted for at least 60 min; we did not detect ERK1/2 phosphorylation in response to IgG-SRBC over this same period (data not shown). In subsequent studies, we examined agonist-induced MAP kinase protein phosphorylation at 2 min for fMLP, 10 min for PMA, and 30 min for opsonized particles. As shown in the representative blots in Fig. 5, phosphorylation of ERK1/2 induced by either fMLP or PMA was markedly reduced in rac2−/− BM neutrophils compared with wild-type neutrophils. Only a small decrease in p38 phosphorylation was typically seen for fMLP or PMA-activated rac2−/− BM neutrophils compared with wild-type cells, although this difference was more prominent in some experiments (compare Figs. 5 and 6A). Activation of p38 by either OpZ or IgG-SRBC was similar in wild-type and rac2−/− cells. Phosphorylation of JNK was also detectable after stimulation with either PMA or fMLP, which was consistently decreased in rac2−/− cells. Previous studies have suggested that Rac is downstream of phosphoinositide-3 kinase (PI3K) activation induced by chemoattractants and other signals (39, 40). In rac2−/− mast cells, PI3K activation by stem cell factor is normal, but phosphorylation of PI3-dependent kinase Akt (41) is decreased (29). Phosphorylation of Akt was not decreased in rac2−/− BM neutrophils stimulated with either PMA or fMLP, and actually appeared to be increased in fMLP-treated rac2−/− cells compared with wild-type cells (Fig. 5), confirming our initial observations of fMLP-activated neutrophils (11). Under the conditions used, Akt phosphorylation was not observed in response to OpZ or IgG-SRBC.

**Effect of MAP kinase inhibitors on superoxide production**

We next investigated whether the decreased phosphorylation of ERK1/2 or p38 observed in rac2−/− BM neutrophils activated by
fMLP or PMA was functionally linked to NADPH oxidase activation. Studies in human neutrophils have found variable results as to whether NADPH oxidase activity can be suppressed by inhibiting phosphorylation of either ERK1/2 using the MEK-1 inhibitor PD098059 or of p38 using SB203580 (42–47). In initial studies we established that phosphorylation of ERK1/2 or p38 could be effectively inhibited by the corresponding inhibitor (Fig. 6A). However, PD098059 or SB203580 did not significantly inhibit PMA-elicited superoxide production in either wild-type or rac2−/− BM neutrophils, either when given alone (Fig. 6B) or in combination (data not shown). fMLP-induced superoxide production in either wild-type or rac2−/− BM neutrophils, either when given alone (Fig. 6B) or in combination (data not shown). However, PD098059 or SB203580 did not significantly inhibit PMA-elicited superoxide production in either wild-type or rac2−/− BM neutrophils, either when given alone (Fig. 6B) or in combination (data not shown). The residual superoxide-generating activity in fMLP-activated rac2−/− BM neutrophils was also partially sensitive to inhibition by PD098059 or SB203580. These results suggest that the effect of Rac2 deficiency on activation of the NADPH oxidase in murine neutrophils may be mediated largely through non-MAP kinase-dependent pathways.

Discussion
Genetic deficiency of Rac2, a hematopoietic-specific Rho GTPase, is associated with a variety of functional defects in murine BM-derived cells due to abnormalities in intracellular signaling pathways (11, 29, 30) despite the presence of the highly homologous Rac1 (Fig. 1) and, probably, Rac3 (18). In neutrophils, the absence of Rac2 was associated with significant defects in chemotaxis, L-selectin-mediated rolling, F-actin generation, and phorbol ester-induced superoxide production. These defects were reflected in vivo by baseline neutrophilia, reduced inflammatory peritoneal exudate formation, and increased mortality infected with Aspergillus fumigatus (11). The importance of Rac2 in regulating neutrophil function has also been highlighted by the recent discovery of a new genetic immunodeficiency syndrome in humans due to a dominant negative mutation of Rac2 (48, 49). Neutrophils isolated from the affected patient exhibited functional abnormalities similar to those seen in rac2−/− neutrophils, with defects in chemotaxis and NADPH oxidase activation in response to some, but not all, agonists along with diminished L-selectin-mediated adhesion. Chemokine-induced superoxide production, chemotaxis, and degranulation were markedly deficient (48, 49) as was fMLP-induced phagocytosis of IgG-SRBC (48). However, only a small decrease was observed in the phorbol ester-activated respiratory burst (48, 49).

In the current study, we examined receptor-mediated activation of the NADPH oxidase in rac2−/− neutrophils to further characterize the role of Rac2 in the regulation of neutrophil superoxide production. NADPH oxidase activity was normal in opsonized zymosan-activated rac2−/− BM neutrophils, but was deficient in response to fMLP or to IgG-opsonized particles despite the expression of Rac1 at substantially higher levels compared with human neutrophils. The agonist-specific defects observed in activation of the respiratory burst in murine rac2−/− neutrophils are consistent with those reported for human neutrophils expressing a dominant negative Rac2 mutant. We had previously observed that the impaired phorbol ester-induced activation of superoxide production in rac2−/− neutrophils could be partly surmounted by in vivo or in vitro priming with inflammatory cytokines (11). Taken together,
these data show that the requirement for Rac2 in superoxide production is not absolute and suggest that specific signaling pathways leading to activation of the neutrophil respiratory burst are selectively regulated by Rac2.

There are at least two mechanisms by which the absence of Rac2 could lead to defects in neutrophil superoxide production. Rac is a component of the enzymatically active NADPH oxidase complex, and either Rac1 or Rac2 is required for enzyme activity in recombinant cell-free oxidase assays. The GTP-bound form of Rac can bind directly to p67phox and to at least one other site in the NADPH oxidase (5, 19, 50). Decreased respiratory burst activity in rac2−/− neutrophils might reflect a preferred role for Rac2 due either to increased affinity to one or more other subunits of the NADPH oxidase and subsequent enhanced enzymatic activity or to differences in activation or subcellular localization of Rac2 compared with Rac1. Alternatively, Rac2 deficiency may lead to signaling abnormalities that affect phosphorylation of p47phox and subsequent translocation of p47phox/p67phox/p40phox and/or recruitment of Rac-GTP to the enzyme complex. At the current time it is unknown whether the predominant effect of Rac2 deficiency on the respiratory burst reflects its function in the NADPH oxidase complex itself or in the preceding steps leading to enzyme assembly. However, since multiple functional abnormalities in other BM-derived cells have been identified in rac2−/− mice (11, 29, 30), we speculate that defects in intracellular signaling pathways account for at least some of the observed decrease in superoxide production in response to specific agonists.

Why Rac1 is unable to substitute for Rac2 in superoxide production, chemotaxis, and other functional responses that are impaired in Rac2-deficient blood cells also remains to be defined. These two isoforms may be selectively activated by specific exchange factors or interact differentially with downstream effectors. For example, the Rac-GEF Tiam-1 (51) is a strong inducer of p21-activated kinase 1 activation, but not of JNK (52), two kinases that are both strongly activated by Rac-GTP. This selectivity could arise either through differences in subcellular localization or in relative affinities for different Rac isoforms with interacting proteins. For example, Rac1 has been shown to bind to and stimulate p21-activated kinase 1 activity more efficiently than Rac2 (53). In addition, studies using the yeast two-hybrid system have suggested that the affinity of Rac2 for p67phox is higher than that for Rac1 (19), and Rac2 is more active than Rac1 in stimulating NADPH oxidase activity in the presence of neutrophil cytosol (28). Another potential mechanism for functional defects in rac2−/− cells is that the overall level of Rac-GTP might be a rate-limiting step in the activation of downstream functions, and signaling cascades triggered by different neutrophil agonists may vary in the amount of activated Rac generated.

Rac has been implicated as a participant in many of the multiple signaling pathways that are triggered upon neutrophil activation by soluble or particulate agonists (1, 39, 54, 55). How these complex and often redundant signals are ultimately linked to specific functional responses, such as activation of the otherwise dormant NADPH oxidase, remains incompletely understood. However,
from studies using biochemical, pharmacologic, or genetic approaches, it is clear that different agonists activate both overlapping and distinct signaling cascades (1, 39, 54, 56–59) that could potentially lead to selective activation of Rac2 and the respiratory burst.

Signaling defects downstream of heterotrimeric G protein-coupled receptors for chemoattractants are a prominent feature of Rac2 deficiency. We previously reported that that chemotaxis and F-actin polymerization in response to fMLP or leukotriene B4 was substantially diminished in rac2−/− neutrophils (11) and now show that fMLP-induced activation of superoxide production also significantly impaired. These features of the rac2−/− phenotype have some overlap with mice deficient in the p110 catalytic subunit of PI3K (57–59). PI3Kγ-deficient leukocytes are unable to generate phosphatidylinositol 3,4,5-trisphosphate upon G protein-coupled receptor stimulation, in association with deficient chemoattractant-induced chemotaxis and NADPH oxidase activity. fMLP-induced Rac activation was reported to be unaffected in PI3Kγ-deficient mice (58). This suggests that PI3Kγ-dependent signaling and a parallel pathway(s) linked to Rac2 activation are both required for inducing a respiratory burst by fMLP and related agonists.

The rac2−/− neutrophils also exhibited a defect in NADPH oxidase activation upon activation through FcγR using Ig-opsonized particles, but not with zymosan opsonized with serum opsonins (C3bi, IgG), which can bind to both the β2 integrin receptor CR3 and FcγR (60–64). This is the first direct evidence that Rac2 plays an important role in regulating the FcγR-dependent respiratory burst in neutrophils, which is likely to occur downstream of the activation of receptor-associated tyrosine kinases, including Syk and Hck, that are prominent proximal events upon FcγR ligation (65). Our data also indicate that signals induced either downstream of the CR3 receptor or through CR3 costimulation of FcγRs (66) are sufficient to activate NADPH oxidase independently of Rac2 in murine neutrophils. Signaling through Rho GTPases has previously been shown to play an important role in the phagocytosis of opsonized particles by macrophages, and different Rho family members appear to play distinctive roles in this process (10, 65, 67). In macrophages, Cdc42 and Rac regulate uptake of Ig-opsonized particles via FcγR, whereas Rho plays a prominent role in CR3-mediated phagocytosis (67). Our findings that superoxide production in rac2−/− neutrophils is impaired in response to FcγR ligation but not when CR3 binding is also involved are consistent with these observations.

Although PMA is not a physiologic stimulus for neutrophil activation, it is noteworthy that PMA-elicited superoxide production was substantially decreased in rac2−/− neutrophils. PMA is a direct activator of protein kinase C (PKC) and is one of the most potent activators of the neutrophil respiratory burst. Neutrophil activation by most physiologic agonists is accompanied by activation of PKC, and specific inhibitors of PKC decrease NADPH oxidase activity (reviewed in Refs. 54 and 55). An early event downstream of agonist binding to chemoattractant or Fcγ receptors is the activation of phospholipase C, which catalyzes the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate by hydrolysis of membrane phospholipids (54). In turn, DAG directly activates PKC, and inositol 1,4,5-trisphosphate promotes the release of intracellular calcium, which is important for activation of many PKC isoforms, including the PKC-β and -δ species present in neutrophils (54, 55). DAG can also be generated through a parallel pathway involving activation of phospholipase D. Direct evidence for PKC-β regulation of NADPH oxidase activity was found in a recent study in which PKC-β activity was ablated either using a specific inhibitor or genetically using a knockout mouse lacking PKC-β (68). In either case, an ∼2-fold reduction in neutrophil superoxide production elicited by IgG-coated particles or PMA was observed. Inhibition of PKC-β expression in HL-60 cells using antisense oligonucleotides also resulted in decreased NADPH oxidase activity (69). The deficient respiratory burst in rac2−/− neutrophils in response to PMA, fMLP, or IgG-opsonized particles may reflect a common defect in Rac2-dependent events downstream of PKC that promote oxidase assembly.

We hypothesized that the differential activation of MAP kinase cascades might explain the agonist-dependent effects on neutrophil NADPH oxidase activation in murine rac2−/− neutrophils. MAP kinase activation is a prominent response in human neutrophils activated by either soluble agonists or opsonized particles (54, 70, 71). ERK2/1 (p42/44) MAP kinase is activated via the Ras/Raf pathway, although cross-regulation with Rac-dependent pathways can also occur (72, 73). Many groups have reported that JNK and p38 MAP kinase pathways are downstream of Rac and Cdc42 in various cell types, suggesting an analogous role to that played by Ras in the ERK MAP kinase cascade (12, 13, 17, 74, 75).

We found that both fMLP and PMA induced phosphorylation of ERK1/2 and p38 in murine BM neutrophils, which was decreased in rac2−/− cells compared with wild-type cells, particularly for ERK1/2. However, activation of these MAP kinases appears to be only in part functionally linked to downstream activation of superoxide production. Pharmacologic inhibition of either ERK1/2 or p38 phosphorylation had no significant effect on PMA-induced superoxide production in wild-type BM neutrophils. Hence, although PMA activated both ERK1/2 and p38 in murine BM neutrophils in a Rac2-dependent manner, these events do not play a role in NADPH oxidase activation and are unlikely to account for the defect in superoxide generation in PMA-activated rac2−/− neutrophils. These results are generally consistent with observations in human neutrophils, where inhibitors of either ERK1/2 or p38 have had variable results on PMA-induced superoxide production (42, 43, 45–47). With regard to the fMLP-activated respiratory burst, inhibition of ERK1/2 has also had inconsistent effects in human neutrophils (43–45, 76), whereas at least a partial decrease in NADPH oxidase activity has been reported in the presence of p38 inhibitors (42, 43, 47). In fMLP-activated wild-type murine BM neutrophils, we found that inhibition of either ERK1/2 or p38 produced only an ∼30% decrease in NADPH oxidase activity. The residual respiratory burst detected in fMLP-stimulated rac2−/− BM neutrophils exhibited a similar partial sensitivity to p38 and ERK1/2 inhibitors. These data suggest that events downstream of ERK1/2 and p38 regulate the fMLP-activated respiratory burst in murine BM neutrophils, but that the absence of Rac2 is likely to affect additional or redundant fMLP-induced signaling pathways important for NADPH oxidase activation. In contrast to studies on human neutrophils (76–78) or murine macrophages (79, 80), we were unable to detect phosphorylated ERK1/2 in murine BM neutrophils stimulated with opsonized particles. However, p38 MAP kinase was activated with either OpZ or IgG-SRBC, but to a similar extent in wild-type and rac2−/− cells. Therefore, although IgG-SRBC-induced superoxide release was markedly decreased in rac2−/− neutrophils, this effect appears to mediated by downstream pathways unrelated to p38.

We also observed activation of JNK in murine BM neutrophils in response to either fMLP or PMA, which was decreased in fMLP-activated rac2−/− neutrophils. Our data agree with previous reports that formyl receptors in neutrophils can couple to the JNK pathway (44, 58) and are also consistent with the important role for Rac/Cdc42 in pathways leading to JNK activation (12, 13). The failure to detect neutrophil JNK activation in response to fMLP in our earlier study (11) may reflect the use of a less sensitive Ab for
detecting the phosphorylated form of JNK MAP kinase. The functional consequences of defective JNK activation in rac2−/− neutrophils are currently unknown. However, JNK is believed to play a role in the activation of stress-induced gene expression and apoptosis (12, 13).

In conclusion, the present study provides direct genetic evidence that Rac2 is a critical regulator of specific signaling pathways that activate the neutrophil NADPH oxidase, particularly those downstream of chemoattractant receptors and Fcγ receptors, despite the concomitant presence of the highly homologous Rac1 isoform. The effect of Rac2 deficiency on NADPH oxidase activity is probably exerted through multiple pathways, including those independent of MAP kinase activation. Ongoing studies are directed at further identifying these pathways and the specific Rac2 sequences important for regulation of NADPH oxidase activation.

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