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Rac2 Regulates Neutrophil Chemotaxis, Superoxide Production, and Myeloid Colony Formation through Multiple Distinct Effector Pathways

Dirk Carstanjen,²,³*, Akira Yamauchi,²,⁴† Annemart Koornneef,*, Heesuk Zang,*, Marie-Dominique Filippi,*, Chad Harris,*, Jason Towe,‡ Simon Atkinson,‡ Yi Zheng,* Mary C. Dinauer,† and David A. Williams⁵*

Polymorphonuclear neutrophils (PMN) are an important component of the innate immune system. We have shown previously that migration and superoxide (O₂⁻) production, as well as some kinase signaling pathways are compromised in mice deficient in the Ras-related Rho GTPase Rac2. In this study, we demonstrate that Rac2 controls chemotaxis and superoxide production via distinct pathways and is critical for development of myeloid colonies in vitro. The Rac2 mutants V36A, F37A, and N39A all bind to both Pak1 and p67phox, yet are unable to rescue superoxide production and chemotaxis when expressed in Rac2⁻/⁻ PMN. In contrast, the N43A mutant, which binds to Por1 (Arfaptin 2), p67phox, and Pak1, is able to rescue superoxide production but not chemotaxis. The F37A mutant, demonstrated to have reduced binding to Por1, shows reduced rescue of fMLP-induced chemotaxis. Finally, the Rac2Y40C mutant that is defective in binding to all three potential downstream effectors (Pak1, p67phox, and Por1) is unable to rescue chemotaxis, motility, or superoxide production, but is able to rescue defective growth of myeloid colonies in vitro. These findings suggest that binding to any single effector is not sufficient to rescue the distinct cellular phenotypes of Rac2⁻/⁻ PMN, implicating multiple, distinct, and potentially parallel effector pathways. The Journal of Immunology, 2005, 174: 4613–4620.

Rac2 is a hematopoietic-specific member of the small Rho GTPases family. Rho family members regulate actin cytoskeletal organization and gene expression (1–7). Despite a high degree of sequence homology with Rac1, Rac2 appears to specifically regulate chemotaxis, endothelial rolling, superoxide production, and kinase activation in neutrophils (1–3, 8), as shown by studies using mice genetically deficient in Rac2. Rho GTPases cycle between active GTP-bound and inactive GDP-bound forms and typically bind effector proteins only in the active, GTP-bound state. The switch I domain of Rho GTPases (aa 32–40 for Rac and Cdc42) undergoes extensive conformational changes upon GTP-binding, facilitating interaction with downstream effector proteins. For Rac2, the specific effector proteins that regulate motility and chemotaxis, as well as superoxide production in primary cells, are largely unknown, with the exception of the p67phox subunit of the NADPH oxidase.

Neutrophils are highly motile cells. As demonstrated in phagocytic syndromes characterized by defective neutrophil migration, this motility is essential for normal innate immune function. Cell motility is a complex process depending on spatio-temporal regulation of actin assembly and disassembly (9). Rac proteins are believed to regulate actin assembly through Pak1 and Lim-kinase 1 (10). In addition, Por1 regulates membrane ruffling in fibroblasts downstream of Rac1 (11). Finally, Rac antagonistically regulates Rho through Pak1 inhibition of myosin L chain kinase (12, 13) and generation of reactive oxygen species (ROS), at least in nonphagocytic cells (14). Therefore, Pak1, Por1, and even p67phox are potential physiological mediators of Rac function regulating neutrophil motility (Fig. 1).

One approach to identify the pathways emanating from Rho GTPases has been the use of mutants disrupting binding to specific effector proteins introduced into heterologous cells (15–18). This work has shown that generation of specific actin structures and cell cycle progression can be separated from MAPK activation for Rac1 and Cdc42. For instance, codon 37 of Rac1 is implicated in membrane ruffling via partner of Rac1 (Por1) (19) and G1 cell cycle progression. Por1 has been implicated as an important downstream mediator for the induction of membrane ruffles. Codon 40 is critical for activation of the JNK MAPK signaling pathway (15). The mutations at position 40 disrupt binding to Cdc42/Rac interactive binding sequence (19)-containing proteins, as illustrated by defective interactions of Y40C mutants of Rac1 and Cdc42 with Pak1. In Drosophila, expression of Rac1 containing the F37A and the Y40C mutation in a functional Rac1/Rac2 null background is associated with defects in axon growth and branching (20). Rac proteins are also essential regulators of the NADPH oxidase complex, which mediates...
phagocyte superoxide production (21). Mutation of codon 40 of Rac1 and other mutations within the insert domain and codons 27, 30, and 36 of Rac2 (22–26) show reduced binding to p67phox or reduced activation of the oxidase complex.

With the exception of studies in Drosophila, most studies to date examining effector domain mutants have used in vitro assays or cellular approaches that require expression of the effector mutant in the context of constitutively activated (ca)5 forms of Rac. ca forms of Rho GTPases, such as L61 and V12, may themselves behave in a fashion qualitatively different from activated wild-type (wt) proteins (27). In addition, most studies have also been conducted in cell lines, mainly fibroblasts, expressing endogenous Rac1, but not Rac2. In hematopoietic cells, despite a high degree of sequence identity between Rac1 and Rac2, it is now clear that Rac2 is a major GTPase involved in migration and superoxide generation (5, 8). In this sense, hematopoietic cells are unique in that they express both Rac1 and Rac2, but the signaling pathways involved in Rac2-mediated functions are unknown.

In the studies presented here, we investigated the proximal downstream signals of Rac2. We studied the ability of Rac2 switch I mutant domain proteins to rescue Rac2 function in bone marrow cells genetically deficient in Rac2 using physiologically relevant primary cell assays (Fig. 1). We demonstrate that single amino acid changes in the switch I domain of wt Rac2 prevents the rescue of migration and superoxide production defects in Rac2−/− neutrophils even though some of these mutants maintain binding to Pak1, Por1, and p67phox in vitro. Our data indicate mutations in amino acid residues that disrupt binding to Pak1 and Por1 lead to loss of wt Rac2 function in primary neutrophils, that binding to Pak1 or Por1 individually is insufficient to reconstitute Rac2 function in neutrophils, and that activation of the NADPH oxidase likely involves both binding to p67phox and a second pathway. Finally, none of the mutations in the Rac2 switch domain interfered with Rac2-mediated growth factor-induced cell survival and proliferation.

Materials and Methods

Construction of retroviral vectors

An improved bicistronic murine stem cell virus-based vector, MIEG3, expressing enhanced GFP has been described previously (28). This vector contains the encephalomyocarditis virus internal ribosomal entry site in its original viral configuration, resulting in improved GFP translation. The vector-expressing Flag-tagged murine Rac2 (FR2) has also been described previously (29). The amino acid point mutations (Fig. 1) were introduced into this vector by site-directed mutagenesis (QuickChange; Stratagene). The primer sequences are available upon request. The mutated sequences were confirmed by automated nucleotide sequencing and subcloned into MIEG3 using EcoRI and XhoI restriction sites yielding V56A-FR2, F27A-FR2, N91A-FR2, Y40C-FR2, N43A-FR2, and F37AY40C-FR2. N-terminal Flag-tagged wt human Por1 (National Center for Biotecnology Information [NCBI] X97567; kindly supplied by L. van Aelst, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (11) was used for PCR amplification and inserted into MIEG3 using EcoRI/XhoI digest for Por1 to generate MIEG3-Por1. wt human Pak1 (NCBI U24152 WT Pak1), ca Pak1 T423E, and dominant-negative (dn) Pak1 (both kindly supplied by J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA) were used for PCR amplification and inserted into MIEG3 using EcoRI for Pak1 and ca Pak1. To generate ecotropic retroviral supernatant, Phoenix eco cells were obtained from American Type Culture Collection, cultured in DMEM with 4.5 g/L glucose (Invitrogen Life Technologies), supplemented with 100 U/ml penicillin and 100 g/ml streptomycin (P/S) and 10% FCS (HyClone) and transduced with either pk2 or expressing wt Rac2), MIEG3 (empty vector), or the respective mutant containing plasmids, using either a liposome-based method (lipofectamine; Invitrogen Life Technologies), supplemented with 100 U/ml penicillin and 100 g/ml streptomycin (P/S) and 10% FCS (HyClone) and transduced with either pk2 or expressing wt Rac2), MIEG3 (empty vector), or the respective mutant containing plasmids, using either a liposome-based method (lipofectamine; Invitrogen Life Technologies) or Calcium Phosphate Transfection (Sigma-Aldrich), according to the manufacturer’s instructions. Retroviral supernatant was harvested 48–96 h after transfection. MIEG3 was harvested from a stable GP + E86 cell line previously described (29).

Binding of Rac2 mutants to Pak1, p67phox, and Por1

The Rac-binding domain of PK or Por1 was cloned into pGEX-5X3 (Pharmacia) vector. p67phox-GST was kindly provided by E. Pick (Julius Friedich Cohnheim-Minerva Center for Phagocytic Research, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel). GST-tagged proteins were expressed in BL21 (DE3) and purified using glutathione-agarose beads (Sigma-Aldrich) in a buffer containing 50 mM HEPES (pH 7.3), 200 mM NaCl, 5 mM MgCl2, protease inhibitor mixture (Roche), and 1 mM PMSF. Cell lysates were prepared from NIH3T3 cells transduced with Flag-tagged wt or mutant forms of Rac2. Transduced cells were isolated through FACS via GFP expression. GTPγS-loading was performed by successively treating the cell lysates with 2 mM EDTA, 0.1 mM GTPγS, and 10 mM MgCl2. The purified GST-fusion proteins were then incubated with the cell lysates containing GTPγS-loaded Rac2 on mutants for 2 h at 4°C. After the incubation, the reaction mixture was washed three times with a buffer containing 50 mM HEPES (pH 7.3), 200 mM NaCl, 10 mM MgCl2, and subjected to SDS-PAGE for immuno blot analysis.

Animals and generation of in vitro-differentiated neutrophils

Rac2−/− mice (B6.129Rac2tmmddw) and their normal littermates have been described previously (1). The animals had been backcrossed into C57BL/6 mice for >12 generations. Six- to 8-wk-old Rac2−/− or wt littermates were treated with 150 mg/kg 5-fluoracil (American Pharmaceutical Partners) i.p. 48 h before harvesting bone marrow. The mononuclear cell fraction was isolated by density gradient centrifugation (histopaque–1083; Sigma-Aldrich) for 30 min at 1500 rpm at room temperature, prestimulated with recombinant rat stem cell factor (rSCF), recombinant human granulocyte CSF (rhuG-CSF), and recombinant human megakaryocyte growth and development factor (rhuMGDF, all 100 ng/ml; Amgen), for 4 h in IMDM (Invitrogen Life Technologies) supplemented with 10% FCS and P/S. Cells were transduced twice with retroviral supernatants on fibroblast

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5 Abbreviations used in this paper: ca, constitutively active; wt, wild type; rSCF, recombinant rat stem cell factor; rhG-CSF, recombinant human granulocyte CSF; rhMGDF, recombinant human megakaryocyte growth and development factor; mIL-3, recombinant murine IL-3; ROS, reactive oxygen species; dn, dominant negative.

FIGURE 1. Schematic representation of Rac2 protein and switch I domain effector mutants. F37 and Y40 mutants which interrupt interactions with POR1, PAK, and p67phox are potential proteins involved in transducing signals downstream of Rac2 are shown (see text and Tables I and II for details). The downstream pathways and critical myeloid functions assayed are shown in the lower part of the figure.
Materials and Methods

Described in the text.

Results

Binding of Rac2 mutants to Pak1, p67phox, and Por1 and induction of membrane ruffling

Pak1, p67phox, and Por1 are intensively studied downstream effectors of Rac1, a Rho GTPase highly homologous to Rac2. Binding of Rac to p67phox in the assembled NADPH oxidase complex is essential for oxidase activity (34), and Pak1 and Por1 may contribute to signals transduced via Rac2 in terminally differentiated neutrophils. To more fully examine the potential role(s) of Rac2 interactions with effectors in neutrophil functions, we studied switch I domain mutants in wt Rac2 expressed in Rac2-deficient primary neutrophils. As few data are available studying interaction of Rac2 switch I domain mutants with these effector proteins, we first performed pull-down assays of these mutants with GST-Pak1, GST-p67phox, and GST-Por1 to determine the extent of protein binding of these effectors to Rac2 (Fig. 2 and summarized in Table I). Binding of Rac2 or mutants of the switch I domain was confirmed by anti-Flag immunoblotting. The effector domain is highly conserved between Rac2 and Rac1. Thus, as expected, wt Rac2 and mutants of Rac2 were treated in a buffer containing 0.1 mM GTP and 7.5 mM glucose (PBSG) were added into each well manually before starting assay, and chemiluminescence was detected in total 200-μl suspension on a 96-well plate using an Lmax microplate luminometer (Molecular Devices). The data are expressed as relative luminescence units by long kinetic mode for over 30 min, and the relative total amount of superoxide produced was determined using SoftMax PRO software (Molecular Devices). Under these conditions, 97.5% of chemiluminescence was inhibited by superoxide dismutase.

Statistics

The one-tailed t test was used for statistical analysis with p < 0.05 considered significant.

Table I. Summary of Rac2 mutant effects on neutrophil function and effector binding

<table>
<thead>
<tr>
<th>Effect/Rac2 Mutant</th>
<th>FR2 (wt Rac2)</th>
<th>V36A</th>
<th>F37A</th>
<th>N39A</th>
<th>Y40C</th>
<th>F37A/Y40C</th>
<th>N43A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony formation</td>
<td>↑↑</td>
<td>ND</td>
<td>↑↑</td>
<td>ND</td>
<td>↑↑</td>
<td>↑↑</td>
<td>ND</td>
</tr>
<tr>
<td>Chemotaxis (Boyden chamber)</td>
<td>↑↑</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Superoxide chemiluminescence (PMA)</td>
<td>↑↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Pak1-GST</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>p67phox-GST</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Por1-GST</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

↑↑, Increased compared to wt/Mieg3; -, no rescue of knockout phenotype; +, binding in GST pull-down; and --, no binding in GST pull-down.
demonstrates binding to Pak1, p67phox, and Por1 (Fig. 2). As previously reported for Rac1 and Cdc42 (15), the Rac2Y40C mutant does not bind to Pak1. The Rac2Y40C mutant also does not bind to p67phox in this in vitro assay, a result similar to that reported previously for the Y40K mutant of Rac1 (15). Residues V36, F37, and N39 have been implicated in binding of Rac1 to Por1 (Arfaptin2) (35). Although F37A and Y40C mutation in Rac2 leads to loss of binding to Por1, the V36A, N39A, and the N43A mutations do not prevent binding to Por1. Loss of binding to Por1 correlated with the inability to potentiate membrane ruffling of these mutants as analyzed after stimulation with platelet-derived growth factor in NIH3T3 cells (data not shown). As previously reported for Rac1, Rac2F37A, Rac2V36A, Rac2N39A, and Rac2G43A, mutants also show binding to p67phox at levels equal to and greater than wt Rac2.

Expression of wt and mutant Rac2 in transduced neutrophils

Neutrophils differentiated in vitro after retroviral transduction of Rac2-deficient myeloid progenitor cells express the mutant Rac2 proteins V36A, F37A, N39A, Y40C, F37A/Y40C, and N43A at levels equal to wt Rac2 (FR2) as judged both by immunoblotting with anti-Flag Ab and by the intensity of GFP expression using flow analysis. Expression of transgenes was 2- to 3-fold higher than endogenous Rac2 as judged by immunoblotting with anti-Rac2 Ab (data not shown), as previously seen using this vector system (8).

Neutrophil chemotaxis does not depend on Pak1 or Por1

Rac2-deficient neutrophils have a well-described defect in directed migration after stimulation with chemotactic agents such as fMLP (1, 8, 28). Migration depends on a complex spatio-temporal change in actin assembly and disassembly, which is thought to be regulated at least in part by Rac, Pak1, Lim-kinase 1, and cofilin (10). In addition, Por1-Rac1 direct interaction has been implicated in actin rearrangement leading to membrane ruffling in fibroblasts (11). As seen in Fig. 3a, when compared with wt Rac2 and empty vector (MIEG3), neither the F37A nor Y40C mutants are able to rescue chemotaxis. Furthermore, neither N43A or V36A Rac2 mutants, each of which retain the capacity to bind to both Pak1 and Por1, rescue chemotaxis when expressed in Rac2−/− neutrophils. These data indicate that binding of Rac2 to either Pak1 or Por1, while potentially necessary, is not sufficient to regulate neutrophil migration. To substantiate these findings, wt Por1 and ca Pak1 were expressed in wt and Rac2−/− neutrophils. Expression ca Pak1 was 5- to 10-fold higher than endogenous Pak1. wt Por1 was detected by anti-Flag Ab (Fig. 3b). As seen in Fig. 3c, neither ca Pak1 nor wt Por1 affected wt neutrophil migration (Fig. 3c). Furthermore, none of those proteins rescue chemotaxis of Rac2−/− neutrophils (Fig. 3c). These data indicate that neither Por1 nor Pak1 alone in the absence of Rac2 mediate neutrophil chemotaxis.

Binding of Rac2 to p67phox and Pak1 is not sufficient for generation of superoxide

As noted previously, Rac2−/− neutrophils display a defect in superoxide generation in response to some agonists, including fMLP. In vitro cell-free assays indicate that superoxide generation in neutrophils depends on binding of a Rac protein (either Rac1 or Rac2) to p67phox. To determine the role of Rac2 binding to p67phox in generation of superoxide in Rac2−/− neutrophils, superoxide generation was stimulated with PMA and analyzed quantitatively in neutrophils derived from myeloid progenitor cells transduced with each Rac2 mutant or wt Rac2. Despite the observation that the F37A and N39A mutants retain the capacity to bind to p67phox in vitro, these mutants do not rescue superoxide generation when

FIGURE 3. Chemotaxis of transduced and in vitro generated neutrophils. A, Neutrophils derived from wt or Rac2−/− mouse bone marrow were analyzed for migration in Boyden chambers in response to 1 µM fMLP, as described in Materials and Methods. For each experiment, three replicates were performed and for each bone marrow harvest, at least three individual assays have been performed. The number of migrating cells on the filter was determined by counting four random ×400 microscopic fields. Data are depicted as mean migrated cells per field ± SEM from three to nine individual experiments. *, Significance (p < 0.05) between wt Rac2 (FR2) expressed in Rac2−/− cells and indicated mutant or vector control. For N43A, p = 0.55. B, Immunoblot of ca Pak1 (upper panel) or flag-tagged POR1 (lower panel) in transduced wt (Rac2+/+) or Rac2−/− neutrophils. β-Actin was used as a loading control.

C, Chemotaxis of neutrophils derived from wt or Rac2−/− mice expressing Pak1 or Por1 cDNAs. n = 3–8, mean ± SEM. p-Values are not significant for any values except wt Rac2 vs MIEG3 (empty vector) in Rac2−/− cells where p < 0.05.
expressed in Rac2−/− cells (Fig. 4a). As noted above, the F37A and N39A also bind Pak1, and N39A but not F37A can bind Por1. Thus, the N39A mutant retains in vitro binding to p67phox, Pak1, and Por1, but is unable to rescue superoxide production in Rac2−/− neutrophils. In direct contrast, Rac2−/− neutrophils expressing N43A Rac2, which binds p67phox, Pak1, and Por1, demonstrate superoxide production similar to Rac2−/− neutrophils expressing wt Rac2. These data indicate that Rac2 binding to Pak1, p67phox, or Por1 alone is not sufficient to effect superoxide formation in neutrophils. In addition, the V36A and N43A mutants bind equally well to Pak1, Por1, and p67phox (Fig. 2), but only N43A supports superoxide production (Table I and Fig. 4a). Interestingly, expression of ca Pak1 increases baseline as well as fMLP-stimulated superoxide production in Rac2−/− neutrophils but is unable to substitute fully for wt Rac2 (Fig. 4b). Expression of wt Por1 does not affect superoxide production (Fig. 4b). Taken together, these data suggest that Rac2 regulates one or more pathways involved in activation of superoxide production that are independent of p67phox.

p160rock and Rac2 regulate chemotaxis in a combinatorial fashion, but p160rock is not involved in superoxide generation

p160rock has been previously described primarily as an effector for Rho. One report has demonstrated that Rac1 binding to p160rock is associated with membrane ruffling (15). Therefore, we investigated the effect of a specific p160rock inhibitor on chemotaxis and superoxide production in wt and Rac2−/− neutrophils expressing each Rac2 mutant described above. As shown in Fig. 5a, pharmacological inhibition of p160rock in wt neutrophils significantly reduces chemotaxis, shown by a ~50% loss of fMLP-induced chemotaxis. In Rac2−/− neutrophils or Rac2−/− neutrophils expressing wtRac2, F37A, Y40C, or F37A/Y40C Rac2 mutants, inhibition of p160rock leads to a further reduction in chemotaxis. These data suggest that p160rock is not a direct downstream effector of Rac2 but likely acts in parallel to Rac2, mediating neutrophil chemotaxis. Finally, we studied the effect of the p160rock inhibitor on the generation of superoxide in wt neutrophils or neutrophils expressing wt or Rac2 mutants generated in vitro. As shown in Fig. 5b, the p160rock inhibitor has no significant effect on PMA-induced

**FIGURE 4.** Superoxide production in transduced and in vitro generated neutrophils. Neutrophils derived from wt or Rac2−/− mouse bone marrow were analyzed for superoxide generation after stimulation with PMA using quantitative measurements (A) or NBT test (B). The response to each stimulus is compared with the background spontaneous activity (see text). A, 1 × 105 cells were stimulated with 0.3 μg/ml PMA in PBSG in the presence of isoluminol, HRP. The signal was read for 30 min at 37°C in a luminescence reader. Data are depicted as mean relative luminescence units ± SEM from four to 12 individual experiments. *, Significance (p < 0.05) between FR2 (wt Rac2) expressed in Rac2−/− cells and the indicated mutant. B, Cells were stimulated by either unstimulated (□) or stimulated with 10−5 mol/liter fMLP (■). The percentage of NBT + cells was determined by evaluating 200 cells in triplicate. In these assays, as previously noted by us (3), there is a degree of spontaneous oxidase activity, which may be increased due to the in vitro culture conditions. p-Values are not significant for any values except wt Rac2 vs MIEG3 (empty vector) in Rac2−/− cells where p < 0.05.

**FIGURE 5.** Chemotaxis and superoxide generation of transduced and in vitro generated neutrophils in the presence of p160rock inhibitor. Neutrophils derived from wt or Rac2−/− mouse bone marrow were analyzed for migration (A) and generation of superoxide (B) (as in Figs. 3 and 4) in the absence (□) or presence (■) of 1 mM p160rock inhibitor Y-27632 (Calbiochem). Cells were preincubated for 30 min at 37°C. A, Chemotaxis assays, two experiments with similar results have been performed. B, Superoxide generation data are depicted as mean ± SEM from six individual experiments. *, Significance (p < 0.05) between wt Rac2 and indicated mutants expressed in Rac2−/− cells vs MIEG3 (empty vector) without inhibitor; #, significance in the presence of inhibitor.
Table II. Comparison of effectors mutants in Rac2 to literature

<table>
<thead>
<tr>
<th>Genotype</th>
<th>V36A/R</th>
<th>F37A/L</th>
<th>N39A</th>
<th>Y40C/K/H</th>
<th>N43A/D</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>L</td>
<td>C</td>
<td>L</td>
<td>C</td>
<td>L</td>
</tr>
<tr>
<td>Binding to Pak1</td>
<td>ND</td>
<td>(+ + +)</td>
<td>ND</td>
<td>(+ + +)</td>
<td>ND</td>
</tr>
<tr>
<td>Binding to Poir</td>
<td>ND</td>
<td>(+ + +)</td>
<td>ND</td>
<td>(+ + +)</td>
<td>ND</td>
</tr>
<tr>
<td>Binding to p67phox</td>
<td>ND</td>
<td>(+ + +)</td>
<td>ND</td>
<td>(+ + +)</td>
<td>ND</td>
</tr>
<tr>
<td>In vitro NADPH oxidase activation</td>
<td>++ c</td>
<td>ND (−)</td>
<td>ND (−)</td>
<td>ND (−)</td>
<td>ND (−)</td>
</tr>
</tbody>
</table>

**L:** Data from the literature; C, current data presented in this manuscript. Current data represents wt Rac2 mutants, see Fig. 1 for semiquantitative binding (+ to ++ +).

**Ref.** 15: Q61Rac1 F37A; Q61Rac1 Y40K; Q61Rac1 Y40C. 16: Q64Rac1 N43D.

**Ref.** 17: G12VRac1 F37L; G12V Rac1 Y40C; Q61L Rac1 N43D.

**Ref.** 22: wt Rac2 Y40K.

**Ref.** 23: wt Rac2 V36R; G12V Rac2; Q61L Rac2 V36R.

**Ref.** 24: Q61Rac1 Y31F; G12 Rac1 Y40H.

**ND:** No data published.
Our findings suggest Rac2 activates the NADPH oxidase complex by a second or several p67phox-independent pathways. Mutations in Rac2 that abolish binding to p67phox, as in the case of the Y40C mutant, clearly affect the capacity of Rac2 to mediate superoxide generation in Rac2−/− neutrophils. However, binding of Rac2 to p67phox, as shown by the F37A and N39A Rac2 mutants, is not sufficient to restore the capacity of Rac2 to rescue superoxide production in Rac2−/− neutrophils. Because the N39A Rac2 mutant also appears to bind to Pak1 and Por1, Rac2/Pak1 and Rac2/Por1 interactions are also apparently not sufficient to mediate full activation of the NADPH oxidase via Rac2. Supporting this view, expression of dn Por1 or dn Pak1 did not influence superoxide production in wt neutrophils (A. Koornneef and D. A. Williams, unpublished results). However, we cannot rule out the possibility that switch I mutants may bind effectors but fail to activate specific effectors in the physiologic setting, which would complicate our interpretation of these data (37). Indeed, while previous studies have demonstrated that Rac2V36R both binds to p67phox and activates NADPH oxidase in a cell-free NADPH oxidase assay (Table II) (23), our study shows that V36A in wt Rac2 background binds p67phox but does not rescue superoxide generation in Rac2−/− neutrophils. This difference may be attributed to the specific amino acid substitution (A vs R), or to the difference in expression of the position mutant in an activated vs wt Rac2 background, or to a role of Rac2V36 for regulating NADPH oxidase activity in intact cells.

Rho GTPases have been implicated in cell proliferation via regulation of G1 entry. Most studies examining the effects of Rho GTPases on cell proliferation have used quiescent fibroblasts, where dn mutants or Rho-specific toxins have been shown to block cell cycle entry (38). Rho regulates cyclin D1 expression during cell cycle progression via antagonism of Rac1 and Cdc42 (36). Rac mutants Rac1V12H40, 37L, 40H, Rac161L40C, and 43D have been demonstrated to impair cell cycle progression via inhibition of Pak1, Por1, and the Rac2F37A mutant does not bind Por1. Interestingly, the Rac1Y40 residue is not part of the active site in Rac1 and Rac2, but does not rescue NADPH oxidase activity, as suggested but not proven by the reduced superoxide generation of the N39A mutant in Rac2−/− cells. Of interest, mutating V36A, N39A, and N43A does not change the binding of Rac2 to Pak2, Por1, and p67phox.

In summary, we established a model system to study, in a physiologically relevant fashion, the proximal Rac2 signal transduction pathways using Rac2 switch I domain mutants expressed in neutrophils genetically deficient in wt Rac2. Our findings indicate that Rac2 regulates neutrophil motility as well as superoxide generation by several parallel and obligate pathways. Expression of mutants that preserve binding to p67phox, Por1, and Pak1 did not rescue chemotaxis or superoxide production in Rac2−/− neutrophils. Finally, Pak1, Por1, and p67phox, as well as other potential effector proteins using signals transduced via the switch I domain, are dispensable for cell growth in response to GM-CSF. Thus, Rac2 function in neutrophils is likely mediated through additional as yet unidentified molecules. Although in vitro binding (as used in this study) and activation studies may not be fully predictive of physiological interactions in intact neutrophils, these data underscore the utility of genetic systems to dissect the functions of Rho GTPases in hemopoietic cells.

Acknowledgments
We thank Amgen and Takara Bio for reagents, Gary Bokoch for Abs, and Linda van Aelst and Jonathan Chernoff for plasmids. We thank members of our laboratories for helpful discussions and Keisha Steward for administrative assistance.

Disclosures
The authors have no financial conflict of interest.

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