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Localization of Rac2 via the C terminus and aspartic acid 150 specifies superoxide generation, actin polarity and chemotaxis in neutrophils

Marie-Dominique Filippi[1], Chad E Harris[1], Jarek Meller[2], Yi Gu[1], Yi Zheng[1] & David A Williams[1]

Despite having a high degree of sequence similarity, the Rho guanosine triphosphatases Rac1 and Rac2 regulate distinct functions in neutrophils. Here we demonstrate that the unique Rac2 localization and functions in neutrophils are regulated by two separate C-terminal motifs, the hypervariable domain and aspartic acid 150, one of which has not previously been linked to the function of Rho GTPases. In addition, we show an unexpected dependence of Rac1 localization on Rac2 activity in these same cells, demonstrating a degree of crosstalk between two closely related Rho GTPases. Thus, we have defined specific sequences in Rac that specify subcellular localization and determine the specificity of Rac2 in neutrophil chemotaxis and superoxide generation.

Polymorphonuclear neutrophils constitute the first line of cellular defense against microorganism invasion. In response to environmental stimuli, polymorphonuclear neutrophils activate various signal transduction pathways, allowing these cells to migrate toward the site of infection and ingest microorganisms, release granule contents and secrete cytokines. The Ras-related small Rho guanosine triphosphatase (GTPase) family, including Rac1, RhoA and CDC42, is key in regulating these responses[1–3]. Among the GTPases, the Rac subfamily is composed of three highly related proteins: Rac1, Rac2 and Rac3. Rac1 and Rac3 are ubiquitously expressed, whereas expression of Rac2 is restricted to hematopoietic cells[4–7]. Therefore, hematopoietic tissues are unique in having overlapping expression of Rac2 and other members of the Rac subfamily. Despite this and a high degree of sequence identity between the GTPases, Rac1 and Rac2 regulate unique and very specific hematopoietic cell functions, including distinct actin processes, proliferation and apoptosis[8–10].

Neutrophils genetically deficient in Rac2 have cellular defects in superoxide production, directed migration and filamentous actin (F-actin) assembly. *Rac2*$^{-/-}$ mice have enhanced susceptibility to the opportunistic pathogen *Aspergillus fumigatus*[11]. In addition, a patient with a mutation in the conserved GTP-binding domain of Rac2 has been described with a severe phagocytic immunodeficiency similar to the neutrophil cellular phenotype of *Rac2*$^{-/-}$ mice[12,13]. In contrast, Rac1 deficiency in mouse polymorphonuclear neutrophils is associated with increased integrin-dependent spreading and a subtle abnormality in uropod tail retraction during cell migration[8]. Therefore, individual Rac proteins can be defined as critical and specific regulators of neutrophil functions.

These observations raise the question of how highly related proteins of the Rho family regulate distinct functions in the same cell lineage. Knowledge of the mechanisms of Rac-controlled specificity may have broad implications for understanding the molecular mechanisms of immune cell functions. Sequences of the known functional domains of Rac1 and Rac2, including the GTP-binding domain, the insert domain, sequences mediating interaction with guanine nucleotide exchange factor and domains essential for binding to effector proteins, are about 98% identical[4–6,14]. The main differences between Rac1 and Rac2 are near the C-terminal prenylation consensus site (CAAX box). This region is composed of six basic amino acids (KKRKRK, designated as a polybasic motif) in Rac1. In Rac2, three of these six amino acids are replaced by neutral amino acids (RQQKRP).

Like the Ras family, Rho GTPases are subject to post-translational modifications at the C terminus by prenylation, proteolysis and carboxymethylation. These post-translational changes are thought to be essential for modification of protein-protein interactions and for membrane-associated protein trafficking[15,16]. The function of sequence divergence between Rac2 and Rac1 in mediating differences in the specificity of function of Rac2 versus Rac1 in neutrophils is unknown.

In this report, we demonstrate that in activated neutrophils, Rac2 has subcellular microlocalization distinct from that of Rac1 and that absence of Rac2 is associated with mislocalization of Rac1. The localization of Rac2 is regulated by specific sequences in the hypervariable region of the C terminus and an additional unique residue outside this region that has not previously been associated with GTPase function. The combination of these two distinct structural determinants determines the specificity of Rac2 function in neutrophil chemotaxis and superoxide generation.

[1]Division of Experimental Hematology and [2]Division of Pediatric Informatics, Cincinnati Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229, USA. Correspondence should be addressed to D.A.W. (david.williams@cchmc.org).

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RESULTS

Rac2 has unique functions in neutrophils

Loss of Rac2 activity is associated with a defect in superoxide production and chemotaxis in neutrophils[11]. To show that the cellular phenotype of *Rac2*$^{-/-}$ neutrophils reflects Rac2 specificity of function but not quantitative alteration of total Rac activity, we reintroduced wild-type Rac2 or wild-type Rac1 into *Rac2*$^{-/-}$ cells through retrovirus-mediated gene transfer. For these studies we used a bicistronic retrovirus vector, MIEG3, which coexpresses enhanced green fluorescent protein (EGFP) via an internal ribosome entry site[12]. We transduced *Rac2*$^{-/-}$ bone marrow–derived hematopoietic progenitors with each vector, sorted for EGFP^{+} cells and cultured the cells in cytokines to induce neutrophil differentiation. We purified cells based on similar intensity of EGFP expression. In these conditions, cells integrated two or three copies of the transgene. Thus, transgenic wild-type Rac2 and wild-type Rac1 were each overexpressed in *Rac2*$^{-/-}$ cells at an amount about twofold higher than that of endogenous total Rac in wild-type cells, as assessed by immunoblot (**Fig. 1a**). *Rac2*$^{-/-}$ progenitors had *in vitro* neutrophil differentiation similar to that of wild-type cells (data not shown).

We analyzed two important functions in these transduced phagocytic cells. We assessed production of superoxide, a product of the phagocytic oxidase complex, through reduction of nitroblue tetrazolium (NBT) in response to 10 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). *Rac2*$^{-/-}$ cell populations transduced with empty vector had decreased numbers of NBT^{+} cells after fMLP stimulation compared with wild-type cell populations (**Fig. 1b**). Reintroduction of wild-type Rac2 but not wild-type Rac1 restored the number of NBT^{+} cells to wild-type amounts. We next studied directed migration in response to fMLP with a chemotaxis assay in Boyden chambers. *Rac2*$^{-/-}$ cells demonstrated a reduction of more than 50% in fMLP-directed migration compared with that of wild-type cells, and wild-type Rac2 but not wild-type Rac1 completely 'rescued' migration to normal amounts (**Fig. 1c**). These data confirm that wild-type Rac1 cannot subserve wild-type Rac2 in correcting the main phenotypic abnormalities in *Rac2*$^{-/-}$ primary neutrophils and that the deficiency in superoxide production and chemotaxis is not simply because of a quantitative reduction in total Rac activity.

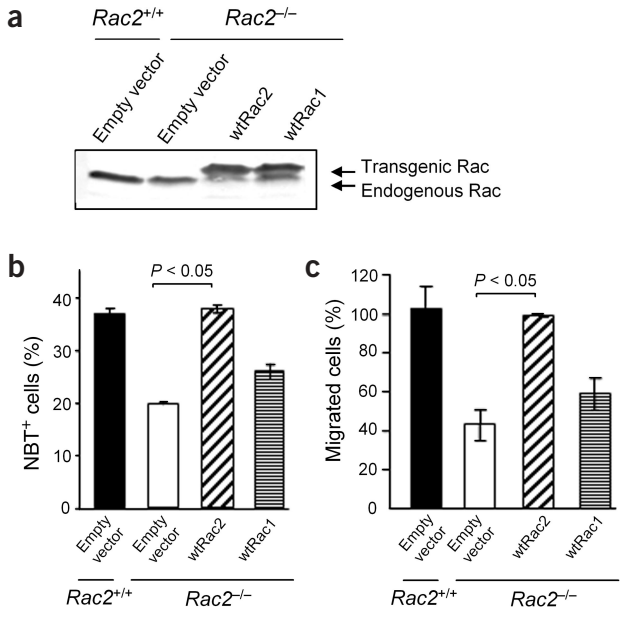

Figure 1 Rac2 has distinct functions compared with Rac1 in primary neutrophils. (**a**) Expression of exogenous Rac proteins in *Rac2*$^{-/-}$ neutrophils assessed by immunoblot with a Rac antibody that recognizes both Rac1 and Rac2. Lysates from equal numbers of cells were loaded. Exogenously expressed Rac proteins migrate more slowly because of the tag. (**b**) *Rac2*$^{-/-}$ cells were transduced with wild-type Rac2 or wild-type Rac1 retrovirus vectors. *Rac2*$^{-/-}$ neutrophils expressing each wild-type Rac protein were assessed for superoxide production using reduction of NBT in response to 10 μM fMLP. Results are expressed as the percentage of cells that are NBT^{+}. Mean ± s.e.m. of three independent experiments; paired *t*-test. (**c**) Migration in response to fMLP. Cells were loaded into the upper well of a Boyden chamber and incubated for 45 min at 37 °C. Results represent the percentage of migrated cells per field compared with *Rac2*$^{-/-}$ cells expressing wild-type Rac2. Mean ± s.e.m. of three to five independent experiments; paired *t*-test. wtRac2, wild-type Rac2; wtRac1, wild-type Rac1.

Rac2 and Rac1 have distinct subcellular distributions

The polybasic sequences immediately upstream of the conserved CAAX box represent the main area of sequence divergence between Rac1 and Rac2, and this domain is essential for Rac2 function in neutrophils[17]. This sequence has also been associated with regulation of GTPase subcellular distribution[18]. We hypothesized that the specificity of function of Rac2 versus Rac1 could be related to distinct subcellular location of Rac proteins. To test this, we generated wild-type Rac2 and wild-type Rac1 with an EGFP tag at the N terminus (EGFP-Rac2 and EGFP-Rac1, respectively). Expression of EGFP-Rac2 in *Rac2*$^{-/-}$ cells completely restored the chemotaxis defect in these cells (data not shown), indicating that the fusion protein was functional. We compared the subcellular distribution of EGFP-Rac2 and EGFP-Rac1 with the distrubution of F-actin in the cells before and after fMLP stimulation (15 s, the point of maximal GTPases stimulation; ref. 19 and data not shown). Before fMLP addition, EGFP-Rac2 showed a mainly central cytoplasmic and perinuclear localization (**Fig. 2a**, top). Treatment with fMLP induced redistribution of EGFP-Rac2 from the perinuclear structure to a peripheral localization. Most EGFP-Rac2 was localized interior to but distinct from cortical F-actin (**Fig. 2a**, bottom). In contrast to Rac2, after fMLP stimulation, EGFP-Rac1 redistributed from dispersed cellular locations with little perinuclear localization (**Fig. 2b**, top) to the cell periphery, showing distinct areas of colocalization with F-actin (**Fig. 2b**, bottom). We obtained similar distribution patterns in experiments with both high and low expression of EGFP-tagged proteins (data not shown). These results provide evidence in primary neutrophils of distinct subcellular distribution of Rac1 versus Rac2 and demonstrate that fMLP stimulation leads to specific redistribution of Rac2.

RQQKRP sequence specifies superoxide production

To further investigate the relationship between specificity of function and the sequence differences of Rac1 and Rac2 at a mechanistic level, we next assessed whether the C-terminal sequences of Rac2 are involved in mediating the two functions of neutrophils that are abnormal in *Rac2*$^{-/-}$ cells. To accomplish this, we replaced the sequence KKRKRK of Rac1 with the analogous sequence RQQKRP of Rac2 (Rac1/2 mutant) and vice versa (Rac2/1 mutant; **Fig. 3a**). The amount of transgenic Rac chimeric protein expression in transduced neutrophils was similar to that of expressed transgenic wild-type Rac1 and wild-type Rac2 (noted above), as assessed by immunoblot (**Fig. 3b**). Expression of Rac1/2 but not Rac2/1 chimeric proteins completely restored the proportion of superoxide-producing *Rac2*$^{-/-}$ neutrophils after fMLP stimulation compared with that of wild-type cells and *Rac2*$^{-/-}$ cells transduced with wild-type Rac2 (**Fig. 3c**). In contrast, neither Rac2/1 nor Rac1/2 restored

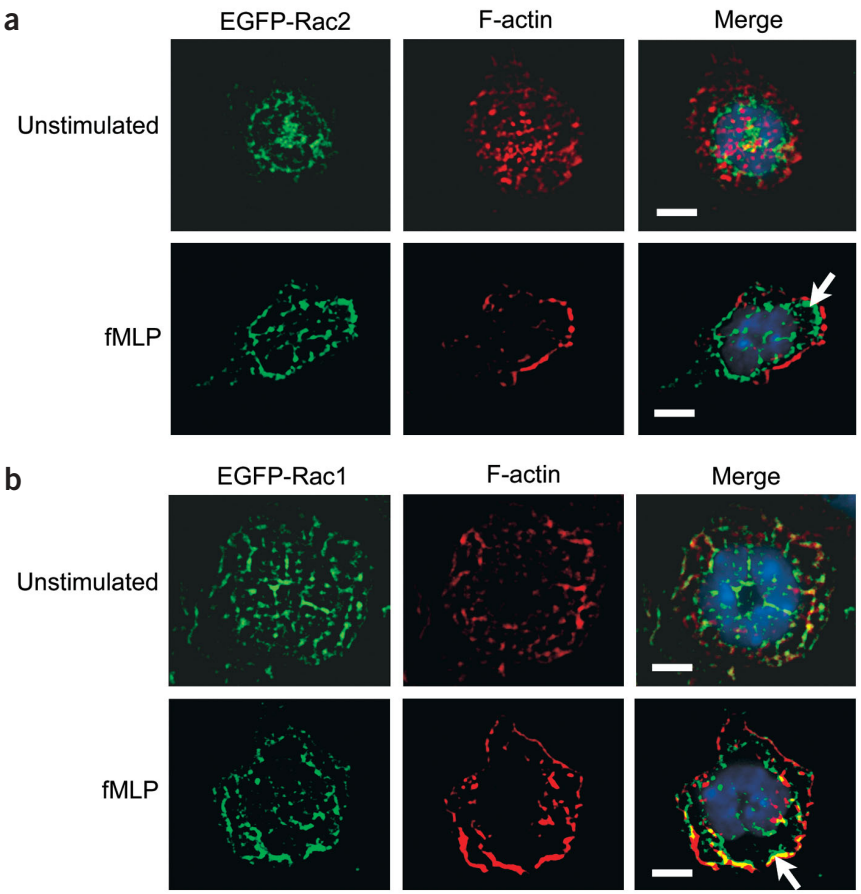

Figure 2 Rac1 and Rac2 show distinct cellular localization. Subcellular redistribution of Rac2 (**a**) versus Rac1 (**b**) compared with that of F-actin in wild-type neutrophils in response to fMLP. Wild-type neutrophils expressing EGFP-tagged Rac proteins were stimulated for 15 s with 10 μM fMLP with a uniform stimulus concentration, fixed and stained with rhodamine-labeled phalloidin. *Z* series of fluorescence images were acquired with a fluorescence microscope equipped with a deconvolution system driven by Openlab software. After stimulation with fMLP, Rac2 has a distinct location versus F-actin (arrow in **a**), whereas Rac1 redistributes to plasma membrane showing colocalization with F-actin (arrow in **b**), compared with that of unstimulated cells. This experiment is representative of at least three independent experiments. Scale bars, 2 μm.

directed migration to wild-type Rac2 migration levels when expressed in *Rac2*$^{-/-}$ neutrophils (**Fig. 3d**). Thus, the specificity of Rac2 in superoxide production is determined mainly by the RQQKRP motif, whereas this sequence does not by itself determine Rac2 specificity in fMLP-mediated chemotaxis.

D150 and RQQKRP specify chemotaxis

Of nine amino acids that diverge between Rac1 and Rac2 in the entire protein outside the RQQKRP motif, three are located between residues 148 and 151 (**Fig. 4a**). Computer modeling of this sequence suggested that these residues may reside on the surface of the three-dimensional folded structure of Rac and thus would potentially be accessible to interact with other proteins (data not shown). To analyze the possible function of this additional divergent sequence in the specification of Rac2-mediated migration, we replaced residues 148–151 (EIGA) of Rac1 with the analogous sequence of Rac2 (DIDS) in the Rac1/2 'tail mutant' (yielding Rac1DIDS/2). Immunoblot analysis confirmed expression of this mutant in *Rac2*$^{-/-}$ neutrophils (**Fig. 4a**). Rac1DIDS/2 completely restored chemotaxis of *Rac2*$^{-/-}$ to wild-type Rac2 amounts in response to fMLP (**Fig. 4b**).

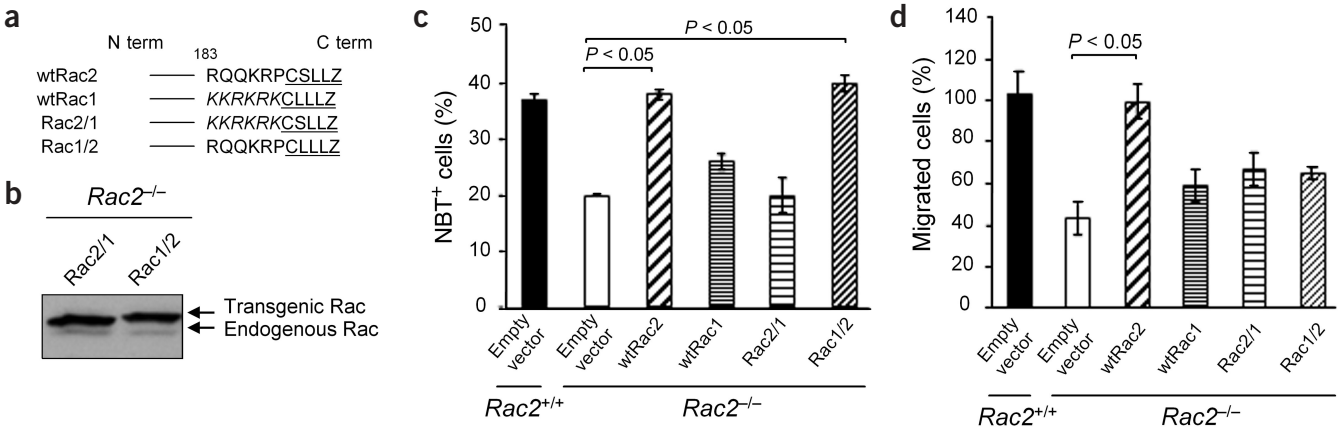

Figure 3 The RQQKRP motif determines the specificity of Rac2 in superoxide production but not directed migration. (**a**) Mutants of Rac1 and Rac2. Number indicates amino acid position. N term, N terminus; C term, C terminus. Underlining indicates conserved CAAX box; italics indicate poly basic motif. (**b**) *Rac2*$^{-/-}$ cells were transduced with a retrovirus vector expressing each Rac mutant and then lysed, and expression of Rac mutants was assessed by immunoblot as described in **Figure 1**. (**c**) Superoxide production of *Rac2*$^{-/-}$ neutrophils reconstituted with each Rac mutant analyzed as described in **Figure 1**. Mean ± s.e.m. of three independent experiments; paired *t*-test. (**d**) Migration of *Rac2*$^{-/-}$ cells expressing each Rac mutant in response to fMLP analyzed with a Boyden chamber as described in **Figure 1**. Results represent the percentage of migrated cells per field compared with that of *Rac2*$^{-/-}$ cells expressing wild-type Rac2. Mean ± s.e.m. of three to five independent experiments; paired *t*-test.

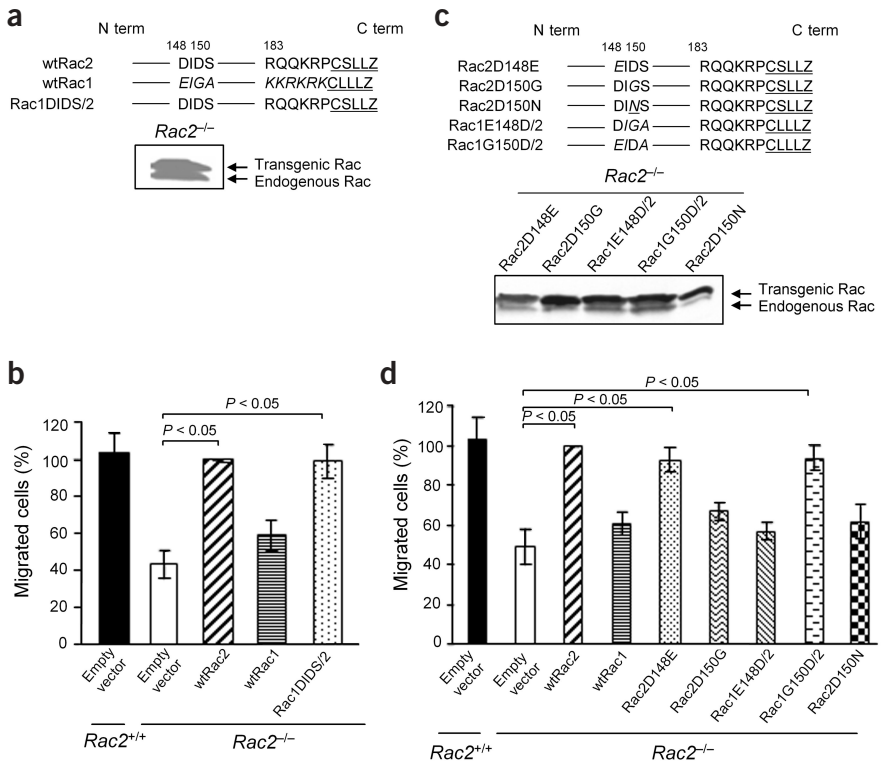

Figure 4 Amino acid D150 and the RQQKRP motif determine the specificity of Rac2 in chemotaxis. (**a**) Rac mutants. Numbers indicate amino acid positions. *Rac2*$^{-/-}$ cells were transduced with each retrovirus vector expressing each mutant. Expression of the mutant in *Rac2*$^{-/-}$ neutrophils was assessed by immunoblot. (**b**) Chemotaxis of *Rac2*$^{-/-}$ neutrophils transduced with Rac1 DIDS/2 mutant compared with that of cells transduced with wild-type Rac1 and wild-type Rac2. Migration was analyzed as described in **Figure 1**. (**c**) Rac mutants in *Rac2*$^{-/-}$ neutrophils assessed by immunoblot. (**d**) Chemotaxis of *Rac2*$^{-/-}$ neutrophils transduced with multiple Rac mutants. Migration was analyzed as described in **Figure 1**. (**b**,**d**) Mean ± s.e.m. of three to four independent experiments; paired *t*-test.

To further clarify the potential function of this sequence in complementing the RQQKRP sequence of Rac2 in mediating neutrophil migration, we assessed the effect of single–amino acid substitution of residues 148 (Rac1E148D/2) and 150 (Rac1G150D/2) and the corresponding replacements of Rac2 with Rac1 sequences (Rac2D148E and Rac2D150G). We confirmed expression of these chimeric proteins in *Rac2*$^{-/-}$ neutrophils by immunoblot (**Fig. 4c**). *Rac2*$^{-/-}$ neutrophils expressing Rac1G150D/2 but not Rac1E148D/2 demonstrated chemotaxis indistinguishable from that of neutrophils expressing wild-type Rac2 (**Fig. 4d**). Confirming the critical nature of residue 150, Rac2D150G failed to rescue chemotaxis of *Rac2*$^{-/-}$ neutrophils to an amount similar to that of wild-type Rac2. In contrast, Rac2D148E rescued *Rac2*$^{-/-}$ neutrophil chemotaxis to wild-type amounts (**Fig. 4d**).

One possible mechanism for the differences in function of Rac2 with aspartic acid versus glycine at position 150 relates to charge, because aspartic acid is acidic, whereas glycine is neutral. To further define the mechanism by which residue 150 complements the RQQKRP motif in Rac2-dependent chemotaxis, we replaced aspartic acid 150 (D150) of Rac2 with the basic amino acid asparagine (N), yielding Rac2D150N.

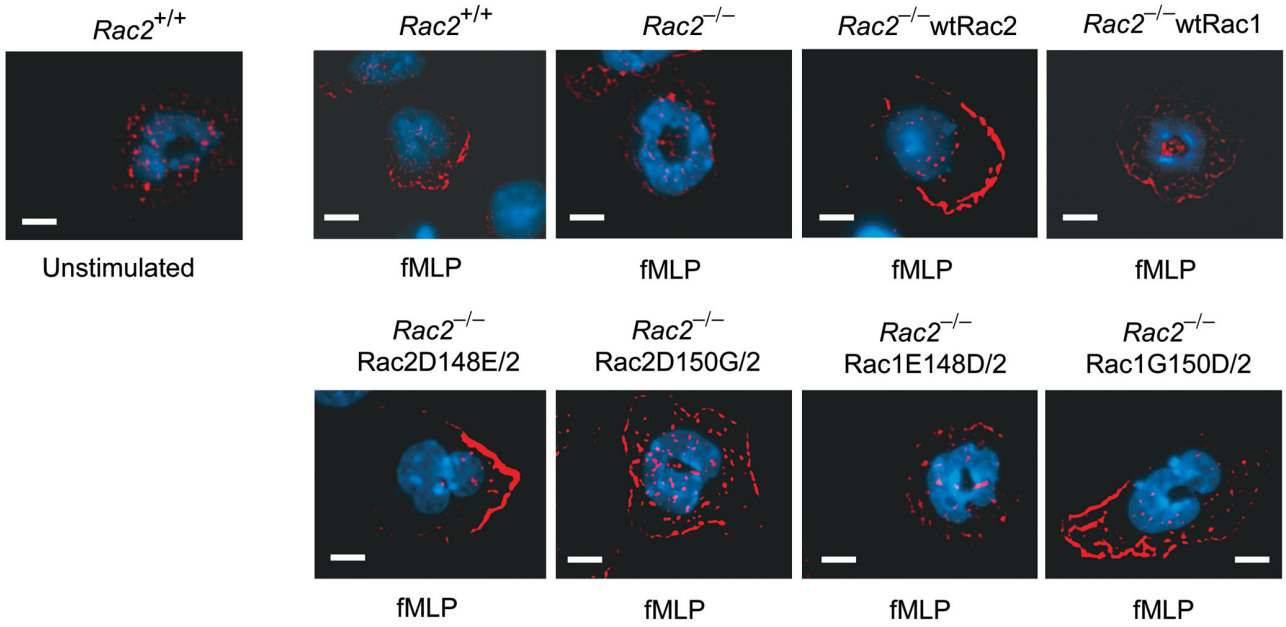

Figure 5 Amino acid D150 and the RQQKRP motif of Rac2 regulate polarized fMLP-induced F-actin assembly. F-actin distribution in fMLP-stimulated *Rac2*$^{-/-}$ neutrophils expressing each Rac mutant compared with that of unstimulated wild-type cells (top row, far left). Cells were stimulated for 15 s with 10 μM fMLP with a uniform stimulus concentration, fixed and stained with rhodamine-labeled phalloidin. A representative cell of each construct is shown. Experiments were done three times with similar results. Scale bars, 2 μm.

Table 1 D150 and the polybasic motif of Rac2 control fMLP-induced F-actin polarization

Genotype	*Rac2*$^{+/+}$	*Rac2*$^{-/-}$					
Vector	Empty	Empty	wtRac2	wtRac1	Rac2E148	Rac2G150	Rac1D148/2	Rac1D150/2
Number of polarized cells/total	12/22	3/20	12/20	3/17	12/20	3/20	4/18	12/21
Cells with polarized F-actin (%)	54	15	60	17	60	15	22	57

Cells expressing each construct were stimulated with a uniform concentration of 10 μM fMLP for 15 s, fixed and stained with rhodamine-phalloidin. Positive cells were assigned scores for asymmetric distribution of F-actin.

This substitution would be predicted to change the charge but conserve the conformation induced by aspartic acid. Rac2D150N was not able to restore chemotaxis of *Rac2*$^{-/-}$ neutrophils (**Fig. 4d**), suggesting the charge at this residue is essential for the function of Rac2 in directed migration. As expected, Rac1E148D/2 and Rac1G150D/2, as well as Rac2D148E, Rac2D150G and Rac2D150N, were able to fully restore superoxide production when expressed in *Rac2*$^{-/-}$ neutrophils (data not shown), confirming that the RQQKRP motif alone is sufficient to rescue Rac2-dependent oxidase complex formation. These results demonstrate that in addition to RQQKRP, D150 is essential for the function and specificity of Rac2 in fMLP-induced chemotaxis in primary neutrophils.

Aspartic acid 150 and RQQKRP specify F-actin assembly

Chemotaxis requires multiple coordinated cellular processes, including actin reorganization. We have demonstrated that Rac2, but not Rac1, is required for cortical F-actin assembly at the neutrophil cell periphery[8]. We next examined the function of residue 150 in F-actin organization. In contrast to no stimulation, fMLP treatment induced a pronounced rim of cortical actin at the periphery of wild-type cells (**Fig. 5**). Most fMLP-stimulated wild-type cells (54%) showed a polarized (asymmetric) organization of F-actin (**Fig. 5** and **Table 1**), producing a pseudopodia-like protrusion of F-actin at one pole of the cell. This F-actin structure was impaired by Rac2 deletion, as only 15% of cells lacking Rac2 showed polarized cortical actin (**Table 1**). Both F-actin generation and polarization was fully restored by expression of wild-type Rac2 in *Rac2*$^{-/-}$ cells. In contrast, although 50% of *Rac2*$^{-/-}$ cells expressing Rac1 had cortical F-actin, the number of cells with a polarized F-actin structure was similar to that of *Rac2*$^{-/-}$ cells transduced with empty vector. Expression of Rac2D150G did not rescue impaired F-actin polarization in *Rac2*$^{-/-}$ neutrophils, whereas Rac2D148E acted like wild-type Rac2 in restoring polarized F-actin. *Rac2*$^{-/-}$ cells expressing Rac1E148D/2 showed some residual cortical actin but minimal polarized F-actin organization. In contrast, expression of Rac1G150D/2 in *Rac2*$^{-/-}$ cells led to polarized cortical F-actin structures similar in frequency and structure to those produced by expression of wild-type Rac2. Thus, residue 150, in addition to RQQKRP of Rac2, specifies polarized cortical F-actin in addition to directed migration in neutrophils.

Correlation of subcellular localizations and functions

We next addressed the function of the polybasic motif and residue 150 of Rac proteins in the agonist-induced subcellular redistribution of Rac and colocalization with F-actin by expression of EGFP-tagged chimeric proteins. The localization of EGFP-Rac2/1, similar to that of wild-type Rac1, was mainly peripheral and was associated with F-actin after fMLP stimulation (**Fig. 6** and **Supplementary Table 1** online). The perinuclear localization seen with wild-type Rac2 was absent from cells expressing EGFP-Rac2/1 but was present with expression of EGFP-Rac1/2. However, this chimeric molecule did not form an obvious organized structure interior to F-actin of the lamellipodium. Instead, Rac1/2 colocalized with cortical actin, like EGFP-Rac1. EGFP-Rac1G150D/2, which

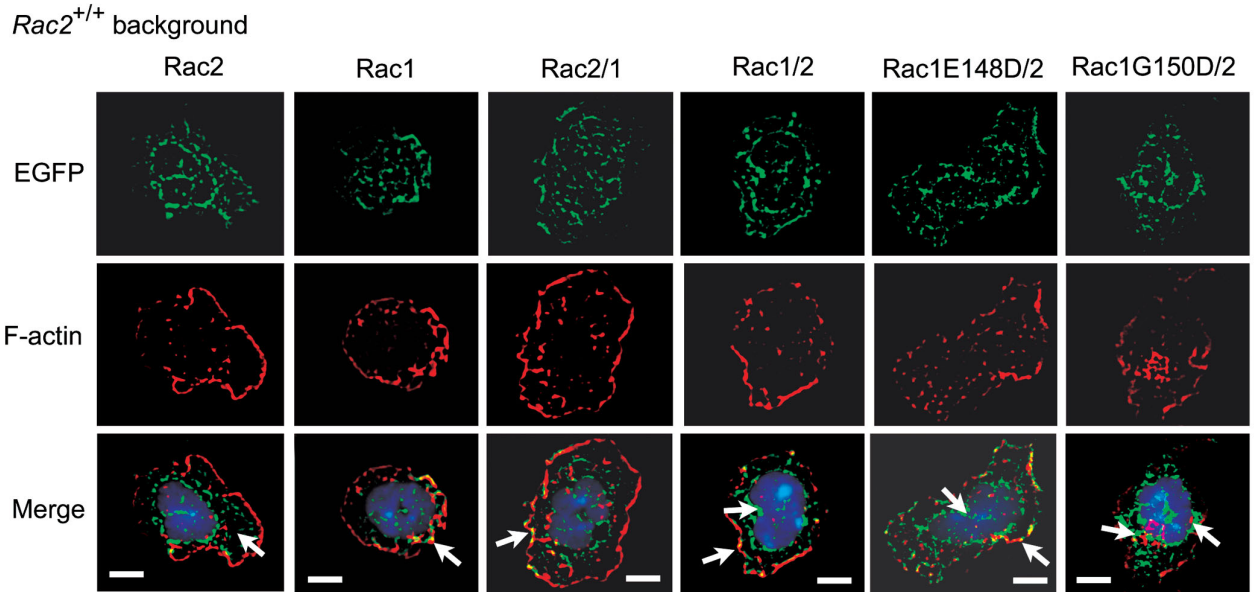

Figure 6 The specificity of Rac2 versus Rac1 is correlated with a specific subcellular distribution. The subcellular distribution of the Rac 'tail mutants' and F-actin in response to fMLP in wild-type (*Rac2*$^{+/+}$) cells was determined as described in **Figure 2**. Arrows indicate areas where Rac mutants overlap with F-actin for Rac2/1, Rac1/2 and Rac1E148D/2 and distinct localization for Rac1G150D/2. One representative cell of each construct from three independent experiments is shown. Scale bars, 2 μm.

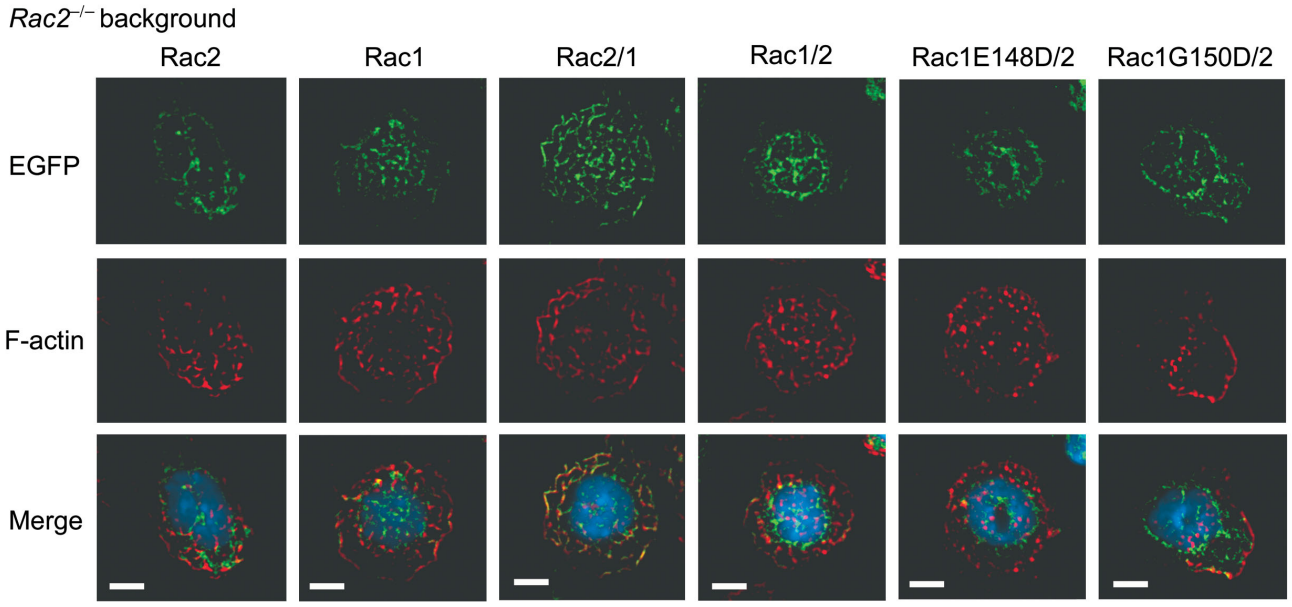

Figure 7 Polarized distribution of Rac2 is necessary for pseudopodia formation. The subcellular distribution of the Rac 'tail mutants' and F-actin in response to fMLP in *Rac2*$^{-/-}$ cells was determined as described in **Figure 2**. There is a lack of Rac organization accompanied by absence of pseudopodium for Rac1, Rac2/1, Rac1/2 and Rac1E148D/2. Scale bars, 2 μm.

rescued both chemotaxis and polarized F-actin assembly, showed a cellular distribution indistinguishable from that of EGFP-Rac2, concentrating interior to polarized F-actin. EGFP-Rac1E148D/2 demonstrated a distribution indistinguishable from that of Rac1/2. We obtained similar results when we analyzed cells by confocal microscopy (data not shown). Confirming the interpretation of the results described above, Rac1 and Rac1/2 and Rac1E148D/2 but not Rac2 colocalized with the plasma membrane marker CD44, as analyzed by confocal microscopy (**Supplementary Fig. 1** online). In contrast, Rac1G150D/2 fully recapitulated Rac2-like localization without overlap with CD44.

We next examined the localization of Rac1 and Rac mutants in *Rac2*$^{-/-}$ neutrophils. In *Rac2*$^{-/-}$ cells after fMLP stimulation, EGFP-Rac1 was poorly organized and was present in only a few areas along with residual cortical F-actin (**Fig. 7** and **Supplementary Table 1** online). Thus, in the absence of Rac2, wild-type Rac1 localization is impaired. Distribution of EGFP-Rac2/1 induced by fMLP in *Rac2*$^{-/-}$ cells resembled that of Rac1 in the same cells. EGFP-Rac1/2 and EGFP-Rac1D148/2 demonstrated the perinuclear distribution seen in wild-type cells but like Rac1 were poorly organized (**Fig. 7** and **Supplementary Table 1** online). The distribution of EGFP-Rac1D150/2 mimicked that of wild-type Rac2, with a perinuclear and polarized pattern interior to F-actin, and this distribution was accompanied by the formation of notable cortical actin in a polarized way. These data suggest that asymmetric redistribution of Rac2 after fMLP stimulation is necessary for pseudopodium formation. As expected, expression of EGFP-Rac2 in *Rac2*$^{-/-}$ neutrophils rescued F-actin assembly and polarization. Expression of EGFP-Rac1 and the mutants EGFP-Rac2/1, EGFP-Rac1/2 and EGFP-Rac1D148/2 in *Rac2*$^{-/-}$ cells failed to rescue cortical F-actin formation in a polarized pattern. Therefore, RQQKRP of Rac2 seems to be necessary and sufficient for the perinuclear distribution of Rac2, whereas the redistribution of Rac2 interior to F-actin in response to fMLP is determined by both RQQKRP and the residue D150, correlating with the ability of Rac2 to mediate migration and F-actin polarization in neutrophils.

DISCUSSION

Rho GTPases are key molecular 'switches' in the control of the biology of immune and hematopoietic cells. Despite having a high degree of identity, Rac1 and Rac2 have distinct functions in both primitive hematopoietic cells and differentiated neutrophils[8–10]. One important unresolved issue is the molecular mechanism by which the specificity of function of Rac2 versus Rac1 is determined in neutrophils.

Here we have presented evidence that the specificity of function of Rac2 versus Rac1 in primary neutrophils is regulated at least in part by distinct subcellular distribution of these GTPases that is in turn determined by the C-terminal RQQKRP sequence and the aspartic acid at position 150 of Rac2. The latter structural site has not been identified as functionally important in any known GTPases, to our knowledge. Our data confirm and extend previous studies. For example, in fibroblasts Rac2 was described to have a perinuclear localization and Rac1, to have a distribution at the cell periphery[18]. In addition, the hypervariable region of Rac has been associated with the regulation of oxidase complex formation in a cell-free system[20] and primary neutrophils[17]. Our study extends those previous studies in several ways. We have demonstrated experimentally that the RQQKRP sequence of Rac2 was sufficient to determine perinuclear location of Rac2 and its specificity in oxidase complex formation compared with that of Rac1. We have also demonstrated that the residue 150, in addition to RQQKRP, determined the relocation of Rac2 in the cytoplasm associated with the formation of polarized F-actin pseudopodium and directed migration. Overall, our results associate the sequence specificity of Rac2 function with subcellular distribution. The spatial pool of active GTP-bound Rac is more restricted than the distribution of the total protein after activation[21]. Therefore, we cannot rule out the possibility that activated Rac proteins show more specific subdomain localization than demonstrated in our studies reported here.

p67^{phox}, one of the main components of the oxidase complex and a known effector of Rac2, has a perinuclear location[22]. This indicates that Rac2 regulates NADPH oxidase complex formation and

chemotaxis by distinct sequences and in different subcellular locations. The difference in subcellular localization probably facilitates interactions with specific target proteins. These results reinforce the idea that spatiotemporal regulation of protein trafficking and intracellular compartmentalization is essential in the regulation of normal cell functions.

Aspartic acid 150 is outside any previously identified functional domains in Rac proteins. D150 is in close structural proximity to the C-terminal domain and on the surface of the protein in its tertiary structure based on computer-assisted modeling (data not shown and refs. 23, 24). The molecular basis of the mechanism leading to distinct localization between Rac1 and Rac2 remains to be determined. Given its function in sequestering Rho GTPases in the cytosol, Rho guanine nucleotide dissociation inhibitor (GDI) proteins may participate in protein trafficking and may determine the subdomain localization of Rac. It is unlikely, however, that this interaction determines the specificity in localization of Rac2 versus Rac1, because no difference in GDI binding has been noted between these two GTPases (data not shown and ref. 25). In addition, the residues directly involved in the interaction of Rho GTPases and GDI are highly conserved[23,24,26]. Aspartic acid 150 and RQQKRP of Rac2 and glycine 150 and KKRKRK of Rac1 are distinct in their local charge. As shown here, the charge is probably essential in the function of the molecule and may passively determine the binding with specific membrane compartments, as seen with Ras proteins. A basic polylysine motif found in Kirsten-Ras allows its association with negatively charged phosphoinositide at the plasma membrane by direct ionic interaction[27], whereas a 'neutralized' motif drives the protein to endomembrane compartments and Golgi-associated structures[28]. In addition, in contrast to Harvey-Ras, Kirsten-Ras, which contains a polybasic domain similar to that of Rac1, does not require lipid modification for membrane targeting[29]. These observations may be relevant for the differences in the cellular localization of Rac1 and Rac2. However, we cannot exclude the possibility that in addition to subcellular localization, interactions with distinct downstream effectors via sequences described in this study are also involved in the specificity of function of Rac2 versus Rac1 in hematopoietic cells, as seen for p21-activated kinase binding and activating properties[30] and phosphoinositide 5-kinase activity[31] in fibroblast cell lines. Thus, an alternative explanation would be that specific guanine nucleotide exchange factor proteins upstream of Rac lead to differences in Rac activation and, by inference, Rac localization[21].

Chemotaxis is a complex process that requires actin rearrangement in an asymmetric way, resulting in a polarized cell. Rac proteins are linked to a positive feedback loop in which phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3), a known substrate of phosphoinositide-3′-kinase (PI3K), is formed and stimulates Rac activation. Activated Rac in turn leads to an increase accumulation of the PtdIns(3,4,5)P_3 at the plasma membrane and formation of the pseudopodium[32–35]. However, those studies did not discriminate between Rac1 and Rac2 in this process, because a dominant negative mutant of Rac was used. In contrast to mast cells[36] and hematopoietic stem cells[8], in neutrophils absence of Rac2 does not affect protein kinase B activation[10,11]. However, inhibition of PI3K activity via the PI3K inhibitor Ly294002 led to impaired F-actin polarization associated with a loss of polarized EGFP-Rac2 localization interior to F-actin. This was also accompanied by misorganization of EGFP-Rac1 (data not shown). Therefore, Rac2 and Rac1 seem to be downstream of PI3K in neutrophils. In addition, in our model, loss of Rac2 was associated with impairment of F-actin and PtdIns(3,4,5)P_3 (data not shown) organization, leading to a loss of pseudopodium and a defect in cell polarization. These results suggest that Rac2 could be an essential component of the feedback loop by regulating the spatial and temporal F-actin assembly, perhaps through proper distribution of target proteins. Rac2 deficiency is also associated with defective organization of Rac1 at the plasma membrane, suggesting that the distribution of Rac1 is correlated to F-actin organization and, by inference, is dependent on Rac2-controlled cell polarity. Downstream of Rac2, Rac1 dysfunction might contribute to the F-actin defect and abnormal chemotaxis described in *Rac2*$^{-/-}$ cells. These points raise additional questions about the function of crosstalk between Rac1 and Rac2 in the biology of cells of hematopoietic origin.

In conclusion, we have identified structural cues of Rac2 that determine its *in vivo* specificity of function in superoxide production and chemotaxis compared with that of Rac1. This specificity seems to depend on the distinct subcellular distribution of each GTPase. These studies have broad implications for the involvement of signaling molecule localization and trafficking in immunological function.

METHODS

Retrovirus vector construction and virus supernatant generation. The C-terminal basic motif KKRKRK of Rac1 was replaced by the analogous sequence RQQKRP of Rac2 (Rac1/2 mutant) and vice versa (Rac2/1 mutant) with a PCR-based technique[37]. The mutants Rac1DIDS/2 (in which the EIGA sequence of Rac1 (amino acids 148–151) was replaced by the DIDS sequence of Rac2), Rac1E148D/2, Rac1G150D/2, Rac2D148E, Rac2D150G and Rac2D150N were generated with the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). The identities of the Rac mutants were verified by sequencing and then the mutants were subcloned into the bicistronic retrovirus vector MIEG3, which encodes EGFP[12]. Wild-type and mutant proteins were tagged at the N terminus with the hemagglutinin epitope.

For generation of the chimeric reporter proteins EGFP-Rac1, EGFP-Rac2, EGFP-Rac2/1 and EGFP-Rac1/2, cDNAs of wild-type rac1, wild-type Rac2, Rac1/2, Rac2/1 were cloned into the pEGFP-C1 vector (Clontech). The resulting fusions were sequenced and subcloned into a mouse stem cell virus–based[12] retrovirus vector. Retroviral supernatants were generated by transfection of ecotropic Phoenix-gp cells (provided by C. Baum, Cincinnati Children's Research Foundation, Cincinnati, Ohio) with a calcium phosphate precipitation kit (Sigma).

Transduction of bone marrow cells and *in vitro* generation of neutrophils. Mouse low-density bone marrow cells were isolated from C57BL/6J mice (Jackson Laboratories) and *Rac2*$^{-/-}$ mice (back-crossed to C57BL/6 (B6.129S6-Rac2<tm1mddw>; N12))[17]. The cells were transduced twice on fibronectin fragment CH296 (provided by Takara Bio, Otsu, Japan) as described[12]. EGFP$^+$ cells were isolated with a fluorescence-activated cell sorter (FACSDiva; Becton Dickinson) 4 d after the second infection and were cultured for another week in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% FBS (HyClone), 2% penicillin-streptomycin (Invitrogen), 100 ng/ml of recombinant rat stem cell factor, 100 ng/ml of megakaryocyte growth and development factor and 100 ng/ml of granulocyte colony-stimulating factor (all supplied by Amgen). For assessment of neutrophil differentiation, cytospin preparations of the cells were stained with Wright-Giemsa and were examined with light microscope. All animals experiments were in accordance with protocols approved by The Children's Hospital Research Foundation Animal Care Committee (Cincinnati, Ohio).

NADPH oxidase activity. Reduction of NBT (Sigma) was used as a quantitative measure of the number of cells producing superoxide and was done as described[17]. Neutrophils were seeded for 1 h at 37 °C onto chamber slides (LabTek) in Iscove's modified Dulbecco's medium to allow cell adherence, then were stimulated for 20 min at 37 °C with 10 μM fMLP (Sigma) in saturated NBT solution. After being washed with PBS, cells were fixed with methanol and were counterstained with Safranin-O (Sigma). At least 200 cells were examined to determine the percentage of NBT$^+$ cells.

Chemotaxis. Chemotaxis in response to fMLP was evaluated in triplicate with a 48-well microchemotaxis chamber (Neuro Probe) as described[11]. After being diluted in Hank's balanced salt solution (Invitrogen) containing Ca^{2+} and Mg^{2+}, 1 μM fMLP was loaded into the lower chamber. Neutrophils (1×10^5) in Hank's balanced-salt solution (50 μl) were placed into the upper chamber. The upper and lower chambers were separated by a 3-μm polycarbonate membrane (Neuro Probe), which allows neutrophil migration. The chamber with cells was incubated for 45 min at 37 °C in 5% CO_2. Cells in three randomly chosen fields were counted for determination of the number of migrated cells. In the presence of a gradient of carrier only or in the absence of a gradient of chemoattractant, the number of migrated cells was 10% that in the presence of gradient of fMLP, indicating that this test is an assay of directed migration (data not shown).

Immunofluorescence. For characterization of the subcellular localization of EGFP-Rac proteins and F-actin, GFP^+ neutrophils (2×10^4) were seeded for 1 h at 37 °C onto chamber slides in Hank's balanced-salt solution to allow cell adherence. The cells were then stimulated for 15 s with 10 μM fMLP, fixed for 20 min with 2% paraformaldehyde, pH 7.6 (Sigma), and then permeabilized with 0.1% Triton X-100 (Sigma). After incubation for 15 min in 2% BSA (Roche), the cells were stained for 1 h at 25 °C with rhodamine-labeled phalloidin (Molecular Probe) and then were washed three times with PBS. Slides were mounted in SlowFade Light Antifade medium containing 4,6-diamidino-2-phenylindole (Molecular Probes).

Z series of fluorescence images were captured with a Leica DMIRB fluorescence microscope (Germany) at ×63 magnification with an ORCA-ER C4742-95 camera (Hamamatsu) equipped with a deconvolution system (Leica) driven by Openlab 3.1 software (Improvision). Excitation wavelengths of 488 and 568 nm and emission filters were used to detect EGFP (510–520 nm) and/or rhodamine (595 nm). In one experiment, the slides were read by experimenters 'blinded' to sample identity. Pictures were analyzed by deconvolution. Additional images were captured with confocal microscopy.

Immunoblot. Wild-type, $Rac2^{-/-}$ and $Rac2^{-/-}$ neutrophils reconstituted with Rac proteins (1×10^5) were lysed for 30 min at 4 °C in a buffer of 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 10 mM Tris-HCl, pH 7.6, 0.1% BSA, 20 μg/ml of aprotinin, 2 mM Na_3VO_4 and 0.01 mM phenylarsine oxide. Cell lysates were recovered by centrifugation for 30 min at 12,000*g* at 4 °C. Total Rac proteins were evaluated with antibody to Rac (102) from Transduction Laboratories, which recognizes both Rac1 and Rac2.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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