

# Spatial and Temporal Analysis of Rac Activation during Live Neutrophil Chemotaxis

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## Summary

The ability of cells to recognize and respond with directed motility to chemoattractant agents is critical to normal physiological function. Neutrophils represent the prototypic chemotactic cell in that they respond to signals initiated through the binding of bacterial peptides and other chemokines to G protein-coupled receptors with speeds of up to 30  $\mu\text{m}/\text{min}$  [1, 2]. It has been hypothesized that localized regulation of cytoskeletal dynamics by Rho GTPases is critical to orchestrating cell movement. Using a FRET-based biosensor approach [3], we investigated the dynamics of Rac GTPase activation during chemotaxis of live primary human neutrophils. Rac has been implicated in establishing and maintaining the leading edge of motile cells, and we show that Rac is dynamically activated at specific locations in the extending leading edge. However, we also demonstrate activated Rac in the retracting tail of motile neutrophils. Rac activation is both stimulus and adhesion dependent. Expression of a dominant-negative Rac mutant confirms that Rac is functionally required both for tail retraction and for formation of the leading edge during chemotaxis. These data establish that Rac GTPase is spatially and temporally regulated to coordinate leading-edge extension and tail retraction during a complex motile response, the chemotaxis of human neutrophils.

## Results and Discussion

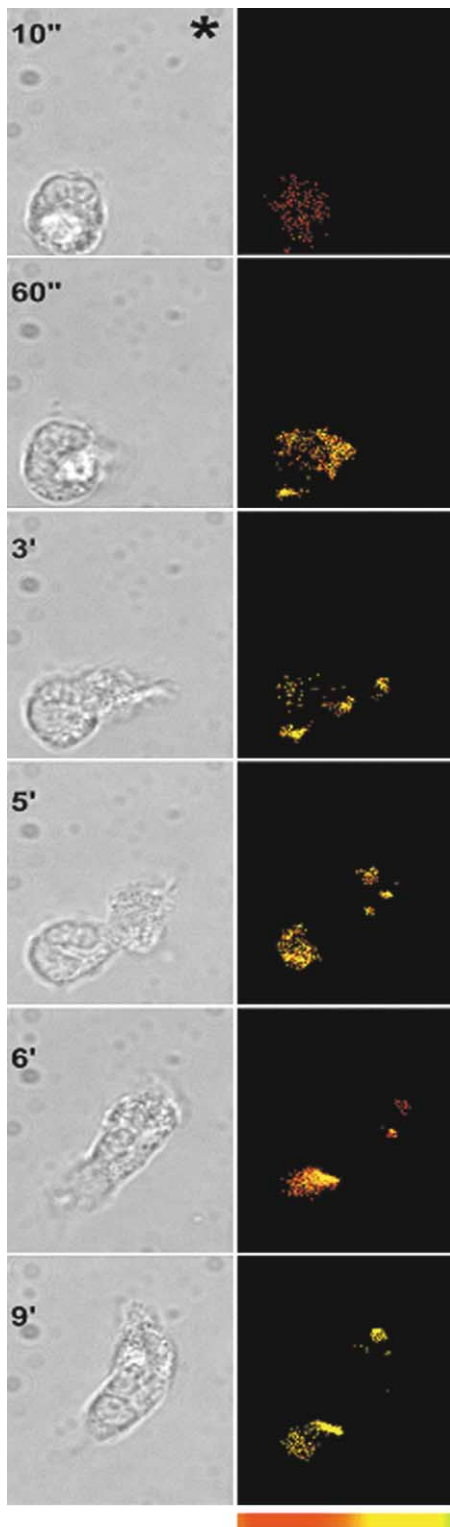
During leukocyte chemotaxis, actin is preferentially assembled at the leading edge to move the cell forward, whereas myosin-mediated contractile forces aid in tail retraction [1, 2, 4]. We have previously shown that Rac GTPase is activated rapidly after stimulation of human neutrophils and HL-60 cells with chemoattractant [5]. Here we examined the dynamics of Rac activation during chemotaxis of primary human neutrophils to the G protein-coupled receptor agonist fMLP by using FLAIR [3, 6]. FLAIR (Fluorescent Activation Indicator for Rho GTPases) utilizes the fluorescence resonance energy transfer between EGFP-tagged Rac1 or Rac2 and Alexa 546-PBD to detect the formation of active Rac-GTP in real time. As shown in Figure 1 (see also the supplementary movies available with this article online), we ob-

served very distinct spatial and temporal activation of Rac at various stages of the human neutrophil chemotactic response. At early times (0–10 s), the neutrophil appears rounded, and there is very little Rac activity. As the neutrophil senses the chemoattractant (60 s to 3 min), the cell begins to extend a lamellipod in the direction of the gradient. This is accompanied initially by a broad area of increased Rac activity localized to the extending lamellipod (60 s) and subsequently by more specific Rac activation localized within the developing leading edge (3 min). As early as 60 s, we additionally observe an intense area of active Rac that remains at the rear of the polarizing cell. As the neutrophil becomes fully polarized (3–6 min), active Rac becomes localized to distinct areas at the established leading edge. These foci of Rac activity are maintained during chemotaxis, although individual foci come and go as the leading edge dynamically advances toward the point source of fMLP (6–9 min). The absence of continuous Rac activity across the leading lamellipodium at this stage is evident. Interestingly, we see that the area of active Rac at the cell rear is less discrete, but changes in intensity in a pattern that appears to match tail retraction and attachment/detachment to the substratum (see the 6–9 min time period). We have observed this pattern of Rac activation in five out of five motile cells examined during a single experiment and overall in ten separate experiments that have used neutrophils prepared from different donors. We have also observed that as individual neutrophils reorient in response to movement of the chemoattractant point source, this process repeats itself. Rac2 is the predominant Rac isoform in human neutrophils [7, 8], but essentially identical results were obtained whether we used EGFP-Rac1 or EGFP-Rac2 in the FLAIR analyses (see Supplementary Movie 2). Control experiments, including use of a GTPase binding-deficient version of the PBD, indicate that we are detecting FRET that is indicative of Rac activation [6] (see Supplementary Movie 3). We cannot rule out the possible existence of activated Rac that is protected from interaction with the PBD probe by tight binding to effector proteins, however.

Using a specific Rac2 antibody, we evaluated whether endogenous Rac2 redistributes in response to chemoattractant [8]. In unstimulated neutrophils, Rac2 was distributed throughout the cell body (see unpolarized cell in Figure 2A). Confocal microscopy showed that upon stimulation with chemoattractant, a fraction of Rac2 translocated and concentrated either at or just behind the leading lamellipodium (or both) (Figure 2A, left panels) and typically exhibited a noncontinuous distribution that overlapped, but did not coincide with, F-actin across the extending edge (Figure 2A, right panels; Supplementary Figure S1). Endogenous Rac2 became localized in discrete regions within the tail as well, although the staining in these areas was usually less intense than at the leading edge. The redistribution of endogenous Rac2 was similar to the localization of active Rac2 detected by FRET (see Figure 1 and Supplementary Movie

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**Figure 1. Dynamics of Rac Activation in Chemotaxing Human Neutrophils**

Rac activation was measured in live human neutrophils by FRET between EGFP-Rac1 and Alexa 546-labeled PBD. A time course from 10 s to 9 min shows bright-field and corresponding FRET images of neutrophils chemotaxing toward a point source of fMLP (asterisk in right lower corner of the 10 s bright-field panel); see text for description. Prior to the neutrophils sensing the fMLP gradient,

2). These data indicate that Rac2 is recruited to areas in the leading edge and retracting tail where actin assembly is occurring and that this recruitment appears to be largely coincident with activation.

We examined the effects of adhesion on Rac activity in fMLP-stimulated neutrophils (Figure 2B). As previously observed, neutrophils in suspension developed a polarized morphology in response to global stimulation with fMLP, although the cells tended to remain more rounded than the adherent cells, with a broad F-actin-rich leading lamellae (see Supplementary Figure S2). Under these conditions, Rac activation was observed in the anterior (actin-rich) region of the polarized cells but was substantially reduced in the cell posterior. The loss of Rac activity in the tail was evident in multiple cells (quantitated in Figure 2C), even when the suspended cells were examined in several focal planes. This observation indicates that the loss was not merely due to a focusing artifact. This contrasted with the polarized adherent neutrophils, where Rac activity was evident in both the anterior and posterior regions of the cell. The amount of FRET seen within the tail of either suspended or adherent cells was significantly different from the main cell body (middle) at  $p \leq 0.0001$ . These data suggest that cell adhesion may play an important role in modulating localized Rac activity, particularly in posterior regions where the cell tightly engages the extracellular matrix.

The effects of various Rac wild-type and mutant proteins on the chemotactic responses of fMLP-stimulated human neutrophils were determined (Figure 3; see also the supplementary movies and Table S1). We initially chose conditions in which GTPases were expressed at levels similar to that of endogenous Rac; this avoided potential overexpression artifacts (see quantitation in Supplementary Figure S3). The introduction of wild-type Rac1 protein had little effect on the chemotactic response to the fMLP gradient when this effect was compared to that in control neutrophils (Figure 3A; Supplementary Table S1). Cells expressing a constitutively active Rac1(G12V) mutant also responded as effectively as control cells. In marked contrast, cells expressing a Rac1(T17N) dominant-negative mutant at levels similar to endogenous Rac were significantly impaired in their ability to chemotax. Identical functional results were obtained for cells expressing the equivalent Rac2 mutants.

Examination of the neutrophils expressing each Rac mutant by time lapse microscopy revealed that the cells containing dominant-negative Rac1- or Rac2(T17N) exhibited a significant tail retraction defect (Figure 3B; Supplementary Movie 7). These cells still detected and polarized toward the fMLP gradient, and they even attempted to move the cell body toward the chemoattractant point source ( $31.1\% \pm 4.1\%$  polarized/motile cells versus  $38.1\% \pm 4.3\%$  in control cells). However, the rear of the cells was unable to retract toward the moving cell body, and this inability caused the cells to stretch

the average FRET intensity is 10 units on a 12-bit scale (range = 0–4096 intensity units) after background and bleed-through correction and varies between 30 and 80 intensity units at various stages of chemotaxis. The intensity scale bar range is from red (5–15 intensity units) to yellow (25–35 intensity units) to green (70–80 intensity units).

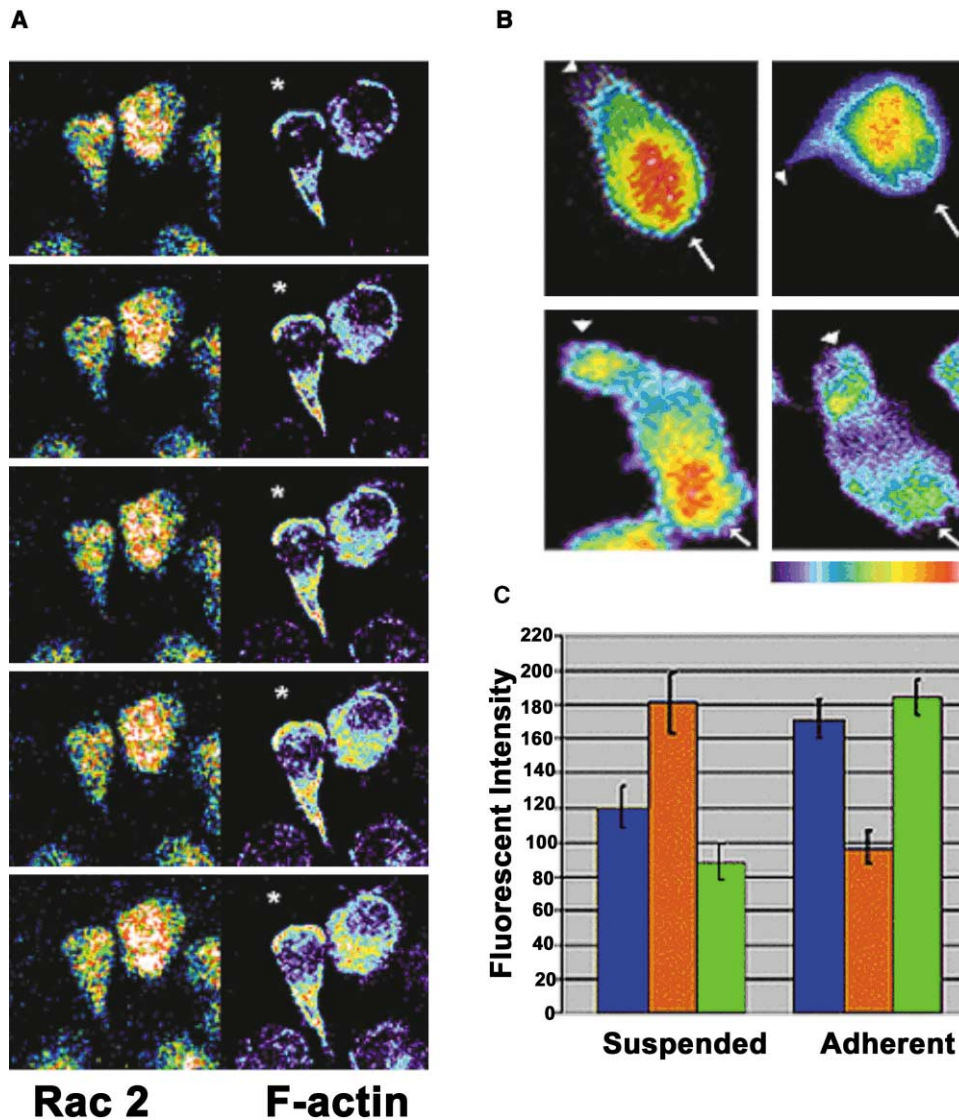
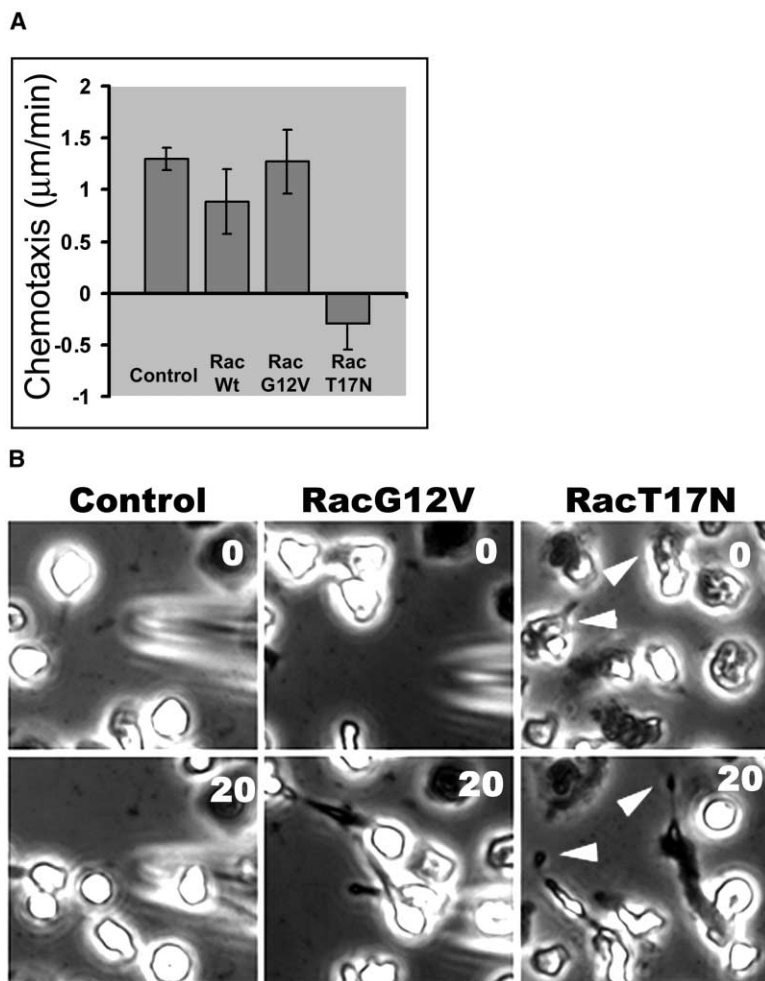


Figure 2. Localization of Endogenous Rac2 and Activation Dynamics in Adherent versus Nonadherent Cells

(A) Confocal images taken in  $0.3\ \mu\text{m}$  sections of Rac2 antibody staining (left-hand panels) and F-actin (right-hand panels). Cells were stimulated for 6 min with an fMLP point source and fixed during stimulation. The images go from the top (uppermost panel) to the bottom (lowest panel) of the cell. Endogenous Rac2 primarily localizes to the front of polarized cells in this series of panels. Less intense Rac2 staining is seen in the tail of some cells as well (see also Supplementary Data 2.1). Images are shown in pseudocolor to highlight intensity differences. The pseudocolor intensity scale ranges from low (blue) to medium (green) to high (red).

(B) Rac activation was measured in suspended (top panels) and adherent (bottom panels) neutrophils after fixation with 6% paraformaldehyde. Two representative cells are shown for each. Suspended cells were stimulated globally with 30 nM fMLP. Adherent cells were plated, stimulated on a marked coverslip with an fMLP point source for 3 min, and then fixed. FRET analyses were performed with the same analysis program and exposure times as for live cells (Figure 1). Examination of several time points during chemotaxis showed that the fixation procedure reproducibly preserved Rac activity, with the fixed adherent cells exhibiting spatial and temporal changes similar to those observed in live adherent cells ( $n = 200$  cells). We note that the FRET signals were usually more intense in the fixed cells, and this greater intensity resulted in an apparent broadening of the area of Rac activation when these images were compared to those obtained in live cells. It was evident, however, that fixed suspended neutrophils polarized in response to global stimulation with fMLP exhibit strong Rac activation in the anterior portion of the cell body (arrow), with significantly less activation in the posterior tail (arrowhead) ( $n = 50$  cells). FRET intensity scale ranges from low (blue) to medium (green) to high (red).

(C) Quantification of FRET localization within the stimulated neutrophil. Suspended cells show prominent Rac activation (FRET) in the leading edge (anterior, blue bar) and cell body (orange bar) but significantly less in the posterior tail (green bar). ANOVA showed that differences between the amount of Rac activation seen in the anterior and posterior of suspended cells were significant ( $p \leq 0.0001$ ). Adherent cells show Rac activation in the anterior (blue bar) of the cell as well as the posterior tail (green bar), with significantly less seen within the cell body (orange bar). Differences in the amount of Rac activation between the anterior and the cell body, as well as differences seen between the posterior and the cell body, were highly significant by ANOVA ( $p \leq 0.00001$ ).



**Figure 3. Effects of Rac Mutants on Chemotactic Response to fMLP**

Either control neutrophils (expressing  $\beta$ -galactosidase) or neutrophils expressing the wild-type (Wt), constitutively active G12V, or dominant-negative T17N Rac1 mutants (added at  $3 \mu\text{g/ml}$ ) were stimulated with a point source of fMLP (the micropipette is evident at the right of each panel) and imaged at 30 s intervals, as described in the Experimental Procedures.

(A) Quantification of the rate of movement of the chemotaxing cells expressing each construct toward the point source of fMLP. Values are an average  $\pm$  standard error for a minimum of five cells of each type from a single representative experiment. The asterisk indicates a statistically significant difference from the control ( $p < 0.01$ ). Similar results were obtained in at least three separate experiments, but data were not pooled because of variations in the rate of movement of individual neutrophil preparations.

(B) Shown are select frames (time in min is displayed in the upper right-hand corner of each frame) at the beginning (upper panels) and toward the end (lower panels) of imaging. Dramatically extended tails are evident in cells expressing Rac (T17N)—see arrowheads. Movies of Rac Wt/G12V/T17N mutants undergoing chemotaxis can be found with the Supplementary Material online.

out toward the point source and form elongated tails. The phenotype was also characterized by the inability of the tails to detach from the substrate (see Supplementary Movie 7). Quantitatively, we found that  $29.8\% \pm 4.3\%$  of the cells expressing low levels of Rac1(T17N) exhibited a tail retraction defect versus  $2.8\% \pm 4.0\%$  of control cells.

Rac clearly plays an additional role to regulate leading-edge extension. When we expressed dominant-negative Rac at levels approximately 2-fold greater than endogenous Rac (i.e., when we added it to cells at greater than  $8 \mu\text{g/ml}$ —see Experimental Procedures and Supplementary Figure S3), a loss of the intense F-actin staining seen in the leading lamellipod of control cells was observed (Figure 4A). The remaining F-actin staining was evident as a band around the periphery of the cell. This was verified by quantitation (Figure 4B) of the F-actin distribution from the anterior edge to the rear of multiple cells. We also observed that neutrophils became increasingly less polar and no longer tried to move toward the fMLP point source in the presence of higher levels of (T17N) Rac ( $8.5\% \pm 3.5\%$  polarized cells versus  $38.1\% \pm 4.3\%$  in controls; see Supplementary Table S1).

It has proven difficult to study chemotactic signaling mechanisms in primary neutrophils because manipulation and transfection of these cells is problematic. How-

ever, current evidence suggests that Cdc42 is important in sensing the chemotactic gradient and in initiating the resulting polarization of leukocyte responses [9, 10]. Rho has been shown to modulate leukocyte tail retraction through the action of Rho kinase on integrin adhesion [4, 11] and, potentially, myosin contractility [12, 13]. Although the requirement for Rac2 in neutrophil chemotaxis has been clearly demonstrated in Rac2 null mice [14], there is little biochemical data on the mechanism of Rac action during the chemotactic response. We show here that Rac undergoes spatially localized and temporally regulated changes in its activation state and that these changes correlate with distinct phases of the chemoattractant response. Endogenous Rac2 becomes localized at or just behind the leading edge of the moving leukocyte (or both), apparently coincidentally with activation, and in areas where dynamic actin polymerization is presumably being regulated through Rac effector proteins. We have previously shown the Rac effector p21-activated kinase 1 to be recruited to the leading edge [15]. Specific Rac activation in the tail is associated with regulation of tail retraction, which requires myosin II-mediated contraction [4]. We observed that treatment of human neutrophils with the myosin light chain kinase inhibitor ML-7 also induced the appearance of elongated, nonretracted tails and decreased overall motility

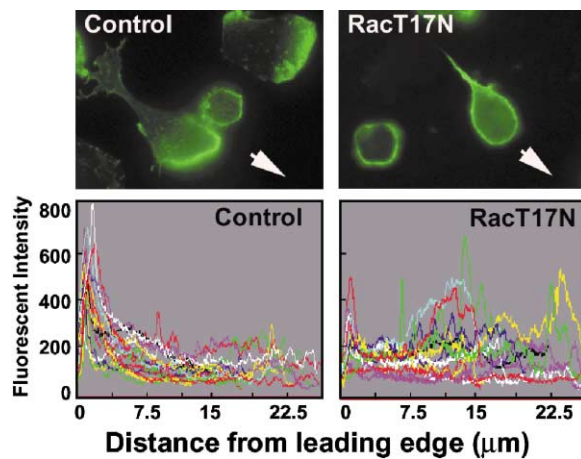


Figure 4. Distribution of Actin in Neutrophils Expressing a Dominant-Negative Rac Mutant

Upper panels: neutrophils expressing dominant-negative Rac1 (added at 30  $\mu\text{g}/\text{ml}$ ) or control cells were globally stimulated with 30 nM fMLP for 5 min, fixed, and stained with Alexa 488-conjugated phalloidin. The arrow indicates the direction of movement. Lower panels: the distribution of fluorescence intensity across the length of 15–20 polarized cells of each type was measured and plotted. Relative fluorescent intensity was plotted along a line drawn from the leading edge to the tail of individual polarized cells. Each colored line represents a single cell.

(our unpublished data). This phenotype only partially recapitulates the Rac (T17N) phenotype, in which the tails additionally often appear to be stuck to the substratum. This suggests that the effects of Rac on tail retraction are likely to be complex. The very localized and dynamic changes in Rac activation observed in the highly motile neutrophils contrasts markedly with the broad gradient of activation previously observed at the front of wounded fibroblast monolayers (see [3]). Interestingly, we show that spatially directed Rac activity is modulated by cell adhesion; this finding suggests that integrin-mediated signals are likely to play important roles in regulating Rac activation during chemotaxis. Recent work has provided evidence that Rho family GTPases participate in a positive feedback loop involving the formation of phosphatidylinositol 3,4,5  $\text{P}_3$  at the leading edge of chemotaxing HL-60 leukocytes [16]. It will be of interest to determine how the Rac activation dynamics we have described relate to localized accumulation of  $\text{PIP}_3$  during various phases of the human neutrophil chemotactic response.

#### Experimental Procedures

##### Chemotaxis Assays

Human neutrophils at approximately 95% purity were isolated from human blood as described [5]. All experimental manipulations were done in chemotaxis buffer (140 mM KCl, 10 mM glucose, 10 mM HEPES, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , and 189  $\mu\text{M}$   $\text{CaCl}_2$  [pH 7.0]). Cells were plated on flamed 25 mm glass coverslips (Corning), placed into Attofluor live-cell chambers (Molecular Probes), and stimulated on the microscope stage with a micropipette point source containing 30  $\mu\text{M}$  formyl Met-Leu-Phe (fMLP) at approximately 30°C. Based on our experimental set up, we have determined that this resulted in an fMLP concentration at the pipette tip of about 30 nM. The percent

of responding motile neutrophils ranged from 25% to 60% in various experiments.

Individual cells were tracked via the nanotrack feature of the ISEE Invision software. The coordinates of the cell path were imported into an Excel worksheet, and a chemotaxis index (the distance traveled by the cells in the direction of the point source per unit time) was determined in  $\mu\text{m}/\text{min}$  by subtracting of the distance between the cell and the pipette at the end of the track from that at the beginning, then dividing this value by the total time taken. The effects of dominant-negative Rac on cell morphology were quantitated by counting the number of cells in a field (60–100 cells) in at least three separate experiments that were (1) polarized and moving toward the pipette; (2) polarized toward the point source but unable to move due to the presence of nonretracting tails, and (3) nonpolarized. Numbers given in the text represent the mean  $\pm$  standard deviation of at least three separate experiments.

##### Purification of GTPases

Rac1 and Rac2 glutathione S-transferase (GST)-fusion proteins were expressed in *E. coli* and purified on glutathione beads, and the GST moiety was removed by thrombin cleavage according to the manufacturer's instructions [17]. The activity of the purified GTPases was determined by the binding of [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  [18].

##### Introduction of Labeled PBD and GTPases into Primary Human Neutrophils

All experimental components were introduced into human neutrophils in chemotaxis buffer. For FLAIR experiments, purification and fluorescent labeling of the PBD was as described previously [6]. Labeled wild-type PBD or PBD(H83,86L) non-GTPase binding mutant were introduced by electroporation with 10  $\mu\text{l}$  of 40  $\mu\text{M}$  labeled PBD in 800  $\mu\text{l}$  chilled chemotaxis buffer containing  $1 \times 10^{-6}$  suspended neutrophils. A BioRad Gene Pulser (Biorad) was used with the following settings: 1.0 kV, infinite resistance, and capacitance = 25. After electroporation, we washed the cells twice in chilled buffer by spinning them for 5 s in a tabletop centrifuge set at 7500 rpm to remove unincorporated PBD. Cells were plated after being washed and were immediately transfected with EGFP-Rac1 or EGFP-Rac2 wild-type via a Semliki Forest virus expression system [19], enabling us to obtain transfection efficiencies of 95% routinely. Virus was left on cells for 45 min, gently removed, and replaced with chemotaxis buffer. EGFP-Rac was detectable within 3 hr after viral infection, and imaging was initiated.

For chemotaxis assays (see above), the purified proteins were mixed with Bioporter reagent, pipetted onto coverslips of spread neutrophils, and incubated at room temperature for 2–4 hr as per the manufacturer's instructions (Gene Therapy Systems, San Diego, CA). Using Rac protein labeled with Alexa 546 succinimide ester (Molecular Probes), we observed uptake by more than 95% of cells in 2 hr in the presence of Bioporter reagent. The amount of Rac protein present in cells after Bioporter treatment was determined by immunoblotting. Cells were incubated with various concentrations of Rac2 protein, and equal amounts of protein lysate were run on 13% SDS-PA gels, then stained with Rac2 antibody R786 at 1:2000. No significant increase in the intensity of the endogenous Rac band was observed at introduced Rac concentrations from 1 to 3  $\mu\text{g}/\text{ml}$  (see Supplementary Figure S3). At higher concentrations (8–30  $\mu\text{g}/\text{ml}$ ), the increase in staining intensity was proportional to the concentration of introduced protein.

##### Imaging and FLAIR Analyses

Imaging experiments were performed on an Olympus IX70 microscope, and time lapse imaging was performed with a Princeton MicroMax 5 MHz 12 bit cooled CCD camera coupled to Invision ISEE software. Invision software was also used to perform FRET analysis. The filters used were as follows: GFP HQ480/40, HQ 535/50, Q505LP, FRET D480/30, HQ 61075, 505DCLP, Alexa 546 HQ545/30, HQ610/75, and Q565LP (Chroma). The methodology for the FLAIR technique has been described in detail [3, 6]. However, image bleed-through correction curves were prepared for our specific imaging system (filter cubes, camera, and microscope) and for the exposure times used. Signals were always substantially above background and much greater than the variation in the background itself.

Backgrounds ranged between 200 and 350, whereas the regions with highest FRET were typically 30–70, and never less than 10 above background. Rac activation as high as 100 above background was also seen. In all experiments, the signal was at least 20% greater than the background, technically good for FRET experiments. Image acquisition was as follows: 5 s exposure in the FRET channel, 3 s exposure in the EGFP channel, a 1 s exposure in the Alexa 546 channel, and a 0.1 s exposure in bright field. Cells were examined with a 60× Plan APO 1.4 NA objective lens. All images in sequence were background corrected and registered to the initial FRET image prior to thresholding and bleed-through corrections. A non-GTPase binding PBD mutant (H83,86L) labeled with Alexa 546 dye was substituted for wild-type PBD, and no FRET signal was observed in human neutrophils expressing EGFP-Rac1 or EGFP-Rac2 (see Supplementary Material). In addition, similar analyses with EGFP (vector control) or RhoA(Q63L) in the presence of wild-type Alexa 546-labeled PBD gave no FRET signals. Confocal imaging of endogenous Rac2 and F-actin was done with a Fluoview 200 scanhead on an Olympus IX70 platform with a 60× Plan APO 1.4 NA oil immersion objective lens. Scanning was first done with a krypton 568 laser to detect the Alexa 568 goat anti-rabbit-conjugated secondary antibody (used at 1:600 dilution) to the specific Rac2 polyclonal antibody (used at 1:300 dilution) [8]. Scanning was then done with an argon 488 laser to detect the Alexa 488-conjugated phalloidin (used at 1:600 dilution). Images were collected in Z at 0.3 μm and are presented as single 0.3 μm sections or rendered composites built from Z stacks (Supplementary Figure S1).

We performed quantitative analysis of FRET in fixed suspended and adherent cells on a per cell basis by using Metamorph (UIC) software to carry out line scans through individual cells. We used phalloidin staining to mark the leading edge in suspended cells ( $n = 19$ ). Cell polarization with regards to the fMLP point source was used for determination of a leading edge in adherent, migrating cells ( $n = 19$ ). Line scans were performed on 12 bit images, with at least 75 intensity points taken for each cell. Anterior, cell body, and posterior designations were done blindly with equal numbers of values for each section so as not to bias the data. Intensity values were averaged for each section of the cell, and standard error of the mean was calculated. We used an analysis-of-variance to compare each data set to determine if there was a significant difference between the anterior, middle, and posterior of adherent and suspended cells.

#### Supplementary Material

Supplementary figures, movies, and tables, as indicated in the text, are available with this article online at <http://images.cellpress.com/supmat/supmatin.htm>.

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