

A Role for Cdc42 in Macrophage Chemotaxis

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Abstract. Three members of the Rho family, Cdc42, Rac, and Rho are known to regulate the organization of actin-based cytoskeletal structures. In Bac1.2F5 macrophages, we have shown that Rho regulates cell contraction, whereas Rac and Cdc42 regulate the formation of lamellipodia and filopodia, respectively. We have now tested the roles of Cdc42, Rac, and Rho in colony stimulating factor-1 (CSF-1)-induced macrophage migration and chemotaxis using the Dunn chemotaxis chamber. Microinjection of constitutively activated RhoA, Rac1, or Cdc42 inhibited cell migration, presumably because the cells were unable to polarize significantly in response to CSF-1. Both Rho and

Rac were required for CSF-1-induced migration, since migration speed was reduced to background levels in cells injected with C3 transferase, an inhibitor of Rho, or with the dominant-negative Rac mutant, N17Rac1. In contrast, cells injected with the dominant-negative Cdc42 mutant, N17Cdc42, were able to migrate but did not polarize in the direction of the gradient, and chemotaxis towards CSF-1 was abolished.

We conclude that Rho and Rac are required for the process of cell migration, whereas Cdc42 is required for cells to respond to a gradient of CSF-1 but is not essential for cell locomotion.

WHEN tissue is injured or becomes locally infected, an inflammatory reaction ensues. There are three principal components of an inflammatory response: (a) increased blood flow, (b) increased capillary permeability, and (c) increased migration of leukocytes into the affected area. Inflammation is characterized by the orderly recruitment and deployment of leukocytes at the focus of tissue necrosis, and the cell types seen in different inflammatory foci vary widely depending on the nature of the stimulus. Acute inflammatory foci contain mostly neutrophils, whereas sites of chronic inflammation and persistent tissue necrosis contain higher proportions of macrophages (Bevilacqua et al., 1994). Movement of cells into sites of inflammation depends initially upon the interactions between leukocytes and endothelia (Dustin et al., 1989; Tedder et al., 1995) and is then largely directed by the process of chemotaxis (Downey, 1994). Mediated by the coordinated activation of the cytoskeleton and associated adhesive integrins (Howard and Watts, 1994; Bokoch et al., 1996; Laudanna et al., 1996), leukocytes will migrate within an extracellular matrix towards a high

point of a concentration gradient of chemoattractant. Leukocytes are extremely sensitive to variations in the concentrations of chemotactic factors, and may detect a difference of as little as 1% across a cell diameter (Zigmond et al., 1981). A variety of products present at the site of inflammation can act as chemoattractants including bacterial products such as formylmethionyl peptides, C5a, platelet activating factor, and leukotriene B4 (for review see Murphy, 1994). In addition, chemokines (Schall and Bacon, 1994) and cytokines (Van Damme, 1991) released by cells at foci of tissue damage are being recognized as important mediators of inflammation due to their proposed ability to enhance the migration of specific subsets of leukocytes.

The actin cytoskeleton is believed to provide both the protrusive and contractile forces required for cell migration, via a combination of actin polymerization, depolymerization, actin filament cross-linking, and the interaction of myosin-based motors with actin filaments (Condeelis, 1993; Stossel, 1993; Howard and Watts, 1994; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). The regulation of these activities within cells has recently become focused on the Rho family of small GTPases. In Swiss 3T3 fibroblasts, Rho regulates the assembly of actin stress fibers and focal adhesion sites, whereas Rac regulates membrane ruffling and the production of protrusive lamellipodia and Cdc42 induces the formation of filopodia (Tapon and Hall, 1997). Rac and Cdc42 also mediate the formation of small focal complexes in fibroblasts that are

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distinct from the Rho-induced focal adhesions (Hotchin and Hall, 1995; Nobes and Hall, 1995).

The roles of Rho, Rac, and Cdc42 in regulating cytoskeletal organization have subsequently been investigated in a variety of other cell types, including leukocytes (for review see Dharmawardhane and Bokoch, 1997). Rho has been reported to regulate leukocyte actin reorganization (Bengtsson et al., 1990; Koch et al., 1994; Ehrengreber et al., 1995) and cell motility (Stasia et al., 1991; Laudanna et al., 1996). We have characterized the roles of Rho, Rac, and Cdc42 in macrophages stimulated with CSF-1, which activates the CSF-1 receptor (CSF-1R),¹ a receptor tyrosine kinase encoded by the protooncogene *c-fms* (Allen et al., 1997; Rohrschneider et al., 1997). As with 3T3 fibroblasts, Cdc42 mediates the formation of filopodia whereas Rac induces lamellar extension and the formation of ruffles. Rho is activated downstream of Rac and induces cell contraction. Our observations on the effects of Cdc42, Rac, and Rho in this motile cell type are consistent with an involvement of these proteins in regulating cell migration during chemotaxis. To study the roles of Rho family proteins in cell migration, we have exploited our previous findings (Webb et al., 1996) of a strong chemoattractive response of Bacl1 macrophages to CSF-1. Macrophage/monocyte chemotaxis is an important parameter in tissue inflammation (Bevilacqua et al., 1994), pregnancy (Wood et al., 1997), and tumour progression (Kacinski, 1997), and a role for CSF-1 in these phenomena is well documented (for review see references above).

To study chemotaxis, most investigators use some variant of the Boyden chamber, which assesses the proportion of a cell population that migrates toward a source of chemoattractant by a defined time point (Wilkinson and Allan, 1993). Using this approach, studying the role of a specific protein in regulating chemotaxis usually involves the derivation of cell lines overexpressing or expressing mutant forms of the protein. Because Rho family proteins are known to regulate long-term changes in cells such as gene expression in addition to their rapid effects on cytoskeletal organization (Hill et al., 1995; Ridley, 1996), we wished to look at immediate effects of activating or inhibiting these proteins rather than long-term effects that would contribute to the properties of cell lines. We have, therefore, microinjected cells with recombinant proteins and analyzed their migration using the Dunn chemotaxis chamber (Zicha et al., 1991). This device allows the migration of microinjected cells to be directly monitored, analyzed for migration rate, persistence, and directionality, and then compared with uninjected neighboring macrophages. It also provides more stable and linear concentration gradients of chemoattractant than can be achieved with Boyden chambers (Webb et al., 1996; Zicha et al., 1997a) with a half-life greater than both the time course of our assays and the half-life of microinjected Rho proteins. Here we report the use of this apparatus to analyze the roles of Rho, Rac, and Cdc42 in the process of chemotaxis-induced macrophage migration.

1. *Abbreviations used in this paper:* cAR1, cAMP chemoattractant receptor; CSF-1R, colony stimulating factor-1 receptor; fMLP, f-Meu-Leu-Phe; GDP, guanosine diphosphate; IL, interleukin; WASp, Wiscott-Aldrich syndrome protein.

Materials and Methods

Cell Culture

The cells used in these experiments were a subclone of the cloned mouse cell line Bacl1.2F5 (Morgan et al., 1987). Unless otherwise stated, all media and supplements were obtained from ICN Flow Laboratories (High Wycombe, UK). Cells were maintained in growth medium consisting of DME supplemented with 10% (vol/vol) FCS (Globepharm, Esher, UK), 1.32 nM CSF-1 (Chiron Corp., Emeryville, CA), 2 mM L-glutamine, 0.15 mM L-asparagine, 15 nM β -mercaptoethanol, 77.5 U/ml streptomycin and 25 U/ml penicillin. Cells were grown at 37°C in T25 tissue culture flasks (Falcon Plastics, Cockeysville, MD) maintained in a humid atmosphere of 5% CO₂. Cells were subcultured twice weekly by dissociation with 0.04% EDTA in PBS and resuspended in growth medium at 2×10^5 cells/ml. To obtain quiescent cultures, cells were maintained for 24 h in medium as above, but lacked CSF-1. Quiescent Bacl1 macrophages were restimulated by the addition of 1.32 nM CSF-1 after 24 h in the absence of CSF-1.

Expression and Purification of Recombinant Proteins

V14RhoA, V12Rac1, V12A35Rac1, N17Rac1, V12Cdc42, N17Cdc42, and C3 transferase were expressed in *Escherichia coli* as glutathione-S-transferase-fusion proteins and purified essentially as described (Ridley and Hall, 1992). Active protein concentrations for GTP-binding proteins were determined by a filter binding assay using [³H]guanosine diphosphate (GDP) (Hall and Self, 1986). As previously reported, N17Cdc42 and N17Rac1 were found to have a low affinity for [³H]GDP (Self and Hall, 1995), compared with their concentrations as determined by gel electrophoresis. The protein concentrations for N17Rac1 and N17Cdc42 were therefore estimated using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). The protein concentration of C3 transferase was also determined by this method.

Microinjection

To obtain cells for microinjection, 2×10^4 cells/well were seeded in 25-mm-diam Petri dishes containing 18-mm square No. 3 glass coverslips (Chance-Propper, Smethwick, UK). For microinjection, coverslips were marked with a glass pen or indelible marker on the reverse side, to facilitate the localization of microinjected cells (see Fig. 1). For analyses of cell migration, cells were microinjected 48 h after seeding. To obtain quiescent, cytokine-starved cells for microinjection, cells were seeded as above and maintained in growth medium for 24 h, followed by 24 h in medium without CSF-1. V12Rac1, V12Cdc42, and V14RhoA proteins were injected at between 200 and 300 μ g/ml (determined by [³H]GDP binding), C3 transferase was injected at ~ 2 μ g/ml, and then N17Rac1 and N17Cdc42 were injected at ~ 0.75 mg/ml (total protein concentration, determined by Bradford assay). To identify microinjected cells, rabbit IgG (Sigma Chemical Co., St. Louis, MO) at 0.5 mg/ml was microinjected together with the recombinant proteins. Cells were incubated for 20 min after microinjection before being used in chemotaxis assays.

Immunofluorescence

For immunofluorescence studies, cells were fixed in 4% formaldehyde in PBS containing 3% sucrose (wt/vol) for 20 min at room temperature. Cells were then permeabilized with 0.5% Triton X-100 in PBS containing 1% BSA for 5 min. For localization of actin filaments, cells were incubated with 0.1 μ g/ml TRITC-labeled phalloidin (Sigma Chemical Co.) for 60 min. For localization of CSF-1R, cells were incubated for 60 min at room temperature with a 1:80 dilution of rat anti-CSF-1R antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in a PBS/1% BSA blocking buffer. This was followed by a second fixation in 4% formaldehyde for 5 min before incubation for 1 h with 1:50 FITC-conjugated goat anti-rat IgG (Caltag, Burlingame, CA) in blocking buffer. Injected rabbit IgG was localized with FITC-conjugated goat anti-rabbit IgG (1:400, Sigma Chemical Co.). Images of cells were obtained using confocal laser-scanning microscopes (either model MRC-500; Bio-Rad Laboratories, Inc. or model TCS NT; Leica, Inc., St. Gallen, Switzerland).

Chemotaxis Assays

Recording and Tracking Cell Movement. Chemotaxis was assessed by direct observation and recording of cell behavior in stable concentration

gradients of CSF-1 using the Dunn chemotaxis chamber (Weber Scientific International Ltd., Teddington, UK). This apparatus permits the directions of migration of individual cells to be measured in relation to the direction of the gradient and the time course of the response to be followed (Zicha et al., 1991, 1997b). Details of the construction and calibration of the apparatus are given in Zicha et al. (1991) and Webb et al. (1996). To set up gradient experiments, both concentric wells of the chemotaxis chamber were filled with CSF-1-free medium and a coverslip seeded with cells was inverted onto the chamber in an offset position leaving a narrow slit at one edge for refilling the outer well (see Fig. 1). After firmly seating the coverslip, it was sealed into position using hot dental wax with the marked region laying over the annular bridge. The medium was next drained from the outer well and replaced with medium containing 1.32 nM CSF-1 and the slit was finally sealed with the hot wax.

Recordings of cell migration typically began within 30 min of assembling the chamber by which time a linear diffusion gradient had been established (Webb et al., 1996). One part of the bridge region with usually 10–15 cells (or in the case of microinjection experiments that part corresponding to the marked region, see Fig. 1 b) was selected. Images of the cells were digitally recorded onto a computer hard disk at a time-lapse interval of 4 min for 3 h using an inverted microscope (model IMT-2 equipped with 10× phase-contrast objective; Olympus, Tokyo, Japan). In microinjection experiments an area of uninjected control cells was also simultaneously recorded. Microscopic images were acquired from a charge-coupled device camera (model TM-765; Pulnix Europe Ltd., Basingstoke, UK) and captured by a Matrox Magic video capture card (Dorval, Quebec, Canada) fitted to an Elonex PC (model 486DX2-66; London, UK). The processed sequences were displayed rapidly as a movie and an interactive tracking of cells with a superimposed mouse pointer resulted in the generation of cell trajectories, each consisting of a sequence of (x_t , y_t) position coordinates obtained from 45 (3 h at 4-min lapse intervals) consecutive images of a single cell. Cell trajectories were then plotted as scatter diagrams showing cell tracks and also as diagrams that displayed the final position of each cell after 3 h of migration, where its origin is fixed at the (0, 0) coordinate. Calculations for rates of cell migration were also derived using the same data set, with individual cell speed being calculated for each of the 45 consecutive images. The mean cell speed and 95% confidence interval was then calculated for each lapse interval, and an overall figure derived for the 3-h period.

At the end of each migration assay, the coverslip was carefully removed from the Dunn chamber and the cells were processed for immunolocalization of a coinjected marker protein, rabbit IgG, as previously described (Allen et al., 1997). This procedure served to confirm the identity of all the tracked cells.

Direction Plots. Statistical analysis of directional data was used for testing the directionality of the chemotactic response (Dunn and Zicha, 1995; Zicha et al., 1997a). Briefly, each cell trajectory was converted to a single angle representing the direction from the starting point of the trajectory to the point at which it first crossed a virtual horizon set at 10 μ m. These data were summarized in a direction plot that is a circular histogram showing the number of cell directions lying within each 20° interval. The Rayleigh test for unimodal clustering of directions (Mardia, 1972) was then applied to the data and a $P < 0.01$ was chosen as the criteria for rejecting the null hypothesis of random directionality. Where there was significant unimodal clustering, we calculated the mean direction and its 95% confidence interval.

All analyses were carried out using Mathematica™ 2.2 for Windows or Mathematica™ 3.0 for Windows 95 (Wolfram Research Inc., Champaign, IL) with appropriate macros (Notebooks) written by D. Zicha.

Results

CSF-1 Stimulates Cell Motility and Chemotaxis

Bac1.2F5 macrophages are known to be completely dependent on CSF-1 for their ability to migrate and CSF-1 is a potent chemoattractant (Boocock et al., 1989; Webb et al., 1996; Allen et al., 1997). As previously reported, cells starved of CSF-1 for 24 h were rounded and exhibited very little motile behavior or translocation (Fig. 2 a and see Fig. 4 a). In comparison, when the cells were restimulated with CSF-1 they became rapidly polarized (Webb et al., 1996;

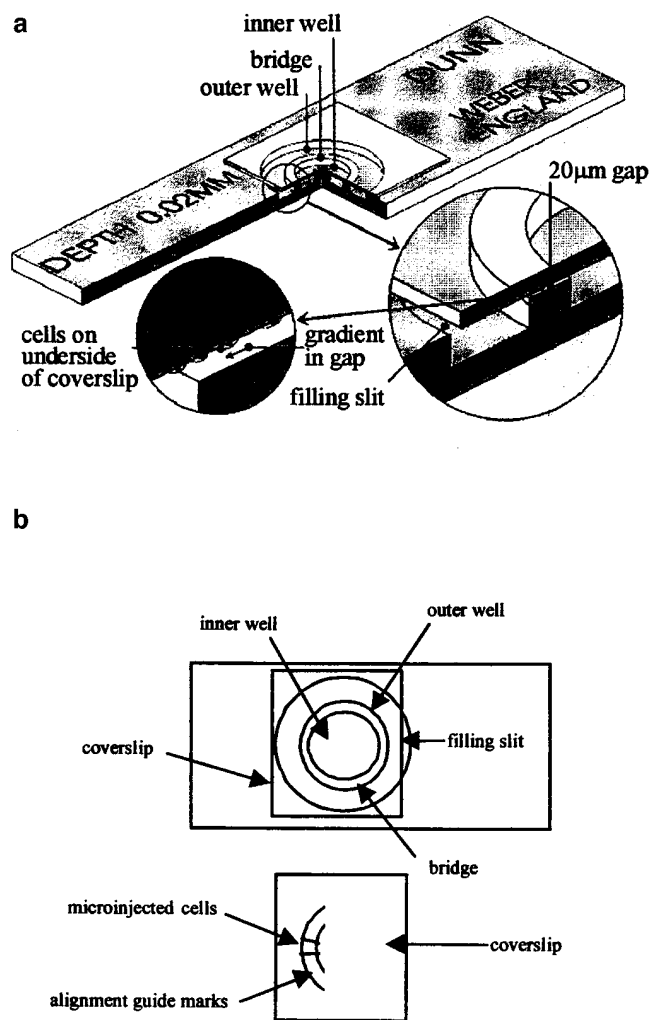


Figure 1. Microinjection and gradient formation in the Dunn chemotaxis chamber. (a) The Dunn chamber is a modified Helber counting chamber slide. Cells are cultured on coverslips that are then inverted onto the slide. Cells that rest over the annular bridge of the chamber can be observed under phase-contrast optics and their migration tracks are recorded automatically by time-lapse frame grabbing. (b) To measure microinjected cells, guidemarks are drawn on the coverslip to mark the limits of the Dunn chamber annular bridge and a small quadrant demarcated within a section of the alignment marks. All the cells within this quadrant are microinjected before mounting the coverslip on the chamber. Measurements of cell migration are taken for all the cells within the quadrant and a number of uninjected cells lying outside the quadrant. The latter measurements serve as internal controls.

data not shown). In the case of cells placed in a gradient of CSF-1, they became even more polarized along the axis of the CSF-1 diffusion gradient (Webb et al., 1996), and showed strong positive chemotaxis toward the source of CSF-1 (Fig. 2 b and see Fig. 4 a; Webb et al., 1996).

To test whether the process of microinjection altered the migratory behavior of Bac1 cells, control experiments were carried out with inactive proteins. Both rabbit IgG and an inactive Rac1 protein (V12A35Rac1) were microinjected into CSF-1-starved cells that were then exposed to a gradient of CSF-1. It was found that neither the micro-

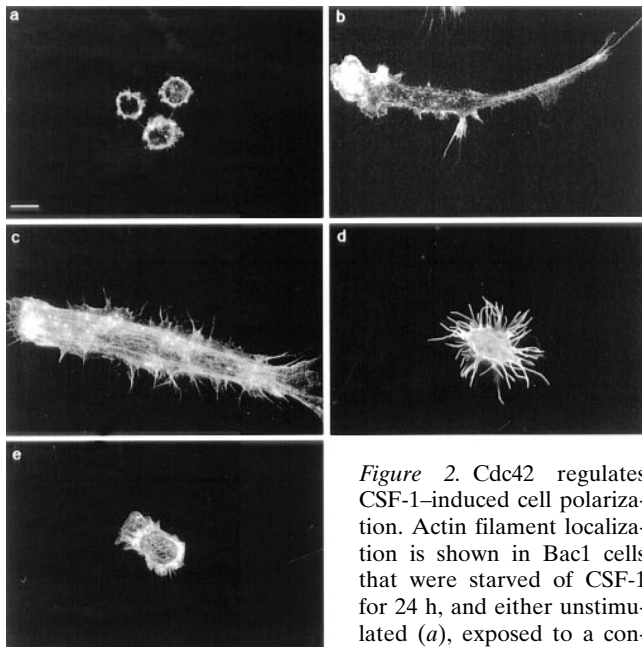


Figure 2. Cdc42 regulates CSF-1-induced cell polarization. Actin filament localization is shown in Bac1 cells that were starved of CSF-1 for 24 h, and either unstimulated (a), exposed to a concentration gradient of CSF-1 for 2 h (b), or microinjected with V12A35Rac1 (c), V12Cdc42 (d), or N17Cdc42 (e) followed by exposure for 2 h to a CSF-1 concentration gradient. Bar, 10 μm .

for 2 h (b), or microinjected with V12A35Rac1 (c), V12Cdc42 (d), or N17Cdc42 (e) followed by exposure for 2 h to a CSF-1 concentration gradient. Bar, 10 μm .

injection procedure nor introduction of the inactive Rac1 protein had any effect on cell morphology or actin organization, and the injected macrophages polarized in parallel with the alignment of the CSF-1 gradient (Fig. 2 c). In addition, microinjection did not alter the ability of the cells to show a chemotactic response to CSF-1, as the Rayleigh test demonstrated highly significant unimodal clustering of cell trajectories in the direction of the source of the chemoattractant (Fig. 3, a and b). Microinjection of rabbit IgG or V12A35Rac1 did lead to a reduction in the speed of cell locomotion, however (Fig. 4 a). This was not due to the proteins or contaminants in the protein preparations, since a similar reduction in cell speed was observed after microinjection of buffer alone (data not shown).

Cdc42 Is Required for Bac1 Cell Chemotaxis but Not for Migration

Microinjection of the constitutively active mutant of Cdc42 (V12Cdc42) into CSF-1-starved Bac1 cells significantly reduced the speed of cell migration in a concentration gradient of CSF-1 when compared with control V12A35Rac1-injected cells (Fig. 4 a). Cell morphology was grossly altered, with V12Cdc42 stimulating the production of numerous filopodia around the cell margin (refer to Fig. 2 d). The production of radial arrays of filopodia correlated with a failure of the microinjected cells to polarize along the CSF-1 gradient. Chemotaxis was also abrogated as shown by the lack of significant unimodal clustering of the cell trajectory data (Fig. 3, c and d).

Surprisingly, cells microinjected with the dominant inhibitory mutant of Cdc42 (N17Cdc42) showed a significant increase in migration speed when compared with control microinjected cells (Fig. 3 e and Fig. 4 a). This increase in

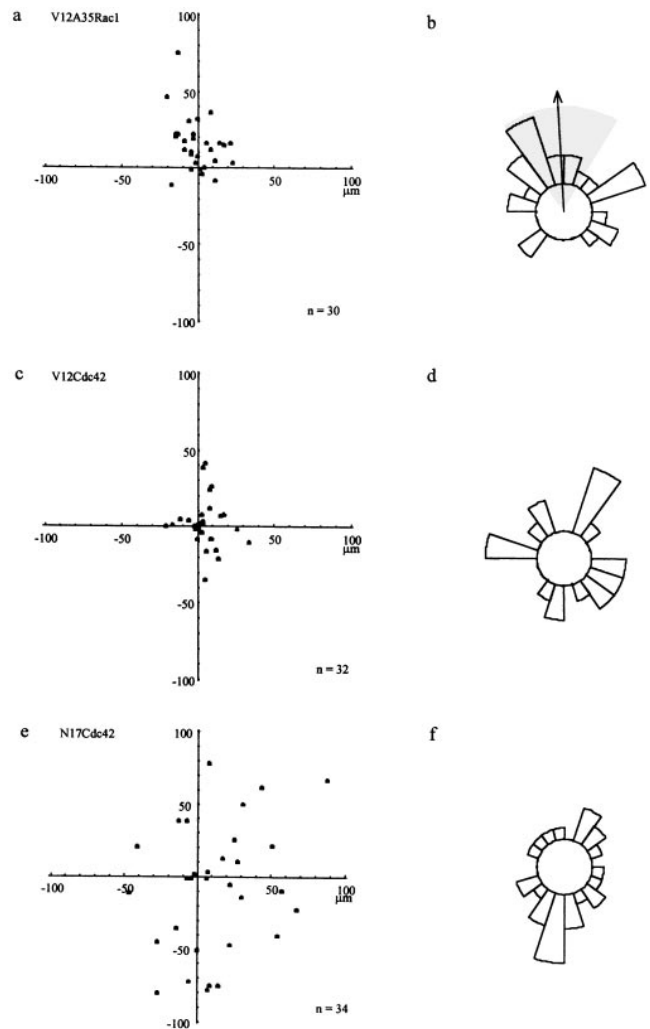


Figure 3. Cdc42 is required for CSF-1-induced chemotaxis but not migration. Bac1 macrophages were starved of CSF-1 for 24 h, microinjected with V12A35Rac1 (a and b), V12Cdc42 (c and d) or N17Cdc42 (e and f) and then exposed to a concentration gradient of CSF-1 for 3 h in a Dunn chamber. The migration tracks for the indicated number of cells were plotted as described in Materials and Methods. The final positions of these cells are indicated, taking the starting point for each cell as the intersection between X and Y axes, and the source of CSF-1 at the top (a, c, and e). Circular histograms (b, d, and f) show the proportion of cells with a direction of migration lying within each 20° interval, again with the source of CSF-1 at the top. Cells that migrated <10 μm are excluded from this analysis. (b) Arrow, mean direction of cell migration; grey segment, 95% confidence interval. In V12Cdc42- and N17Cdc42-injected cells (d and f), no significant directionality of migration was observed.

cell speed is obvious from even the earliest measured time intervals. The Mathematica-generated mean cell speeds for the first four intervals are calculated as 38.25, 35.76, 34.40, and 31.65 $\mu\text{m}/\text{h}$, respectively. Examination of the cell tracks generated by injected cells shows that the pattern of migration is not disturbed so the results shown here are not due to any abnormal turning behavior (Fig. 4, b and c). This increase could be a result of the morphology assumed by N17Cdc42-injected cells. In a gradient of CSF-1,

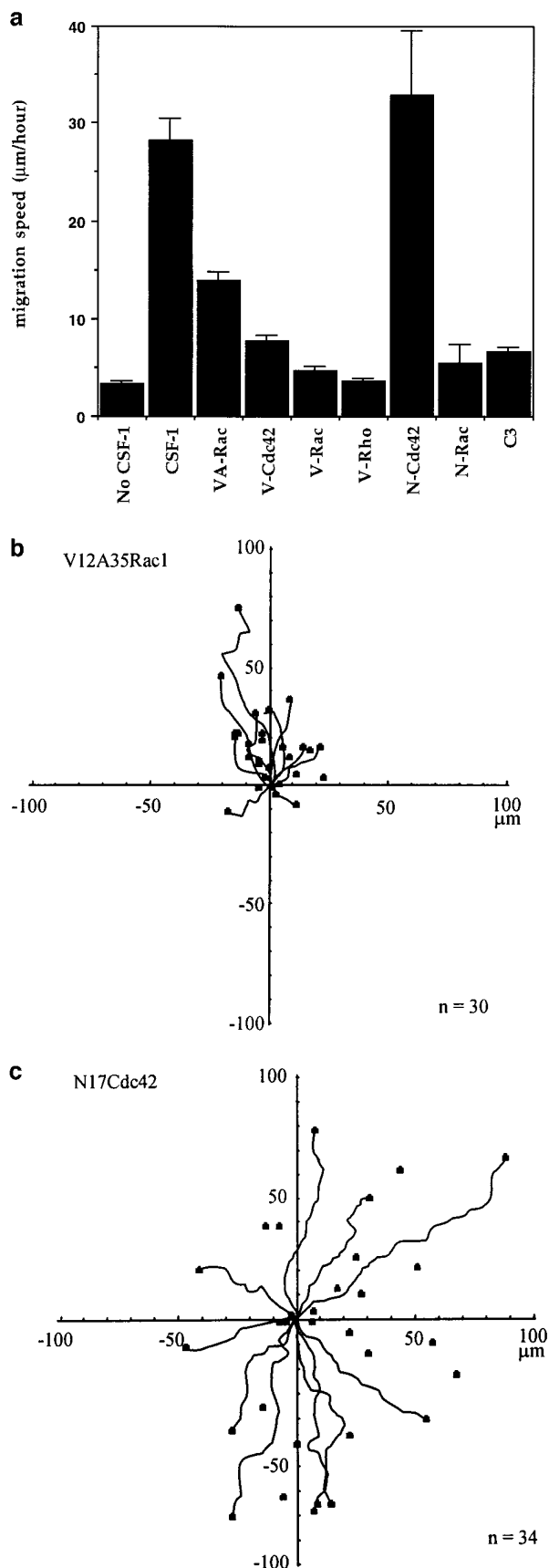


Figure 4. Effects of Rho, Rac, and Cdc42 proteins on the migration speed of macrophages. *Bac1* macrophages were starved of CSF-1 for 24 h, microinjected with the indicated proteins, and

the N17Cdc42-injected macrophages had a rounded, weakly polarized morphology with broad leading lamellae and truncated tails (refer to Fig. 2 *e*) compared with control cells (refer to Fig. 2, *b* and *c*). The lamellae of migrating N17Cdc42-injected cells also showed a great deal of expansion and ruffling activity as judged through observation of the digital recordings and, as expected, there was no evidence of filopodia (data not shown). Despite their polarized morphology and active migration, these macrophages did not sense the chemotactic gradient of CSF-1 as can be seen from Fig. 3 *f* and Fig. 4 *c* that demonstrate cell tracks were randomly dispersed and no unimodal clustering of trajectories could be discerned using the Rayleigh test. A Mathematica notebook was written to analyze the directional bias of cell migration at each of the 45 consecutive frames within an experiment. Analyses of the data for N17Cdc42-injected cells show clearly that the gradient component of the cell velocity never becomes biased in the direction of increasing CSF-1 concentration, even at the beginning of the experiment. In contrast, control-injected cells (V12A35Rac1-injected) showed upgradient velocity from the first reading onwards (ΔY values of $+3.8 \mu\text{m/h}$ at the first reading and $+7.2$, 8.7 , and $8.4 \mu\text{m/h}$, respectively, at the following three recording intervals. Similarly, visual observation of recordings and the Rayleigh analysis using a horizon of just greater than one cell diameter ($10 \mu\text{m}$) confirmed that N17Cdc42-injected cells never show preferential directional orientation with reference to the cytokine gradient: this analysis is independent of time (compare Fig. 3, *f* with *b* [control]). Repeating the analysis with reduction of the horizon to any smaller value also fails to give a significant directionality aligned with the gradient (data not shown). Thus, N17Cdc42-injected cells fail to polarize in response to a CSF-1 concentration gradient even at the earliest time points, but rather do so in a random, stochastic manner. Cdc42 is therefore not required for migration and actually exerts a restraint on the speed of migration, but it is essential for detection of the CSF-1 gradient.

Rac Regulates Bac1 Cell Migration

Rac has previously been shown to regulate CSF-1-induced formation of lamellipodia and membrane ruffles in *Bac1* cells and to be required for the assembly of focal complexes (Allen et al., 1997). Microinjection of the constitutively active mutant V12Rac1 into CSF-1-starved cells led to the rapid development of radial arrays of lamellae around the cell margins with concomitant cell spreading (Fig. 5 *a*). No filopodia were observed, and although much lamellar ruffling was observed in microinjected cells, no true cell translocation ensued (Fig. 6, *a* and *b*). Titration of

then exposed to a gradient of CSF-1 for 3 h. The rate of cell locomotion is calculated for each cell at consecutive 4-min intervals for 3 h (*a*), with the mean and 95% confidence limit calculated using a Mathematica notebook. The migration tracks of 10 randomly chosen cells are plotted for V12A35Rac1-injected control cells (*b*), and N17Cdc42-injected cells (*c*) onto vector diagrams showing the final positions of all tracked cells. It is evident that N17Cdc42 does not disturb the linearity of cell migration observed in the control cells.

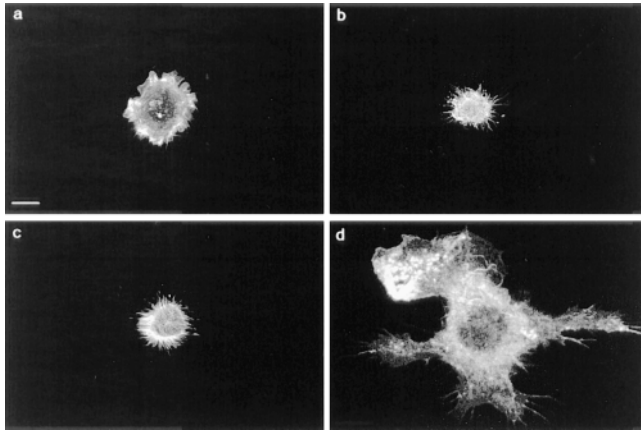


Figure 5. Effects of Rac1 and RhoA proteins on CSF-1-induced cell polarization. Bac1 macrophages were starved of CSF-1 for 24 h, microinjected with V12Rac1 (a), N17Rac1 (b), V14RhoA (c), or C3 transferase (d) and then exposed to a gradient of CSF-1 for 2 h in a Dunn chamber. Cells were fixed and stained to reveal actin filaments. Bar, 10 μm .

the V12Rac1 protein showed that when it was injected at 200 ng/ml or 100 ng/ml, cell spreading and ruffling was observed, and this response was diminished significantly at 50 ng/ml, indicating that this is the threshold concentration required for actin reorganization, similar to the threshold observed in Swiss 3T3 cells (Ridley, 1995). At no concentration of V12Rac1 was cell migration in the absence of CSF-1 stimulated (data not shown).

After 3 h in a gradient of CSF-1, the morphology of cells injected with V12Rac1 (200 ng/ml) appeared essentially similar to unstimulated cells injected with V12Rac1. The cells spread radially and ruffled, but failed to polarize or migrate (Fig. 4 a and Fig. 5 a). The effect of lower doses of V12Rac1 on cell polarity and migration was investigated in the presence of a CSF-1 gradient. At 100 ng/ml the response was similar to that at 200 ng/ml. At 50 ng/ml, the cells also spread radially, but a small proportion of these did eventually polarize weakly and migrate after 4–5 h. When a dose of 25 ng/ml was used, the pattern of cell behavior was similar, with the addition that by 5 h after injection, the weakly polarized cells began to clearly migrate up the gradient of CSF-1 (data not shown). Since the half-life of Rac1 proteins after microinjection is ~ 4 h (Ridley et al., 1992), it is likely that this migratory response at low concentrations of V12Rac1 is due to loss of the injected protein and subsequent reversion to the behavior of uninjected cells. At no concentration did V12Rac1 stimulate an increase in migration rate, either with or without CSF-1.

Cells injected with the dominant-negative mutant N17Rac1 (Fig. 5 b) also failed to polarize in a gradient of CSF-1. No lamellae were observed on injected cells, but a combination of retraction fibers and filopodia were continuously produced, the latter presumably due to the action of endogenous Cdc42. This behavior was reflected in the speed of cell movement, which was very similar to the values observed with CSF-1-starved cells (refer to Fig. 4 a). Thus, N17Rac1, which has been shown to inhibit CSF-1-induced formation of lamellipodia (Allen et al., 1997), led to an almost total inhibition of Bac1 migration even though the

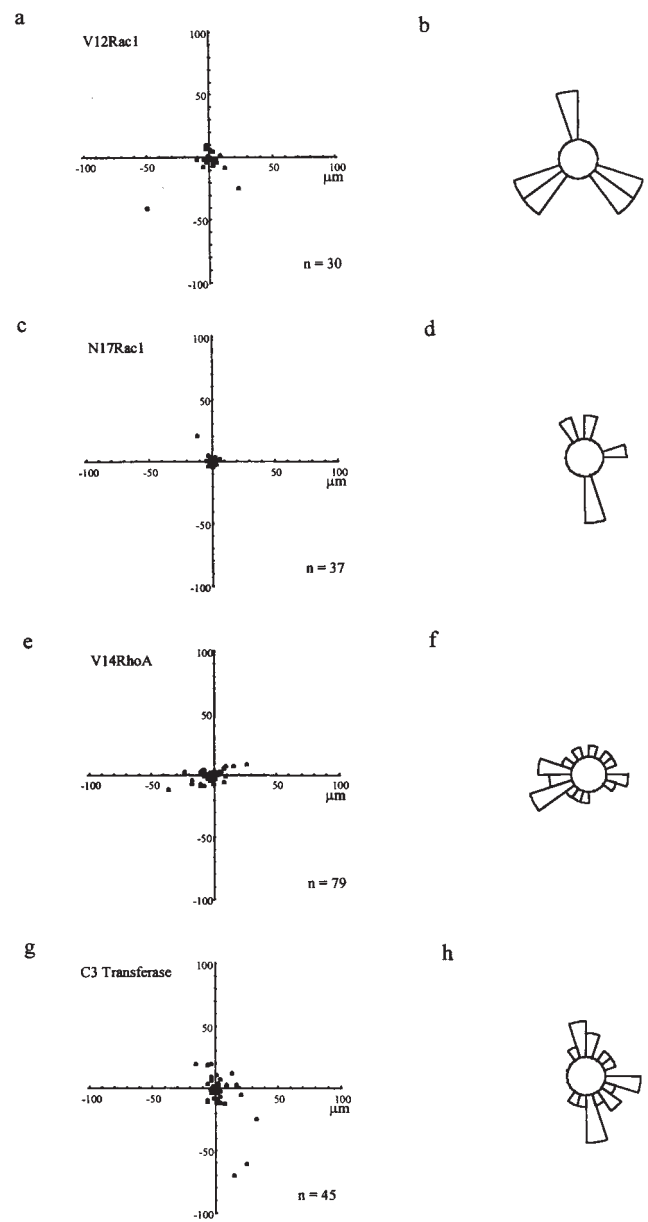


Figure 6. Rac and Rho are required for cell migration. Bac1 macrophages were starved of CSF-1 for 24 h, microinjected with V12Rac1 (a and b), N17Rac1 (c and d), V14RhoA (e and f), or C3 transferase (g and h) and then exposed to a gradient of CSF-1 for 3 h. The migration tracks for the indicated number of cells were plotted and the final positions of these cells are indicated with the starting point for each cell at the intersection between X and Y axes, with the source of CSF-1 at the top (a, c, e, and g). Circular histograms (b, d, f, and h) show the proportion of cells migrating in each direction, again with the source of CSF-1 at the top. A Rayleigh test on these data showed that there was no significant directionality of migration in any case.

microinjected cells displayed many filopodia (Fig. 5 b; Fig. 6, c and d).

Rho Is Required for Cell Migration

Rho is known to regulate the formation of stress fibers and focal adhesions in many adherent cultured cells, and this

may explain the ability of activated Rho to inhibit the migration of MDCK epithelial cells (Ridley et al., 1995). Bac1 macrophages, however, do not have stress fibers, and in these cells Rho induces cell contraction and rounding up rather than stress fiber formation (Allen et al., 1997). Similarly, Bac1 cells do not possess focal adhesions, and Rho is not required for the clustering of integrin-associated proteins (Allen et al., 1997). To determine the role of Rho in macrophage migration, Bac1 cells were injected with either a constitutively activated mutant protein, V14-RhoA, or with C3 transferase, a bacterial exoenzyme that ribosylates and inhibits the function of Rho proteins (von Eichel-Streiber et al., 1996). In a concentration gradient of CSF-1, V14RhoA-injected cells remained rounded and unpolarized, with many retraction fibers (Fig. 5 *c*). No filopodia, lamellipodia, or membrane ruffles were detected, suggesting that activated Rho acts antagonistically to Rac and Cdc42. Conversely, cells injected with C3 transferase extended filopodia and multiple lamellae in response to CSF-1, and the cells spread significantly more than control cells (Fig. 5 *d*), consistent with an enhancement of Rac- and/or Cdc42-mediated responses. V14RhoA abolished the chemotactic response of Bac1 cells to CSF-1 (Fig. 6, *e* and *f*) and reduced their mean speed of migration to that of CSF-1-starved cells (refer to Fig. 4 *a*), consistent with the inability of V14RhoA-injected cells to polarize. C3 transferase also reduced migration speed, but not to the degree observed with V14RhoA (refer to Fig. 4 *a*; Fig. 6, *g* and *h*). Some evidence of cell polarization was observed in C3-injected cells after 3 h in a gradient of CSF-1, but the net cell translocation was too limited to be measured as significant chemotactic behavior (Fig. 6, *g* and *h*). Increasing the time course of the chemotaxis assay to assess any delayed chemotactic response proved futile because C3 transferase-injected cells later assume a highly dendritic morphology (Allen et al., 1997). Taken together, these results suggest that Rho plays an important role in cell migration via its effects on cell contractility, and that Rho activity must be precisely regulated in order for cells to migrate effectively.

Localization of the CSF-1R in Migrating Bac1 Cells

The effects of CSF-1 on cells are mediated through its binding to a single class of high-affinity transmembrane tyrosine kinase receptor, CSF-1R (Sherr, 1990). In neutrophils, serpentine G protein-linked receptors have been reported to relocalize to the leading edge when stimulated by ligand binding in a chemotactic gradient, suggesting that cell polarization may involve receptor redistribution to the highest source of signal within a cell (Sullivan et al., 1984; McKay et al., 1991). To investigate whether CSF-1R becomes similarly redistributed, we have examined its localization on Bac1 cells. Unpolarized, CSF-1-starved Bac1 cells displayed a diffuse distribution of CSF-1R on the plasma membrane (Fig. 7 *a*). Within 2 min of CSF-1 addition, the diffuse staining was lost and CSF-1R became concentrated on cytoplasmic vesicles (Fig. 7 *b*). These data correlate with the observation based on binding studies using radioiodinated CSF-1 that CSF-1R is rapidly internalized after addition of CSF-1 (surface retention half-life of 1.6 min), leading to effective clearance of CSF-1 binding

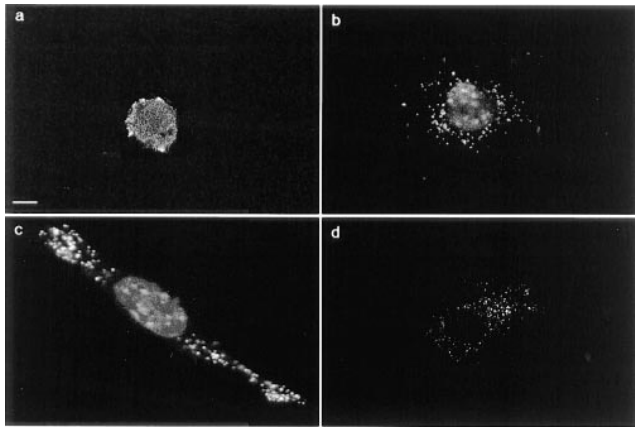


Figure 7. Localization of the CSF-1 receptor in migrating cells. The localization of CSF-1R (c-fms) is shown in CSF-1-starved Bac1 macrophages (*a*), in cells stimulated with CSF-1 for 2 min (*b*), in cells exposed to a concentration gradient of CSF-1 for 2 h (*c*), or in cells microinjected with N17Cdc42 and then exposed to a CSF-1 gradient for 2 h (*d*). Cells were starved of CSF-1 for 24 h before stimulation and then fixed and CSF-1R-localized with a rat anti-CSF-1R antibody followed by FITC-conjugated goat anti-rat IgG. Bar, 10 μ m.

sites from the cell surface for at least 30 min after CSF-1 addition (Li and Stanley, 1991). On the basis of past studies (Boocock et al., 1989), we conclude that CSF-1R is endocytosed after stimulation as a complex with bound CSF-1, which was also observed on cytoplasmic vesicles. In cells polarized along the axis of a CSF-1 concentration gradient, CSF-1R was mainly concentrated in cytoplasmic vesicles at the leading and trailing edges, but vesicles were also present throughout the cell body (Fig. 7 *c*). Although it was not possible to detect CSF-1R on the plasma membrane of CSF-1-stimulated cells, the distribution of CSF-1R-containing vesicles strongly suggests that the receptor is not relocalized to the leading edge of migrating macrophages. As Cdc42 was required for cells to migrate up a concentration gradient of CSF-1, it was possible that it might act by altering the distribution of CSF-1R. The lack of detectable surface CSF-1R in cells exposed to CSF-1 meant that it was not possible to determine whether it was present on filopodia. However, in cells microinjected with N17Cdc42 and subsequently exposed to a gradient of CSF-1, CSF-1R was still observed on vesicles throughout the cytoplasm (Fig. 7 *d*). In addition, a more diffuse distribution of CSF-1R was consistently detected in N17Cdc42-injected cells, suggesting that Cdc42 may directly or indirectly participate in the process of endocytosis.

Discussion

Leukocyte migration plays a critical role in inflammatory and immune responses, and thus there has been a considerable amount of effort directed towards elucidating the nature of the cellular motors (Howard and Watts, 1994; Zigmond, 1996), adhesive contacts (Hogg and Berlin, 1995; Lasky, 1995; Tedder et al., 1995), and migratory signals (Wilkinson, 1990; Murphy, 1994; Negus, 1996) that regulate leukocyte behavior. To migrate, cells have to be

come morphologically polarized in the direction of migration. This polarization is not dependent on the presence of a gradient of external stimulus and can occur stochastically (Lauffenburger and Horwitz, 1996). Alternatively, the direction of polarization can be driven by external cues, either matrix-bound or diffusible, which induce cell migration along a gradient of attractant or repellent. Examples of this behavior are seen in many developmental programs such as axonal outgrowth (Tessier-Lavigne and Goodman, 1996), neural crest cell migration (Perris, 1997), and myoblast migration (Bischoff, 1997), as well as in the chemotactic responses of leukocytes (Bokoch, 1995). Despite its importance, our understanding of how cells establish and maintain polarity during migration is rudimentary. We have used the SV-40 immortalized mouse macrophage cell line, Bacl.2F5, to study leukocyte migration because these cells resemble primary macrophages in many ways, including their requirements for CSF-1 for cellular viability and proliferation (Morgan et al., 1987). For this reason, these cells have been used by several groups investigating the role of CSF-1R and downstream cytoplasmic signaling molecules in regulating cell proliferation (Roussel et al., 1991; Roussel, 1997). In addition to stimulating mitogenesis, CSF-1 induces rapid morphological changes in these macrophages, including cell spreading, the production of F-actin-rich ruffles, and the formation of filopodia (Boockock et al., 1989; Allen et al., 1997). In a concentration gradient of CSF-1, the cells subsequently polarize and migrate towards the source of CSF-1 (Webb et al., 1996). Based upon our recent findings that Rho, Rac, and Cdc42 regulate CSF-1-induced actin reorganization (Allen et al., 1997), we have examined whether these proteins play a major role in determining the polarization and directional migration of cells.

We have found that microinjection of constitutively activated RhoA or Rac1 proteins reduces net cell translocation in a CSF-1 gradient to the levels observed with CSF-1-starved cells, and that activated Cdc42 also inhibits cell migration speed significantly. It has previously been reported that some cell lines overexpressing activated or wild-type Rac constructs show increased migration (Khosravi-Far et al., 1995; Hooshmand-Rad et al., 1997). This could well be a consequence of long-term changes in gene expression induced by Rac that have yet to be characterized. As far as we are aware, this communication is the first report on the short-term effects of Rac and Cdc42 on cell migration. With activated Rho, Rac, and Cdc42, the lack of cell migration can be attributed to the phenotype induced by the injected proteins. As we have shown before (Allen et al., 1997), V14RhoA induces cell rounding and contraction in macrophages, and these cells do not extend lamellae. V12Rac1 causes radial spreading of lamellae with active ruffling around the margins of injected cells, whereas V12Cdc42 stimulates the extension of filopodia in all directions, and subsequent cell flattening. These cells are not able to establish a polarized morphology in response to CSF-1, so it is not surprising that these differently acting GTPases have a very similar effect on migration since they lead to the generation of circular cell profiles. In addition, as Rac1 and Cdc42 have previously been shown to regulate the formation of adhesion complexes in Bacl macrophages (Allen et al., 1997), it is possi-

ble that activated Rac1 or Cdc42 enhance cell-substratum adhesion, which would also be expected to inhibit cell migration (Lauffenburger and Horwitz, 1996; Palecek et al., 1997).

The process of microinjection itself does not interfere with the chemotactic response of cells, although there is an effect on the speed of migration. The inactive V12A35Rac1 protein has no significant effect on cell polarization or directed migration but did reduce migration speed in a CSF-1 gradient by ~50% compared with uninjected cells. However, this reduced migration rate is still far greater than the limited movement of CSF-1-starved, uninjected macrophages. The reason why microinjection inhibits migration rate is unclear, but may possibly reflect changes in membrane organization and/or cell volume as a consequence of the microinjection process.

Inhibition of endogenous Rho or Rac proteins also prevents CSF-1-induced cell migration. In the presence of CSF-1, inhibition of Rho activity by C3 transferase initially leads to cell flattening and radial spreading, presumably reflecting the activities of endogenous Cdc42 and Rac (Allen et al., 1997). Within the time course of our chemotaxis experiments, microinjected cells take on a dendritic morphology elaborating several elongated cellular extensions each terminating in a small lamellipodium. It is apparent from observation of the time-lapse sequences that C3 transferase-injected cells are motile, showing active ruffling at the lamellar fronts, but net translocation is retarded since the cell bodies fail to follow after the ruffling lamellae (data not shown). As a consequence of this behavior, the cells do not establish a polarized morphology. Each extension seems capable of sustaining itself in competition with the remaining extensions on the cell, at least over the time-course of these experiments. This clearly differs from the normal response of Bacl macrophages to CSF-1, where the early circular spreading phase changes to a strongly polarized morphology within 10–15 min (Webb et al., 1996). Thus, it could be speculated that one function of Rho in these cells is to enforce the collapse (contraction) of “minor” lamellar extensions that would have the consequence of allowing a single large lamella to develop as the leading edge of a polarized cell. This implies that Rho is directly involved in the generation of cell polarity via its ability to stimulate contractility. A role for Rho in cell contractility has already been documented in Bacl macrophages, smooth muscle cells, and in other cell types (Brock et al., 1996; Chrzanowska-Wodnicka and Burridge, 1996; Allen et al., 1997), and may be mediated via Rho-kinase (also known as ROCK or ROK α) that can induce the phosphorylation of myosin light chain (Narumiya et al., 1997). This phosphorylation is hypothesized to lead to the bundling of actin and myosin filaments into contractile fibers (for reviews see Burridge and Chrzanowska-Wodnicka, 1996; Ridley, 1996). In addition to a role in the initial process of cell polarization, Rho may also be involved in the forward translocation of the cell body during cell migration, which is proposed to be driven by contraction of an actin-myosin network (Lauffenburger and Horwitz, 1996; Svitkina et al., 1997).

Like C3 transferase, microinjection of the dominant-negative N17Rac1 protein reduces cell migration speed in a CSF-1 gradient to a level that is not statistically different

from CSF-1-starved macrophages. The morphology of N17Rac1-injected cells is characterized by a failure to produce any lamellae or membrane ruffles as would be predicted from the known function of Rac (Ridley et al., 1992; Allen et al., 1997). Over the 3-h period of exposure to a CSF-1 gradient, no cell spreading was observed other than that induced by the extension of filopodia. As the forward protrusion of lamellae is believed to be an essential step in cell locomotion (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996), the lack of lamellae in N17Rac1-injected cells correlates well with the inhibition of locomotion. In the absence of lamellar extension, the cells are clearly unable to become morphologically polarized in response to the CSF-1 gradient.

In contrast to the effects of inhibiting Rac and Rho, N17Cdc42 does not inhibit cell migration, and in fact N17Cdc42-injected cells migrate in a CSF-1 gradient at almost twice the speed of control V12A35Rac1-injected cells. The loss of filopodia in N17Cdc42-injected cells is accompanied by a marked reduction in the degree of cell polarity and a broadening of the leading lamellae of migrating cells. This increase in lamellar size could account for the enhanced migration rate of these cells, and may be a result of losing a restriction on lamellar spreading normally imposed by filopodia. Alternatively, as we have previously shown that in N17Cdc42-injected cells focal adhesion complexes containing $\beta 1$ integrin become dispersed (Allen et al., 1997), it is possible that the increase in migration speed reflects a decrease in cell adhesion to the substratum, since the rate of cell migration is critically dependent on the strength of adhesions (Lauffenburger and Horwitz, 1996; Palecek et al., 1997). A decrease in cell-substratum adhesions could also explain the more rounded morphology of these cells.

Despite retaining the capacity to migrate, N17Cdc42-injected macrophages completely lose the ability to move towards a source of CSF-1, strongly suggesting that filopodia have a significant role to play in gradient perception. Our earlier work on the proinflammatory cytokine tumor necrosis factor- α (TNF- α) has shown that it can abrogate the chemotactic response induced by CSF-1 without inhibiting cell migration (Webb et al., 1996). We have found that TNF- α can suppress the formation of filopodia in Bac1 macrophages (Peppelenbosch, M., G.E. Jones, and A.J. Ridley, unpublished data), an observation that again supports a role for filopodia in the sensing of chemotactic gradients. It may be that filopodia extended in response to chemoattractants act as a scaffold for signal sensors such as the CSF-1R. As we have described earlier, Bac1 macrophages initially respond to CSF-1 stimulation by rapid, radial spreading mediated by lamellae containing filopodia (Boocock et al., 1989), and this response is also observed when CSF-1 is supplied in a concentration gradient (Webb et al., 1996). Filopodia initially extend within 2–3 min as a star-shaped array around the cell margin. This time course coincides with the kinetics of CSF-1 binding to its receptor and subsequent internalization (Boocock et al., 1989; Li and Stanley, 1991). This rapid internalization of CSF-1R, also demonstrated in this paper by immunolocalization, makes it unlikely that CSF-1R act as continuous surface-located sensors of a gradient. Indeed, once a gradient of CSF-1 is perceived, further sensing may not be in-

involved, since a Bac1 cell that initially lies directly behind another cell appears to sense the gradient incorrectly and moves away from the source of CSF-1 even after it is far away from the blocking cell (Webb et al., 1996). It is more likely that the initial receptor activation is sufficient to generate cell polarization. The rapid extension of filopodia will increase the effective diameter of CSF-1-stimulated cells, and may thereby enhance the difference in CSF-1 concentration detected across each cell. The filopodia also have the potential to carry considerable numbers of CSF-1R because of their large surface area, so that once the filopodia retract into the cell body and the ligand-receptor complexes are internalized, a concentrated zone of activated receptor may be created that may still be active some time after being endocytosed (Baass et al., 1995). A cell then senses the higher signal intensity generated on its upgradient side and reorganizes the cytoskeleton accordingly, so that it becomes polarized along the axis of the CSF-1 gradient. Cdc42, in stimulating the formation of filopodia, thus initiates gradient detection and cell polarization.

In this model, cells without filopodia will fail to detect a gradient of chemotactic agent and subsequently fail to polarize along the axis of the gradient. Macrophages stimulated by isotropic CSF-1 are significantly less polarized than those stimulated under gradient conditions despite having normal rates of migration (Webb et al., 1996). In the present study we have found that N17Cdc42-injected cells have broader lamellae and the cells are markedly less polarized. Their morphology is therefore reminiscent of normal cells stimulated by isotropic CSF-1, where cell polarization can be explained by the stochastic processes leading to lamellar growth in one direction (Lauffenburger and Horwitz, 1996).

In contrast to the rapid internalization of CSF-1R, the f-Met-Leu-Phe (fMLP) receptor is not significantly internalized after stimulation of neutrophils with fMLP and has in fact been reported to accumulate at leading edge of migrating neutrophils (McKay et al., 1991) or to be depleted from the tail (Sullivan et al., 1984). The localization of the cAMP chemoattractant receptor (cAR1) of *Dictyostelium discoideum*, however, is not altered during chemotaxis and remains evenly distributed on the plasma membrane (Xiao et al., 1997). Similarly, the chemoattractant receptors C5a and interleukin (IL)-8 also remain dispersed on the surface of activated human blood neutrophils (Gray et al., 1997). Although the internalization of the CSF-1R has precluded analysis of its plasma membrane distribution during chemotaxis, the localization of the internalized vesicles throughout the cytoplasm suggests that it is not concentrated at the leading edge. The fact that fMLP, cAR1, C5a, and IL-8 receptors remain on the plasma membrane indicates that, unlike the CSF-1R, they can act as continuous sensing devices for detection of a concentration gradient, and indeed *Dictyostelium* rapidly repolarize in response to changes in the direction of cAMP delivery (Devreotes and Zigmond, 1988). The fMLP, C5a, and IL-8 receptors and cAR1 all belong to the class of serpentine G protein-coupled receptors, whereas the CSF-1R, *c-fms*, is a tyrosine kinase receptor. It is possible, therefore, that the mechanism of gradient perception differs depending on the class of ligand receptors and/or type of cell.

Cdc42 has also been implicated in generating cell polarity in other systems (for review see Drubin and Nelson, 1996). Cdc42 was first characterized in *S. cerevisiae*, where it was isolated as a cell cycle mutant defective in budding (Johnson and Pringle, 1990). Cdc42 is essential for polarization of the actin cytoskeleton in response to bud site selection, and is also a component of the signaling pathway leading to the polarization of cells towards a mating partner (Chant, 1996). Similarly, in the wing-disc epithelium of *Drosophila*, Cdc42 is required for epithelial cell elongation (Eaton and Simons, 1995) and hair outgrowth on the wing (Eaton et al., 1996). A further example is seen in T cells, where Cdc42 is required for the polarization of the cytoskeleton towards antigen-presenting cells (Stowers et al., 1995).

The molecular mechanism whereby Cdc42 generates a polarized phenotype in response to a chemoattractant awaits further study, but is likely to involve one or more of the downstream targets for Cdc42 that have recently been implicated in mediating its effects on the actin cytoskeleton (Van Aelst and D'Souza-Schorey, 1997). Potential candidates include members of the PAK family of protein kinases, which are targets for Cdc42 and Rac. An increase in filopodia has been observed in cells injected with PAK1 protein (Sells et al., 1997), and in *S. cerevisiae* the PAK homologue Ste20 is thought to act on the heavy chains of the myosin I homologues Myo3 and Myo5 (Goodson et al., 1996). Another effector of Cdc42 of particular significance in relation to our work on macrophages is the Wiskott-Aldrich syndrome protein (WASp), which induces the formation of Cdc42-dependent actin clusters in several cell types (Aspenstrom et al., 1996; Kolluri et al., 1996; Symons et al., 1996). Patients suffering from this rare X-linked disorder have been shown to be deficient in T cell surface microvilli (Molina et al., 1992) and have defective leukocyte chemotactic responses as judged by Boyden chamber (Ochs et al., 1980) and Dunn chamber (Zicha et al., manuscript submitted for publication) analyses. In addition, N-WASp, a relative of WASp, has recently been shown to induce the formation of filopodia when expressed in COS cells (Miki et al., 1998). Taken together, these results suggest that WASp could mediate at least some of the effects of Cdc42 on the cytoskeleton though a direct demonstration of a role for WASp in Cdc42-induced filopodium formation has yet to be demonstrated. We are currently investigating whether WASp contributes to Cdc42-mediated responses in macrophages.

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