

Requirements for Both Rac1 and Cdc42 in Membrane Ruffling and Phagocytosis in Leukocytes

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Summary

Specific pathways linking heterotrimeric G proteins and Fc_γ receptors to the actin-based cytoskeleton are poorly understood. To test a requirement for Rho family members in cytoskeletal events mediated by structurally diverse receptors in leukocytes, we transfected the full-length human chemotactic peptide receptor in RAW 264.7 cells and examined cytoskeletal alterations in response to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP), colony stimulating factor-1 (CSF-1), IgG-coated particles, and phorbol 12-myristate 13-acetate (PMA). Expression of Rac1 N17, Cdc42 N17, or the GAP domain of *n*-chimaerin inhibited cytoskeletal responses to FMLP and CSF-1, and blocked phagocytosis. Accumulation of F-actin-rich “phagocytic cups” was partially inhibited by expression of Rac1 N17 or Cdc42 N17. In contrast, PMA-induced ruffling was not inhibited by expression of Rac1 N17, but was blocked by expression of Cdc42 N17, indicating that cytoskeletal inhibition by these constructs was nonoverlapping. These results demonstrate differential requirements for Rho family GTPases in leukocyte motility, and indicate that both Rac1 and Cdc42 are required for Fc_γ receptor-mediated phagocytosis and for membrane ruffling mediated by structurally distinct receptors in macrophages.

Members of the Rho family of GTPases have been implicated in cytoskeletal alterations mediated by many agonists (for review see references 1 and 2). In particular, Rac1 has been shown to be required for membrane ruffling induced by growth factors (3, 4). A role for Cdc42 has been demonstrated for the formation of filopodia (5), as well as for phagocytosis of *Salmonella* in COS-1 cells (6). Other roles, such as the formation of stress fibers, have been proposed for RhoA (for review see reference 7), although there are also reports of a requirement for this GTPase in the invasion of *Shigella* in sessile cells (8, 9).

Leukocytes have long served as model systems for cell motility. Among the best characterized model systems are responses to the chemotactic peptide FMLP, the prototypic G protein-linked receptor agonist, in human neutrophils, and phagocytosis via Fc_γR in macrophages. Studies in primary cells and transfected cell lines indicate a requirement for a conserved immunoreceptor tyrosine activation motif and Syk tyrosine kinase in phagocytic signaling (for review see references 10 and 11). However, the identities of downstream effectors that lead to cytoskeletal alterations in response to Fc_γR ligation are unknown. The superficial resemblance of submembranous accumulations of F-actin that accrue during phagocytosis (“phagocytic cups”) and mem-

brane ruffles mediated by growth factors suggests that one or more Rho family GTPases are required for phagocytic signaling in leukocytes, but this has not been reported. In addition, a role for GTPases in motile events occurring in human neutrophils has been difficult to test since these cells are difficult to microinject, and there are no reports that neutrophils are capable of expressing recombinant proteins. For the FMLP receptor, the pathways coupling receptor-activated G proteins to the actin-based cytoskeleton are poorly understood.

We were interested in testing whether Rac1 or Cdc42 were required for the cytoskeletal alterations triggered by diverse stimuli in a single cell type. A uniform requirement for these GTPases would suggest that the signal transduction pathways that ensue upon receptor ligation converge at a single point, the activation of the GTPase. On the other hand, differential requirements for individual GTPases by structurally diverse receptors would suggest that these proteins have nonoverlapping functions. To test whether these GTPases are required for leukocyte motility, we derived stable clones of a leukocyte cell line that inducibly expressed epitope-tagged versions of GTPases defective in binding guanine nucleotides. We also expressed a GAP domain that would be predicted to limit the cellular accumulation

of GTP-bound, and hence active, forms of Rac and Cdc42. We tested the ability of these cells to undergo membrane ruffling and phagocytosis.

Materials and Methods

Cells and Reagents. RAW 264.7 mouse macrophage cell line (RAW)¹ cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in RPMI medium containing 10% FCS, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Anti-Myc antibody (clone 9E10) was a gift of James Angelastro (Columbia University, New York). mAb C4 against actin was a gift of James Lessard (University of Cincinnati, Cincinnati, OH). Rabbit anti-sheep erythrocyte IgG was from Diamedix (Miami, FL). Rhodamine-phalloidin and formyl-Nle-Leu-Phe-Nle-Tyr-Lys, a tetramethylrhodamine derivative, were from Molecular Probes (Eugene, OR). FITC-conjugated anti-mouse IgG and Cy5- and rhodamine-conjugated anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). Hygromycin B and G418 sulfate were from GIBCO BRL (Gaithersburg, MD). Murine CSF-1 was from PharMingen (San Diego, CA). Puromycin and pertussis toxin were from Sigma Chemical Co. (St. Louis, MO).

Construction of Plasmids and Transfection of Cells. All transfections were done using CaPO₄ precipitation. To establish stable RAW cell clones that expressed the Lac repressor protein, RAW cells were transfected with p3'SS (Stratagene, La Jolla, CA) and stable clones were isolated in the presence of 0.5 µg/ml hygromycin B. Expression of the Lac repressor protein was verified by indirect immunofluorescence. A single clone was selected and transfected with the full-length human FMLP receptor (gift of Philip Murphy, National Institutes of Health, Bethesda, MD) subcloned into pApuro (reference 12; provided by Tomohiro Kurosaki, Kansai Medical University, Noriguchi, Japan). Single clones were selected in the presence of 5 µg/ml puromycin and expression was verified using a rhodamine-derivatized chemotactic peptide, formyl-Nle-Leu-Phe-Nle-Tyr-Lys, and fluorescence microscopy. A single clone (RAW LacR/FMLPR.2) was used for further transfections with Myc-tagged Rho family GTPases subcloned into pCMV3Rluc, a plasmid containing multiple copies of the Lac repressor binding site (gift of Len Stephens, Babraham Institute, London, UK). Myc-tagged human Rac1 N17 and Cdc42 N17 were constructed as described (13, 14). Myc-tagged Chimaerin-GAP, containing residues 106–299 of *n*-Chimaerin, was constructed using PCR techniques and inserted into pCMV3RLuc. Myc-tagged *n*-Chimaerin was provided by Roman Herrera (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI). All constructs were verified by DNA sequencing. After transfection of RAW LacR/FMLPR.2 with pCMV3RLuc containing the indicated constructs, single clones of cells were isolated in the presence of 1 mg/ml G418 using limiting dilution, and were screened for expression of the Myc epitope after an overnight incubation in 10 mM IPTG, 2 mM sodium butyrate and 50 µM zinc acetate were routinely added to further enhance expression of the induced proteins. Between 50 and 200 individual clones transfected with each construct were screened for expression. Three to six clones were selected for further analysis using the following criteria: first, lack of significant basal (uninduced) expression. Depend-

ing on the clone, a small percentage of the adherent cell population stained positive with anti-Myc mAb even when maintained in the absence of IPTG. Although these cells appeared very bright, they accounted for only a small percentage of the population (<1%). Second, clones were selected for high levels of induced expression, both in terms of the percentage of cells expressing the Myc epitope and the level of expression of individual cells, as indicated by bright immunofluorescent staining using anti-Myc mAb. Clones which demonstrated variable intensities of staining with mAb anti-Myc were not analyzed further. Finally, clones were selected for stability of expression (i.e., cells could be maintained in culture for several months without losing inducible expression of the Myc-tagged constructs).

Cell Stimulation, Immunofluorescence Microscopy, and Immunoblotting. 10⁵ cells were plated on round glass coverslips in a 24-well plate in complete medium containing 50 µM zinc acetate and 2 mM sodium butyrate, and in the presence or absence of 10 mM IPTG. After an overnight induction period, adherent cells were washed, serum-starved in RPMI for 1 h, and incubated at 37°C for a further 10 min in a buffer containing 125 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 5 mM glucose, 10 mM NaHCO₃, 1 mM MgCl₂, 1 mM CaCl₂, and 20 mM Hepes, pH 7.4. Maximal ruffling was induced by the addition of 100 nM FMLP for 1 min, 10 ng/ml CSF-1 for 5 min, or 100 nM PMA for 5 min before fixation.

For phagocytosis assays, 5 × 10⁶ sheep erythrocytes opsonized with rabbit IgG (IgG-RBCs) were added to adherent cell cultures for 30 min as previously described (15). To assess submembranous accumulations of F-actin beneath attached IgG-RBCs (phagocytic cup formation), adherent cells were incubated with IgG-RBCs for 30 min at 4°C to allow particle attachment and, after washing with ice-cold buffer, cells were incubated for a further 5 min at 37°C before fixation.

Adherent cells were fixed in 3.7% formaldehyde and stained for the presence of the Myc epitope with mAb 9E10, for F-actin with 0.33 µM rhodamine-phalloidin, and for IgG-RBCs with anti-rabbit IgG, when indicated. Immunofluorescence microscopy was performed as previously described (16). For photomicroscopy, the stained cells were imaged using a confocal scanning system equipped with a Krypton-Argon laser (MRC 600; Bio-Rad Laboratories, Hercules, CA). Stacked confocal Z sections were collected. For quantitation of Myc expression, microspectrofluorometry using an Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a photomultiplier tube was performed as previously described (15). All fluorescence values were corrected for cellular autofluorescence, which was negligible, and fluorescence of stained untransfected controls not expressing the Myc epitope, which was typically between 5 and 10% of the total fluorescence signal. Immunoblotting was performed as previously described (17).

Quantitation of Ruffling and Phagocytosis. Ruffling was defined by the presence of F-actin-rich submembranous folds using fluorescence microscopy. The extent of ruffling of each cell was scored using a scale of 0–2, where 0 indicates that no ruffles were present, 1 indicates that ruffling was confined to one area of the cell only (<25% of the cell's circumference), and 2 indicates that two or more discrete areas of the cell contained ruffles. The ruffling index was recorded as the sum of the ruffling scores of 100 cells. For all agonists, the ruffling index of cells incubated in the absence of IPTG was not significantly different than the ruffling index of cells that were incubated with IPTG but did not express the Myc epitope. A separate ruffling index was calculated for cells expressing the Myc epitope (usually 20–60% of the total adherent

¹Abbreviations used in this paper: Chimaerin-GAP, the GAP domain of *n*-chimaerin; IgG-RBCs, sheep erythrocytes opsonized with rabbit IgG; IPTG, isopropyl β-d-thiogalactopyranoside; RAW cells, RAW 264.7 mouse macrophage cell line.

cell population). At least five separate fields were analyzed for the presence of the Myc epitope, and all cells expressing Myc were analyzed for extent of ruffling. Ruffling assays were performed in duplicate.

The phagocytosis index was measured by counting the number of ingested erythrocytes per 100 RAW cells after a 30-min incubation with IgG-RBCs. Ingested erythrocytes appeared as phase-lucent vacuoles which also stained positive for the presence of rhodamine anti-rabbit IgG (18). Phagocytosis assays were performed in duplicate.

All experiments depict data obtained from single clones of the indicated constructs; similar results were obtained for three to six additional clones of each construct.

Results

Expression of the Human FMLP Receptor in RAW Cells Results in a Functionally Intact Cytoskeletal Response to FMLP. Although specific macrophage subpopulations respond to the chemotactic peptide, FMLP (19, 20), murine macrophage cell lines lack this responsiveness. To attempt to reconstitute functionally intact FMLP receptors in a murine macrophage cell line, we transfected cDNAs encoding the full-length human FMLP receptor in RAW cells and derived several resultant stable clones. All clones expressed functional FMLP receptors at their surfaces since they bound rhodamine-tagged chemotactic peptide (results not shown). In contrast to quiescent cells, which demonstrated relatively few surface projections (Fig. 1 a), addition of 100 nM FMLP resulted in the appearance of F-actin-rich membrane ruffles in essentially 100% of the transfectants within 15 s of stimulation. No ruffling was seen in untransfected cells incubated with FMLP (results not shown). The ruffling re-

sponse in the transfectants peaked at 1 min (Fig. 1 b), and had declined by 20 min (not shown). Membrane ruffles appeared diffusely throughout the cell. The extent of ruffling by FMLP, but not by CSF-1, was reduced to control levels in the presence of pertussis toxin (Fig. 1 d), indicating that cytoskeletal coupling by the transfected receptors was mediated by G_i , or a structurally similar heterotrimeric G protein. These data suggest that RAW cells expressing the human FMLP receptor use signal transduction pathways that are similar to those triggered by this serpentine receptor in primary leukocytes.

In contrast, addition of CSF-1 induced membrane ruffles in these cells, but the response was slower, peaking at 5 min, and the ruffles appeared in distinct patches (Fig. 1 d). The ability of RAW cells to respond to CSF-1 by membrane ruffling is reminiscent of similar responses in primary mouse macrophages (21). Thus, RAW cell transfectants incubated with two structurally disparate agonists, FMLP and CSF-1, behave like primary macrophages and display morphologically similar, but not identical, responses.

Expression of Dominant-negative Rho Family GTPases in RAW Cell Transfectants. To test a requirement for Rac1 and Cdc42 on membrane ruffling in RAW cells, we expressed inducible GTP binding-deficient versions of these proteins in RAW cells and selected stable clones. As a complementary approach, we also utilized the GAP domain of *n*-chimaerin (Chimaerin-GAP), previously shown to display GAP activity towards Rac1 and Cdc42 (22). Expression of Chimaerin-GAP would be expected to decrease the accumulation of GTP-bound (active) forms of Rac1 and Cdc42. We used a RAW cell clone (RAW LacR/FMLPR.2) that stably expressed both the human FMLP receptor and the Lac repressor protein as a recipient cell line. This line was used to generate further clones expressing Myc-tagged human Rac1 N17, Cdc42 N17, and Chimaerin-GAP. Between 50 and 200 clones expressing each construct were isolated. The clones varied in their "leakiness" (i.e., expression of the Myc epitope in the absence of the inducing agent, IPTG) and level of inducible expression. Therefore, we determined optimal conditions for the expression of the constructs, and selected three to six clones that displayed a high level of inducible expression for each construct. For example, in the absence of IPTG, minimal expression of the Myc epitope was detected, whereas IPTG caused a time-dependent appearance of fusion protein expression. The addition of zinc acetate and sodium butyrate further enhanced protein expression (Fig. 2 a). Fluorescence microscopy revealed a bimodal population of cells that either did or did not express the Myc epitope. In uninduced cells, <1% of the cells expressed the Myc epitope, whereas addition of IPTG, zinc, and butyrate induced expression of the Myc epitope in 20–60% of the total cell population. Clones were selected on the basis of demonstrating uniformly bright fluorescence in those individual cells that stained positive for the Myc epitope (i.e., cells within a selected clone showed a sharp contrast between Myc expression and a lack of expression, and the variation of intensities of Myc-positive cells was relatively small). We quantitated expres-

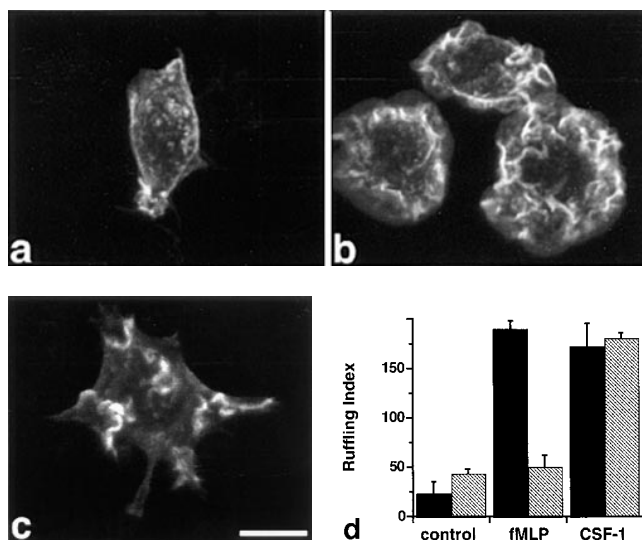


Figure 1. FMLP or CSF-1 induces F-actin-rich ruffles in RAW LacR/FMLPR.2 cells. Cells were fixed and stained with rhodamine-phalloidin as described in Materials and Methods. (a) Unstimulated. (b) Cells incubated with 100 nM FMLP for 1 min. (c) Cells incubated with 10 ng/ml CSF-1 for 5 min. Bar = 10 μ m. (d) Inhibition of FMLP-induced ruffles by pertussis toxin (1 ng/ml for 24 h) in RAW LacR/FMLPR.2 cells. Solid bars, - pertussis toxin; striped bars, + pertussis toxin.

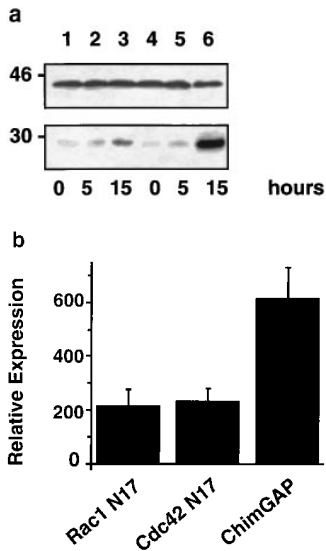


Figure 2. Induction of expression of Myc-tagged fusion proteins in stable RAW cell transfectants. (a) 10^6 RAW cells (derived from a clone of RAW LacR/FMLPR.2 cells transfected with Myc-tagged Chimaerin-GAP) were incubated for 0, 5, or 15 h in the presence or absence of 10 mM IPTG, 50 μ M zinc, and 2 mM butyrate. Cells were subjected to detergent lysis, SDS-PAGE, and immunoblotting with either anti-Myc or antiactin mAbs. Lanes 1 and 4, uninduced; lanes 2 and 3, induced with IPTG only; lanes 5 and 6, induced with IPTG, butyrate, and zinc. (b) Average levels of expression of Myc-tagged fusion proteins in individual cells. Induction of protein expression was performed as described in

Materials and Methods and cells were fixed and stained with anti-Myc mAb and processed for microspectrofluorometry. Expression levels are shown in arbitrary fluorescence units. Nonspecific fluorescence (i.e., of uninduced cells or untransfected cells) was <10% of the total fluorescence signal. Data are expressed as the mean fluorescence (\pm SEM) of 150–200 Myc-positive cells and represent all Myc-positive cells observed in 5–10 high power fields from three separate experiments.

sion of the Myc-tagged fusion proteins on a single cell basis by measuring the fluorescence intensities of Myc-positive cells using microspectrofluorometry. Clones that inducibly expressed Rac1 N17 or Cdc42 N17 showed similar levels of expression, whereas Chimaerin-GAP consistently showed a greater level of expression on a single cell basis ($P < 0.05$, when compared to any other clone; Fig. 2 b). In the 40–80% of the population that did not stain positive with mAb anti-Myc despite the presence of inducing agents, the mean fluorescence was similar to that of uninduced cells. Although we cannot explain why only a proportion of cells of a given clone expressed the fusion proteins, the nonexpressors of a given clone provided ideal controls to test the effects of fusion protein expression on cytoskeletal responses.

Rac1 N17, Cdc42 N17, and Chimaerin-GAP Inhibit Membrane Ruffling Induced by FMLP or CSF-1. The morphology of cells expressing the various Myc-tagged fusion constructs was dependent on the specific construct expressed and its level of expression. Relatively high levels of expression of Rac1 N17, obtainable by the addition of zinc and butyrate, were associated with cell rounding (for example, see Fig. 3 b), whereas moderate expression of either construct did not lead to an altered cell morphology (results not shown). Expression of Cdc42 N17 did not significantly affect the morphology of the cells (Fig. 3 c). Cells expressing Chimaerin-GAP frequently appeared more spread than control cells (Fig. 3 d).

Addition of FMLP to controls (Fig. 1 b) or to uninduced cell lines transfected with various Myc-tagged fusion proteins led to the formation of membrane ruffles (results not

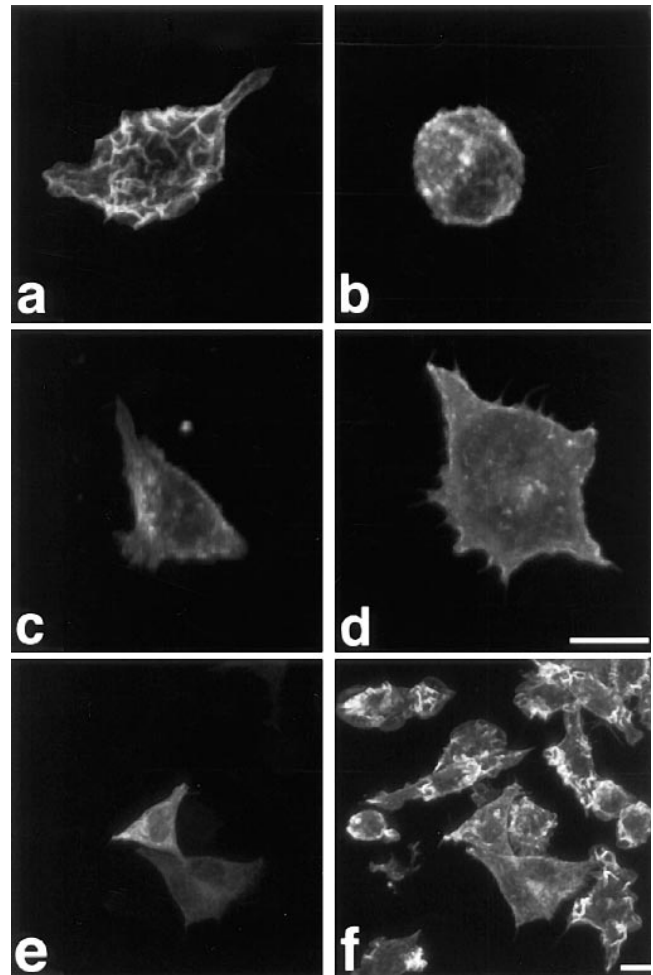


Figure 3. Expression of Myc-tagged Rac1 N17, Cdc42 N17, or Chimaerin-GAP inhibits ruffling in response to FMLP. Cells were incubated with 100 nM FMLP for 1 min before fixation and staining with rhodamine-phalloidin to detect F-actin and mAb anti-Myc followed by FITC anti-mouse IgG to detect the indicated fusion proteins, as described in Materials and Methods. Rhodamine-phalloidin staining of a control cell is shown in a and of representative Myc-positive cells are shown in b–d. A field of cells, some of which express Chimaerin-GAP, is depicted in e (stained with anti-Myc) and f (stained with rhodamine-phalloidin). a, Control; b, Rac1 N17; c, Cdc42 N17; d–f, Chimaerin-GAP. Bar = 10 μ m.

shown). Addition of IPTG to cells transfected with either Rac1 N17, Cdc42 N17, or Chimaerin-GAP led to expression of the fusion proteins and inhibition of FMLP-induced membrane ruffling (Figs. 3, b–f, and 4 a). The inducing agents had no effect on FMLP-induced membrane ruffling in controls (results not shown).

Similar results were obtained using CSF-1 as a ruffle-inducing agent. Expression of Rac1 N17, Cdc42 N17, or Chimaerin-GAP inhibited CSF-induced membrane ruffling by 88–96%. The inhibitory effects on membrane ruffling of Rac1 N17 and Cdc42 N17 were apparent in terms of the number of cells that demonstrated no ruffling response and in the extent of ruffling in those cells that did respond to either FMLP or CSF-1 (Fig. 4 c).

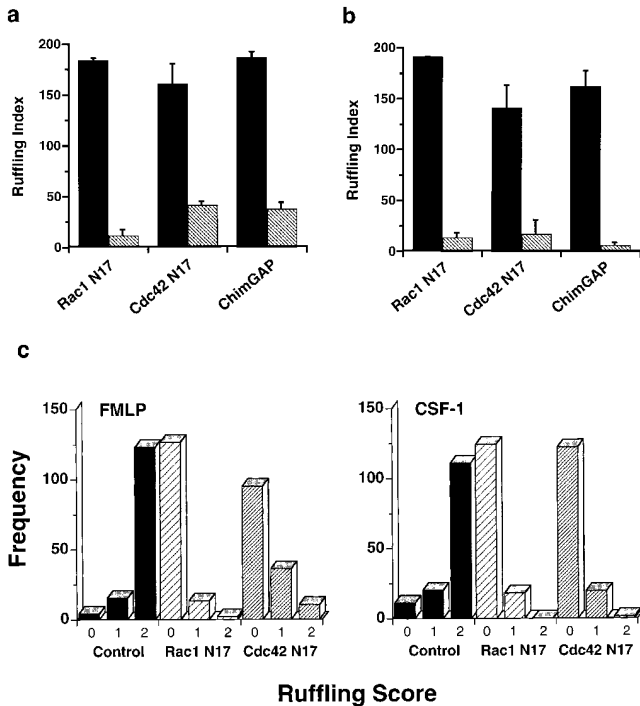


Figure 4. Ruffling indices and scores of FMLP- or CSF-1-stimulated RAW cells transfected with the indicated constructs. *Narrow striped bars*, Myc-expressing cells; *solid bars*, controls, derived from the same clones but which did not express the Myc epitope by indirect immunofluorescence. Ruffling indices of cells incubated in the absence of IPTG, zinc, and butyrate were indistinguishable from cells that were incubated with these agents but did not express Myc. *a*, FMLP; *b*, CSF-1. Data are expressed as the mean \pm SEM, $n = 3$. The reduction of ruffling by Rac1 N17, Cdc42 N17, or Chimaerin-GAP was statistically significant ($P < 0.005$). *c* Histograms of ruffling scores of individual cells stimulated with the indicated agonist. The extent of ruffling of each cell was scored using a scale of 0–2, where 0 indicates that no ruffles were present, 1 indicates that ruffling was confined to one area of the cell only (<25% of cell circumference), and 2 indicates that two or more discrete areas of the cell contained ruffles. 140 cells were counted for each construct.

Rac1 N17, Cdc42 N17, and Chimaerin-GAP Inhibit Fc γ R-mediated Phagocytosis. Fc γ R-mediated phagocytosis requires the net assembly of actin which forms the structural scaffolding that constitutes phagocytic cups. To determine whether Rac1 or Cdc42 were required for phagocytosis, we performed phagocytosis assays on cell populations induced with IPTG and compared ingestion of IgG-RBCs in cells that either did or did not express the various fusion proteins. For example, expression of Cdc42 N17 blocked ingestion of IgG-RBCs, despite the presence of surface-bound erythrocytes (Fig. 5). Expression of Rac1 N17, Cdc42 N17, or Chimaerin-GAP profoundly inhibited phagocytosis (Fig. 6). In contrast, expression of the fusion constructs had a modest effect on particle binding. For example, expression of Rac1 N17 led to a $27 \pm 2.7\%$ reduction in particle binding, whereas expression of Cdc42 N17 reduced binding by $38 \pm 9.7\%$. In contrast, Chimaerin-GAP had no effect on binding efficiency (i.e., the average number of IgG-RBCs associated with either induced or uninduced cells was seven). Thus, inhibition of Rac1 and Cdc42 func-

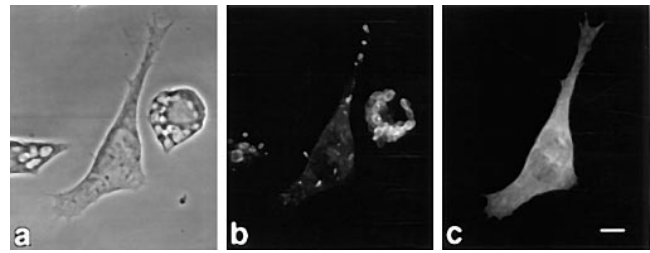


Figure 5. Inhibition of Fc γ R-mediated phagocytosis by Cdc42 N17. Phagocytosis assay was performed as described in Materials and Methods. *(a)* Phase-contrast micrograph. *(b)* Fluorescence micrograph of rhodamine anti-rabbit IgG-stained cells to indicate presence of IgG-RBCs. After fixation and permeabilization, uningested erythrocytes appear crenated. *(c)* Fluorescence micrograph of anti-Myc-stained cell to indicate expression of Myc-tagged Cdc42 N17. Note ingestion of IgG-RBCs in neighboring cells not expressing the Myc epitope.

tion by either dominant-negative versions of these proteins or by GAP expression led to a disproportionate decrease in phagocytosis, as compared to binding, of IgG-RBCs.

To determine whether the inhibition of phagocytosis correlated with an inhibition of the submembranous accumulations of F-actin, we fixed and stained various clones of RAW cells undergoing early stages of phagocytosis. 5 min after the onset of phagocytosis, F-actin-rich phagocytic cups were clearly visible in control cells (Fig. 7, *a* and *b*), confirming earlier data (15). Expression of Rac1 N17 or Cdc42 N17 (Fig. 7, *c–f*) inhibited the appearance of distinct F-actin-rich cups, although some focal accumulations of F-actin beneath the test particles did occur in these cells. The inhibition of phagocytic cup formation by Cdc42 N17 was usually incomplete, whereas expression of Rac1 N17 abolished much of the focal appearance of F-actin beneath the test particles (compare Fig. 7 *e* with *d*). The effects of Chimaerin-GAP resembled that of Cdc42 N17 (results not shown).

Cdc42 N17, but Not Rac1 N17, Inhibits PMA-induced Ruffling. The qualitatively similar effects of Cdc42 N17 and Rac1 N17 on responses mediated by structurally distinct ligands (i.e., FMLP, CSF-1, and IgG) raised the possibility that expression of Rac1 N17 or Cdc42 N17 led to nonspecific toxic effects on RAW cells, rendering them incapable of responding to any F-actin-mobilizing agonist. This was

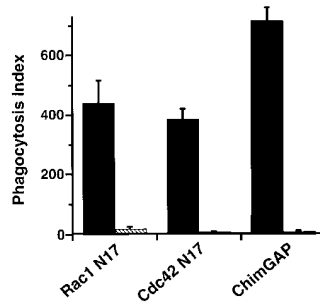


Figure 6. Expression of Myc-tagged Rac1 N17, Cdc42 N17, or Chimaerin-GAP inhibits Fc γ R-mediated phagocytosis. *Striped bars*, Myc-expressing cells; *solid bars*, controls, which are derived from the same clones but did not express the Myc epitope by indirect immunofluorescence. Phagocytosis indices of cells incubated in the absence of IPTG, zinc, and butyrate were indistinguishable from cells that were incubated with these agents but did not express Myc. Data are expressed as the mean \pm SEM, $n = 3$. The reduction of phagocytosis by Rac1 N17, Cdc42 N17, or Chimaerin-GAP was statistically significant ($P < 0.005$).

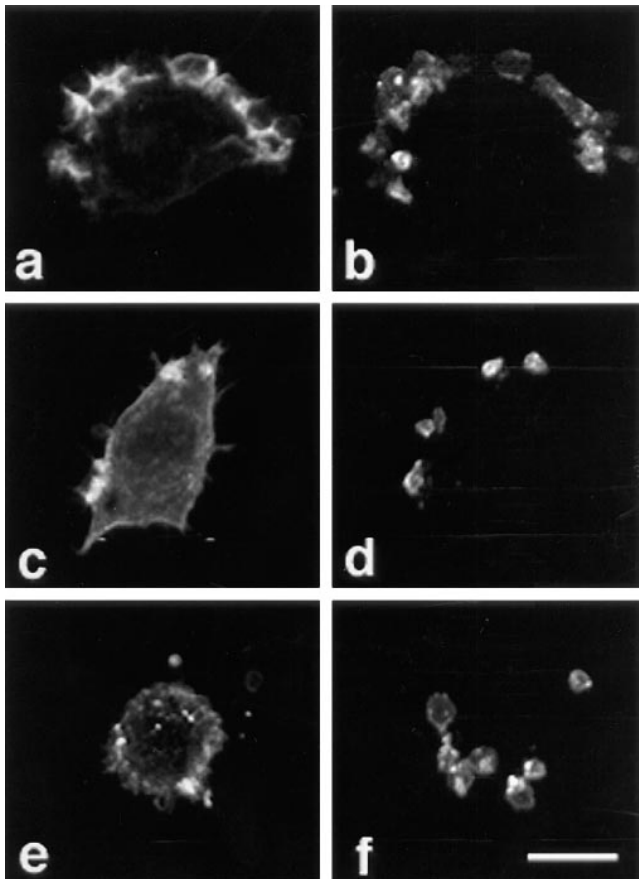


Figure 7. Phagocytic cup formation is inhibited by expression of Rac1 N17 or Cdc42 N17. Cells were incubated with IgG-RBCs for 5 min at 37°C before fixation. Immunofluorescence using rhodamine-phalloidin and anti-rabbit IgG was performed as described in Materials and Methods. *a*, *c*, and *e*, rhodamine-phalloidin; *b*, *d*, and *f*, anti-rabbit IgG. *a* and *b*: control; *c* and *d*, Cdc42 N17; *e* and *f*, Rac1 N17. Bar = 10 μ m.

of particular concern in cells expressing high levels of Rac1 N17, which caused cell rounding. We tested several other ligands for their ability to induce cytoskeletal changes in these cells, but could not detect morphological changes after addition of lysophosphatidic acid, bradykinin, platelet activating factor, or ATP (results not shown). However, addition of PMA caused dramatic changes in cell shape, leading to cell spreading and membrane ruffling (Fig. 8), both of which were inhibited by 1 μ M cytochalasin D (results not shown). In marked contrast to the above results using FMLP, CSF-1, or IgG-RBCs, expression of Rac1 N17 did not inhibit PMA-induced ruffling (compare Fig. 8 *b* with *c*). However, expression of Cdc42 N17 blocked PMA-induced ruffling (Fig. 8 *d* and Fig. 9). These results indicate that expression of Rac1 N17 did not lead to a global lack of responsiveness in these cells. PMA-induced cellular responses are often attributed to activation of one or more protein kinase C isoforms. We could not confirm or refute this, since addition of 5 μ M calphostin C inhibited PMA-induced membrane ruffling, whereas other inhibitors of protein kinase C, such as chelerythrine chloride (10 μ M) and staurosporine (1 μ M), did not (results not shown).

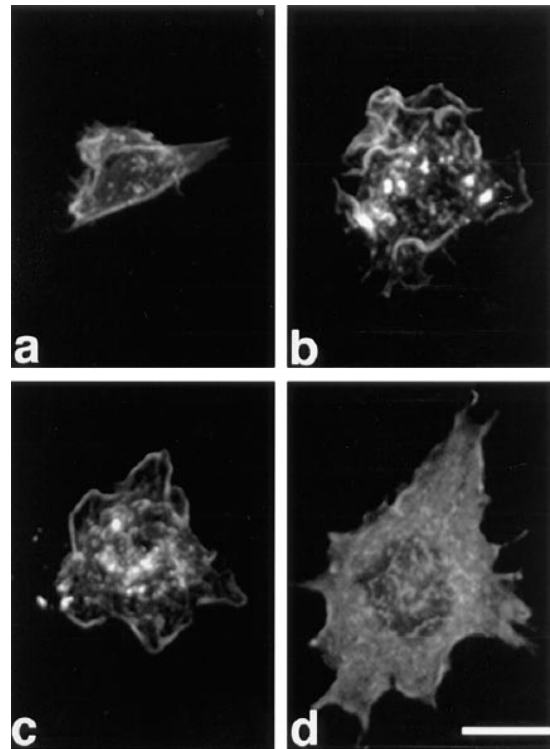


Figure 8. PMA-induced ruffling is a Rac1-independent event in RAW cell transfectants. Cells were incubated with vehicle (*a*) or with 100 nM PMA (*b-d*), and stained with anti-Myc mAb and rhodamine-phalloidin as described in Materials and Methods. Rhodamine-phalloidin fluorescence is pictured. *a*, control; *b*, control incubated with PMA; *c*, Rac1 N17 incubated with PMA; *d*, Cdc42 N17 incubated with PMA. Bar = 10 μ m.

Discussion

The data presented here demonstrate a requirement for both Rac1 and Cdc42 in membrane ruffling mediated by several agonists, and in phagocytosis mediated by Fc γ Rs. The inhibition of these responses by GTP binding-deficient versions of both GTPases raises the possibility that Rac and Cdc42 bind to an identical pool of guanine nucleotide exchange factors, and that expression of either N17 GTPase would necessarily lead to a functional inhibition of both proteins. This is unlikely for the following reasons: first, the inability of Rac1 N17 to inhibit PMA-induced ruffling demonstrates that Rac1 N17 and Cdc42 N17 behave differently in these cells, and thus are unlikely to inactivate the same spectrum of GEFs; and second, Cdc42 N17-expressing cells appeared morphologically normal, unlike those expressing high levels of Rac1 N17. This further supports the concept that Rac1 and Cdc42 have distinct cytoskeletal-altering functions in these cells.

The lack of inhibition of PMA-induced ruffling by Rac1 N17 contrasts with earlier data in Swiss 3T3 cells (3). A report of the effects of PMA on PC12 cells indicates that Rac1 is only partly responsible for migration mediated by this agent (23). We cannot explain these discrepancies, except to point out that the cellular targets of PMA are poorly understood, and may include enzymes other than the con-

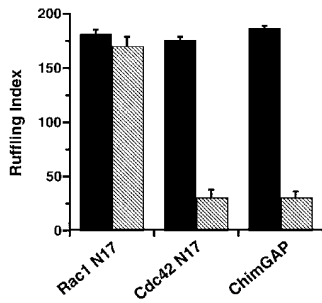


Figure 9. Ruffling indices of PMA-stimulated RAW cells transfected with the indicated constructs. *Striped bars*, Myc-expressing cells; *solid bars*, controls, which are derived from the same clones but did not express the Myc epitope by indirect immunofluorescence. Ruffling indices of cells incubated in the absence of IPTG, zinc, and butyrate were indistinguishable from cells that were incubated with these agents

but did not express Myc. Data are expressed as the mean \pm SEM, $n = 3$. The reduction of ruffling by Cdc42 N17 or Chimaerin-GAP was statistically significant ($P < 0.005$).

ventional isoforms of protein kinase C (24). In addition, the effects of PMA on the cytoskeleton and the signaling requirements that underlie these effects may be cell-type specific.

These data question whether a true hierarchy of GTPases exists in these cells; for example, in Swiss 3T3 cells, the appearance of Cdc42-mediated focal complexes and lamellipodia is blocked by coinjection of Rac1 N17, suggesting that Rac1 lies downstream of Cdc42 in these cells (25). However, the disparate requirements for Rac1 and Cdc42 in membrane ruffling stimulated by PMA in macrophages suggest that Cdc42 does not require the participation of Rac1 to mediate ruffling. It is possible that Cdc42 activates Rac1 in these cells, but that activated Rac1 does not participate in PMA-stimulated membrane ruffling.

Expression of either Rac1 N17 or Cdc42 N17 led to a similar extent of inhibition of membrane ruffling in response to CSF-1 or FMLP. Somewhat puzzling was the finding that their expression did not ablate the focal appearance of F-actin beneath adherent IgG-RBCs, although both proteins blocked phagocytosis and inhibited the appearance of well-formed phagocytic cups. There are several potential explanations for these results. One, which we favor, is that multivalent or clustered stimuli serve as particularly effective ligands for receptors whose actual affinity for univalent ligands (i.e., monomeric IgG) may be low. Engagement of the cytoskeletal machinery by these particulate agonists relies on their ability to recruit signal-transducing elements to a highly concentrated region beneath the plasma membrane. Thus, we would predict that equivalent levels of expression of potential inhibitory constructs may not be

as potent in inhibiting solid phase (e.g., IgG-RBCs), as opposed to soluble (e.g., FMLP) ligands. Incomplete efficacy of inhibition may explain why the expression of Rac1 N17 or Cdc42 N17 did not completely abolish the focal appearance of F-actin beneath the attached IgG-RBCs. Another potential explanation is that multiple GTPases contribute to $Fc\gamma R$ -directed actin assembly, including additional GTPases that have yet to be identified, and that all are required for the coordinated extension of pseudopods and closure of phagosomes. The comparatively less severe effects of Cdc42 N17 on $Fc\gamma R$ -directed cytoskeletal alterations suggest that Cdc42 may modulate the cytoarchitecture of pseudopods, rather than actually initiate actin filament nucleation or uncapping, although this remains to be formally tested. Other possible explanations for the inhibitory effects of Rac1 N17 and Cdc42 N17 on cytoskeletal responses include decreased substrate adherence or other nonspecific toxic effects. We think these possibilities are unlikely since expression of Cdc42 N17 did not lead to detectable changes in the cell surface area or adhesion to the substrate. Expression of Chimaerin-GAP, which led to similar inhibitory effects on cytoskeletal responses, frequently led to enhanced cell spreading, an effect more consistent with increased cell-substratum attachment. Attesting to a lack of global toxicity, expression of Rac1 N17 did not affect PMA-mediated membrane ruffling, and expression of both N17 GTPases or Chimaerin-GAP did not ablate some aspects of signal transduction mediated by LPS (our manuscript in preparation), indicating that not all receptor-mediated responses are inhibited by these constructs.

Based on the above results, we suggest that receptor-mediated cytoskeletal events in these cells trigger activation of multiple Rho family GTPases, and that they function in a coordinated, but not necessarily hierarchical, manner. This model implies that the enzymes are not interchangeable with respect to their abilities to induce alterations in the cytoskeleton, and it is consistent with differences in their subcellular localization (26) and ability to interact with distinct effectors (1). This is also supported by functional differences between effector domain mutants of Rac and Cdc42. Mutation at position 37 of Rac1 abolishes its ability to mediate membrane ruffling and interact with Por1 (27), whereas the analogous mutation in Cdc42 does not produce this effect (28). We are currently examining the subcellular localization and potential protein-protein interactions of Rac1 and Cdc42 that occur during membrane ruffling and phagocytosis in order to further address their functions.

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