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

By Dianne Cox,* Peter Chang,* Qing Zhang,* P. Gopal Reddy,* Gary M. Bokoch,‡ and Steven Greenberg*

From the *Pulmonary Division, Department of Medicine, Columbia University College of Physicians and Surgeons, New York 10032; and ‡Department of Immunology and Department of Cell Biology, Scripps Research Institute, La Jolla, California 92037

Summary

Specific pathways linking heterotrimeric G proteins and Fcγ 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 Fcγ receptor-mediated phagocytosis and for membrane ruffling mediated by structurally distinct receptors in macrophages.
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 (RAW1) 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 Dianomed (Miami, FL). Rhodamine-phalloidin and formyl-N-leu-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 Laboratories (West Grove, PA). Hygromycin B and G418 sulfate were from Gibco BRL (Gaithersburg, MD). Mouse CSF-1 was from Pharmingen (San Diego, CA). Puromycin and pertussis toxin were from Sigma Chemical Co. (St. Louis, MO).

Constructions 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 p3SS (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 pCMV3RLuc. Constructs were verified by DNA sequencing. After transfection in the presence of 5 μg/ml puromycin and expression was verified using a rhodamine-derivatized chemotactic peptide, formyl-N-leu-Leu-Phe-Nle-Tyr-Lys, and fluorescence microscopy. A single clone (RAW LacR/FMLP·2) was used for further transfections with Myc-tagged Rho family GTPases subcloned into pCMV3R luc, a plasmid containing multiple copies of the Lac repressor binding site (gift of Lent Stevens, Babraham Institute, Cambridge, UK). Mouse CSF-1-resistant RAW cells were transfected with pCMV3R luc. Myc-tagged human Rac2 was a gift of Bruce Stillman (Cold Spring Harbor, NY). Myc-tagged Myc-CDC42 was provided by Roman Herrera (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI). Myc-tagged human Rac1 was a gift of Bruce Stillman (Cold Spring Harbor, NY). Myc-tagged Myc-CDC42 was provided by Roman Herrera (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI).

Restriction digestion of pCMV3R luc produced multiple myc-CDC42, myc-Rac1, and myc-Rac2 constructs. Myc-tagged Myc-Rac1 and Myc-Rac2 constructs were inserted into the pCMV3R luc vector, and the correct orientation was confirmed by DNA sequencing. Stable clones were isolated in the presence of 0.5 μg/ml puromycin and expression was verified using an Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a photomultiplier tube. Photomicroscopy 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 quantification of Myc expression, microspectrophotometry 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 not 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

1 Abbreviations 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

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. 1a), 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 response in the transfectants peaked at 1 min (Fig. 1b), 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. 1d), indicating that cytoskeletal coupling by the transfected receptors was mediated by G_{1}, 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. 1c). 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. 2a). 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-
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 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).
Cells were counted for each construct. Indicates that two or more discrete areas of the cell contained ruffles. The extent of ruffling of each cell was scored using a scale of 0–2, with a score of 0 indicating no ruffling, 1 indicating ruffling confined to one area of the cell only, and 2 indicating ruffling 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. 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 c). 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
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 μM 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 d). However, expression of Cdc42 N17 blocked PM A-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. PM A-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 μM calphostin C inhibited PM A-induced membrane ruffling, whereas other inhibitors of protein kinase C, such as chelerythrine chloride (10 μM) and staurosporine (1 μM), did not (results not shown).

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 receptors. 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 PM A-induced ruffling by Rac1 N17 contrasts with earlier data in Swiss 3T3 cells (3). A report of the effects of PM A 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 PM A are poorly understood, and may include enzymes other than the con-
of expression of potential inhibitory constructs may not be
membrane. Thus, we would predict that equivalent levels
of PMA-stimulated membrane ruffling.

These data question whether a true hierarchy of GTPases
exists in these cells; for example, in Swiss 3T3 cells, the ap-
pearance of Cdc42-mediated focal complexes and lamelli-
 podia is blocked by coinjection of Rac1 N17, suggesting
that Rac1 lies downstream of Cdc42 in these cells (25). How-
ever, the disparate requirements for Rac1 and Cdc42 in mem-
brane 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
PM A-stimulated membrane ruffling.

Expression of either Rac1 N17 or Cdc42 N17 led to a
similar extent of inhibition of membrane ruffling in re-
sponse to CSF-1 or FMLP. Somewhat puzzling was the
finding that their expression did not ablate the focal appear-
ce 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 particu-
larly effective ligands for receptors whose actual affinity for
univalent ligands (i.e., monomeric IgG) may be low. En-
gagement 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 op-
posed 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 appear-
ance of F-actin beneath the attached IgG-RBCs. Another
potential explanation is that multiple GTPases contribute to
FcR-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 FcR-directed cytoskeletal alterations suggest that
Cdc42 may modulate the cytoarchitecture of pseudopods,
rather than actually initiate actin filament nucleation or un-
capping, 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 spread-
ing, an effect more consistent with increased cell-substratum
attachment. Attesting to a lack of global toxicity, expres-
sion of both N17 GTPases or Chimaerin-GAP did not ablate some aspects of signal transduction
mediated by LPS (our manuscript in preparation), indicat-
ing 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 func-
tional 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 pro-
duce this effect (28). We are currently examining the sub-
cellular localization and potential protein–protein interac-
tions of Rac1 and Cdc42 that occur during membrane ruffling
and phagocytosis in order to further address their functions.

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