

# Overexpression of a Novel Rho Family GTPase, RacC, Induces Unusual Actin-based Structures and Positively Affects Phagocytosis in *Dictyostelium discoideum*

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Rho family proteins have been implicated in regulating various cellular processes, including actin cytoskeleton organization, endocytosis, cell cycle, and gene expression. In this study, we analyzed the function of a novel *Dictyostelium discoideum* Rho family protein (RacC). A cell line was generated that conditionally overexpressed wild-type RacC three- to fourfold relative to endogenous RacC. Light and scanning electron microscopy indicated that the morphology of the RacC-overexpressing cells [RacC WT(+) cells] was significantly altered compared with control cells. In contrast to the cortical F-actin distribution normally observed, RacC WT(+) cells displayed unusual dorsal and peripheral F-actin-rich surface blebs (petalopodia, for flower-like). Furthermore, phagocytosis in the RacC WT(+) cells was induced threefold relative to control Ax2 cells, whereas fluid-phase pinocytosis was reduced threefold, primarily as the result of an inhibition of macropinocytosis. Efflux of fluid-phase markers was also reduced in the RacC WT(+) cells, suggesting that RacC may regulate postinternalization steps along the endolysosomal pathway. Treatment of cells with Wortmannin and LY294002 (phosphatidylinositol 3-kinase inhibitors) prevented the RacC-induced morphological changes but did not affect phagocytosis, suggesting that petalopodia are probably not required for RacC-induced phagocytosis. In contrast, inactivating diacylglycerol-binding motif-containing proteins by treating cells with the drug calphostin C completely inhibited phagocytosis in control and RacC WT(+) cells. These results suggest that RacC plays a role in actin cytoskeleton organization and phagocytosis in *Dictyostelium*.

## INTRODUCTION

Small- $M_r$  GTPases are molecular switches that function to regulate a diverse array of biological processes, including vesicular trafficking, cell cycle progression, oxidative burst, transcriptional activation, and actin cytoskeleton organization (reviewed in Van Aelst and D'Souza-Schorey, 1997). The Rho proteins (one of five subfamilies of Ras-like GTPases) are divided into three classes; Rho, Rac, and

Cdc42. Members of the Rho family are primarily involved in the regulation of the actin cytoskeleton of the cell; Rho was shown to control the formation of actin stress fibers (Ridley and Hall, 1992); Cdc42 was shown to regulate filopodia formation (Kozma *et al.*, 1995; Nobes and Hall, 1995); and Rac was shown to coordinate lamellipodia formation (Ridley *et al.*, 1992). More recent data suggest that these GTPases regulate other cellular functions, including gene expression (Coso *et al.*, 1995), tumorigenic activation (Qiu *et al.*, 1995), the oxidative burst of phagocytes (Bokoch, 1995), cell cycle progression

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(Ridley, 1995; Westwick *et al.*, 1997), and cellular transformation (Qiu *et al.*, 1995, 1997; Roux *et al.*, 1997).

Several lines of evidence suggest that the actin cytoskeleton of many different types of cells plays an important role in regulating processes such as phagocytosis and fluid-phase pinocytosis. For instance, it has been demonstrated that disrupting the actin cytoskeleton inhibited the internalization of the  $\alpha$ -factor pheromone and prevented receptor-mediated endocytosis in mammalian cells (Lamaze *et al.*, 1997). Furthermore, studies using cytochalasin A and latrunculin B in *Dictyostelium* have demonstrated that disrupting the actin cytoskeleton inhibits the processes of phagocytosis and fluid-phase pinocytosis (Temesvari *et al.*, 1996; Hacker *et al.*, 1997).

The fact that the actin cytoskeleton appears to play such an important role in endocytosis implies that proteins that regulate actin distribution, such as Rho family GTPases, may also be important in the regulation of this process. In fact, a recent study has shown that expression of a constitutively active form of Rac1 or Rho inhibited receptor-mediated endocytosis of the transferrin receptor in HeLa cells (Lamaze *et al.*, 1996). In addition, two independent groups have demonstrated that Rho family GTPases play a role in regulating phagocytosis in macrophages and leukocytes (Cox *et al.*, 1997; Hackam *et al.*, 1997).

The simple unicellular eukaryote *Dictyostelium discoideum* is an ideal system in which to study the function of GTPases because, like the *Saccharomyces cerevisiae*, *Drosophila*, and *Caenorhabditis elegans* systems, it is amenable to genetic and biochemical manipulations. Furthermore, it functions in motility and phagocytosis in a manner similar to that observed for neutrophils. Our laboratory has identified seven Rho family genes in *D. discoideum* (*rac1A*, *rac1B*, *rac1C*, *racA*, *racB*, *racC*, and *racD*) (Bush *et al.*, 1993). On the basis of protein homology searches, we have provisionally named these Rho family members "Rac" proteins; however, it remains to be demonstrated whether these small- $M_r$  GTPases function like mammalian Rac proteins and if the proteins in this family share overlapping roles in *Dictyostelium*. By comparison, three *rac*-like genes have been identified in mammalian cells (*rac1*, *rac2*, and *rac3*) (Didsbury *et al.*, 1989; Haataja *et al.*, 1997), as well as five Rho-like GTPases (*rhoA*, *rhoB*, *rhoC*, *rhoD*, and *rhoE*), and a *cdc42*-like gene (reviewed in Van Aelst and D'Souza-Schorey, 1997). The three *D. discoideum* Rho family proteins, Rac1A, Rac1B, and Rac1C, share at least 81% homology to human Rac1, whereas the other Rac-like proteins from *D. discoideum* share between 58 and 74% homology to human Rac1; therefore, these proteins have been classified as "novel" Rac-like GTPases. Another laboratory has also recently identified an additional *D. discoideum* rho fam-

ily gene (*racE*) that appears to play a role in cytokinesis (Larochelle *et al.*, 1996).

As one approach to investigate the function of novel Rho family proteins, we have generated cell lines that modestly and conditionally overexpress the novel *D. discoideum* GTPase RacC, which is 61% identical to human Rac1 and Cdc42 in amino acid sequence. We report that RacC WT(+) cells displayed unusual F-actin-based structures on their surface that we have termed "petalopodia," because they resemble the petals of a flower. Furthermore, the rate of phagocytosis in RacC WT(+) cells was stimulated threefold, whereas the rate of fluid-phase pinocytosis was reduced threefold (probably as the result of an abrogation of macropinocytosis). Finally, the exocytosis of fluid-phase and lysosomal hydrolases was inhibited in RacC WT(+) cells. These results indicate that RacC may function at discrete steps along the endolysosomal pathway, perhaps to regulate actin-based processes, including phagocytosis, pinocytosis, and endolysosomal vesicle trafficking.

## MATERIALS AND METHODS

### Organism

*Dictyostelium* strains were grown axenically in HL5 medium (1% oxoid proteose peptone, 1% glucose, 0.5% yeast extract [Difco, Detroit, MI], 1.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6.5) in 175-cm<sup>2</sup> tissue culture flasks (Sarstedt, Newton, NC) at 19°C. To generate RacC WT(+) cell lines, the parental Ax2 cells were transformed with the *D. discoideum* RacC expression vector HA-RacC-pVEII $\Delta$ ATG. To create this vector, full-length *racC* cDNA was cloned into the *SacI* site of the *D. discoideum* expression vector HA-pVEII $\Delta$ ATG to generate the new vector pDS7, thus placing RacC in-frame with a 10-amino acid-encoding epitope tag from the hemagglutinin (HA) protein of influenza virus. This vector contained the *discoidin I* promoter, which can be induced with prestarvation factor (Rathi *et al.*, 1991) and repressed by growing cells in the presence of 1 mM folate. Stably transformed cells (Early and Williams, 1987) were maintained under G418 selection (100  $\mu$ g/ml) and folic acid (1 mM) to ensure that the *discoidin I* promoter was turned off. Individual G418-resistant clones (a total of four) were then grown in HL5 media in the absence of both folate and G418; after 2 d the cells were harvested and prepared for Western blot or prepared for functional analysis (see below). All four clones showed identical phenotypic properties, and we selected one for more detailed studies.

### Antibody Generation

To N-terminally tag *racC* with GST, *racC* cDNA was cloned into the *XhoI* site of the bacterial protein expression vector pGEX (Pharmacia, Piscataway, NJ) 4T-1, thus generating the new vector pDS20. This vector was then transformed into the Stratagene (La Jolla, CA) *Escherichia coli* strain XL-1 blue, and clonal isolates were grown in the presence of 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside to induce expression of GST-RacC protein. The recombinantly expressed protein was purified using affinity chromatography with glutathione-Sepharose beads, and 100  $\mu$ g of protein were used to immunize two female white New Zealand rabbits (Cocalico Biologicals, Reamstown, PA), followed by two boosts of 50  $\mu$ g each at 2-wk intervals. After the second boost, polyclonal antisera was obtained and affinity purified using Sepharose beads that were covalently coupled to recombinantly expressed GST-RacC using a cyanogen bromide coupling system (Pharmacia).

### Subcellular Fractionation and Western Blot Analysis

Ax2 cells were collected by centrifugation ( $1000 \times g$  for 5 min) and resuspended at a density of  $2 \times 10^8$  cells/ml in MESES buffer (20 mM 2-[N-morpholino]ethane sulfonic acid, 1 mM EDTA, 250 mM sucrose, pH 6.5) supplemented with a protease inhibitor mixture (leupeptin [50  $\mu\text{g}/\text{ml}$ ], pepstatin A [10  $\mu\text{g}/\text{ml}$ ], benzamidine [2 mM], PMSF [1 mM]). Cells were homogenized on ice using a Dounce homogenizer (30 strokes), and light microscopy was performed to ensure that at least 95% of the cells were broken. Cells were then subjected to the following conditions: no treatment, 15 min incubation with 1% Triton X-100, or 15 min incubation with 100 mM sodium carbonate (peripherally associated membrane protein stripping agent). Membranes and supernatants were separated by differential centrifugation ( $100,000 \times g$  for 30 min), and the samples were resuspended in  $2\times$  (final) sample buffer (Laemmli, 1970). Proteins in the pellets and supernatants were resolved using discontinuous SDS-PAGE and then blotted to a nitrocellulose membrane (Towbin *et al.*, 1979). Western blots were developed as described (Buczynski *et al.*, 1997a,b).

### Scanning Electron Microscopy

Sterile, glass coverslips were inserted into 25-cm<sup>2</sup> tissue culture flasks and submerged in 10 ml of HL5 growth medium. Cells at an initial titer of  $10^5$  cells/ml were grown for 24 h to allow cell attachment to coverslips. The coverslips were removed and fixed in 1.25% glutaraldehyde buffered in 50 mM sodium cacodylate (pH 6.8). The attached cells were dehydrated in a graded acetone series, and critical point dried with liquid carbon dioxide. The coverslips with adhering dried cells were mounted on specimen stubs, sputter coated with 10 nm gold, and viewed with an ISI DS 130 scanning electron microscope.

### Confocal Microscopy Staining

For phalloidin staining, cells were attached to a glass coverslip and grown overnight in HL5 medium, and the coverslip was removed and clamped between the aluminum blocks of a circular Rose chamber (Rose *et al.*, 1958). The medium was replaced by fixing solution (1% formaldehyde, 0.1% glutaraldehyde, 0.01% Triton X-100 in HL5) containing 1  $\mu\text{M}$  rhodamine-phalloidin. The cells were imaged by a Bio-Rad (Richmond, CA) MRC-600 laser scanning confocal microscope equipped with a 25-mW krypton-argon laser (Ion Laser Technology, Salt Lake City, UT) attenuated with a 1% neutral density filter. A  $100\times$  (1.30 numerical aperture) Neofluar objective (Carl Zeiss, Thornwood, NY) was used. Fluorescence and differential interference contrast images were collected simultaneously, and confocal z-sections were acquired at 0.72- $\mu\text{m}$  intervals. Images were further analyzed and processed using NIH Image (written by Wayne Rasband, National Institutes of Health, Bethesda, MD, and available from the internet by anonymous FTP from zippy.nimh.nih.gov) and Adobe (Mountain View, CA) Photoshop.

### Phagocytosis, Fluid-Phase Pinocytosis, and Exocytosis Assays

Phagocytosis assays were performed as described using fluorescent latex beads (Buczynski *et al.*, 1997a). Fluid-phase endocytosis assays were performed according to the methods of Aubry *et al.* (1993a). Fluid-phase flux assays were performed as described using FITC-dextran (Buczynski *et al.*, 1997a). Intravesicular pH was measured as described (Cardelli *et al.*, 1989).

### Lysosomal Hydrolase Secretion Assays and Radiolabel Pulse-Chase Experiments

Cells in growth media were harvested from T-175 flasks, washed, resuspended in fresh media or 10 mM phosphate buffer at a titer of

$5 \times 10^6$  cells/ml, and placed in shaking suspension. At various times, cells were centrifuged, and the subsequent pellets and supernatants were assayed for  $\alpha$ -mannosidase activity. Radiolabel pulse-chase analysis of  $\alpha$ -mannosidase processing was performed as described (Mierendorf *et al.*, 1983).

## RESULTS

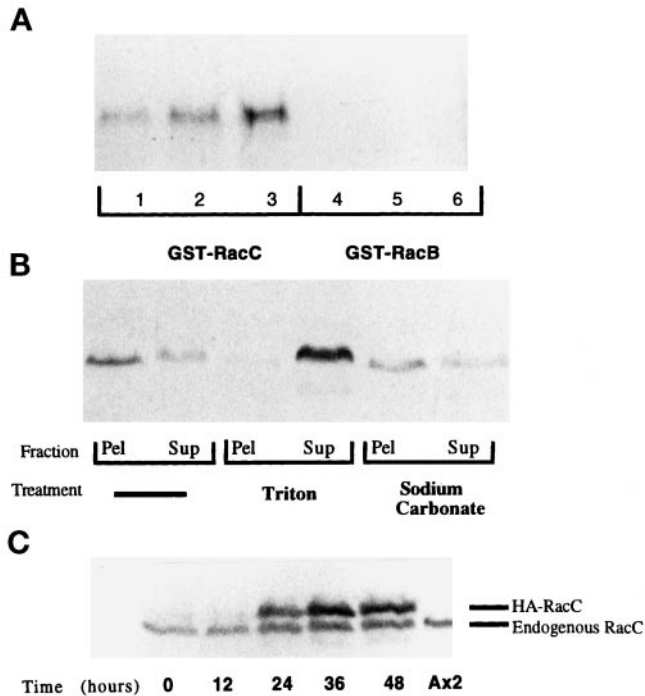
### Endogenous RacC Is Primarily Localized to the Membrane Fraction of *D. discoideum*

To examine the intracellular distribution of RacC in wild-type cells, an affinity-purified polyclonal antibody against recombinant RacC was generated (see MATERIALS AND METHODS). As shown in Figure 1A, anti-RacC polyclonal antibodies did not recognize bacterially expressed GST-RacB (lanes 4–6), the most closely related *Dictyostelium* Rho family protein to RacC. In contrast, these antibodies recognized a species of  $\sim 51$  kDa in the lanes loaded with GST-RacC (lanes 1–3), which is the predicted size of the GST-RacC fusion protein. RacE, which is 49.5% identical to RacC in amino acid sequence (Larochelle *et al.*, 1996), was also not recognized by these antibodies when expressed in *Dictyostelium* cells as a GFP-RacE fusion protein (unpublished results).

Published data indicate that GFP-RacC localizes to the plasma membrane of vegetative *D. discoideum* amoebae (Larochelle *et al.*, 1997), but these studies did not determine what percentage of cellular RacC was actually membrane associated. To answer this question, differential centrifugation of *Dictyostelium* lysates that were treated with various agents, followed by Western blot analysis of the resultant pellet and supernatant fractions using anti-RacC antibodies, was performed. As shown in Figure 1B, 70% of endogenous RacC protein was localized to the membrane fraction of cellular extracts, whereas 30% of endogenous RacC was cytosolic. To test whether RacC was associated with the F-actin cytoskeleton, cell lysates were incubated with Triton X-100 to solubilize membranes, and the Triton-insoluble F-actin cytoskeleton was pelleted by centrifugation. As shown in Figure 1B, Triton treatment released 100% of endogenous RacC into the supernatant, indicating that RacC was not associated with the F-actin cytoskeleton of *D. discoideum*. Furthermore, RacC behaved more like an integral membrane protein and not like a peripherally associated membrane protein, because treatment of lysed cells with sodium carbonate did not release additional RacC from the membrane fraction.

### RacC Overexpression Induces Changes in Cell Morphology and F-Actin Organization in a Phosphatidylinositol (PI) 3-Kinase-dependent Manner

To begin to define the function of RacC in *D. discoideum*, cell lines were created that conditionally over-



**Figure 1.** RacC is localized in the membrane fraction of lysed *D. discoideum* cells and can be conditionally overexpressed. (A) Western blot analysis of recombinantly expressed RacB and RacC-GST fusion proteins using LSU37 anti-RacC polyclonal antibodies. The lanes were loaded with increasing amounts of the GST-RacC and GST-RacB fusion proteins, separated by SDS-PAGE, and blotted to nitrocellulose membranes (lanes 1 and 4 contain 3.25 ng fusion protein; lanes 2 and 5 contain 6.5 ng fusion protein; lanes 3 and 6 contain 13 ng fusion protein). The LSU37 anti-RacC antisera reacted specifically with GST-RacC but not GST-RacB. (B) Western blot analysis of membranes and supernatants of *D. discoideum* Ax2 cells using rabbit polyclonal anti-RacC antisera. Homogenates were prepared as described in MATERIALS AND METHODS. Cells were then incubated with 1% Triton X-100, 100 mM sodium carbonate, or left untreated for 15 min. The cell lysates were then pelleted by differential centrifugation to separate the membranes from the supernatants and then prepared for SDS-PAGE followed by electrophoretic transfer to nitrocellulose membranes. As indicated in the text, 70% of endogenous RacC is located in the membrane fraction (lane 1), whereas 30% remains soluble (lane 2). None of the endogenous RacC was associated with the Triton X-100-insoluble F-actin cytoskeleton (lanes 3 and 4). Finally, RacC is intimately associated with the membranes, because sodium carbonate treatment did not release it from membranes. (C) Western blot analysis of an HA-RacC conditionally overexpressing cell line using *D. discoideum* RacC-specific LSU37 rabbit polyclonal antisera. Cells were transformed with the pVEII $\Delta$ ATG plasmid harboring *racC* cDNA, and G418-resistant clones were obtained. To induce expression of HA-RacC (under control of the *discoidein I* promoter), folate was removed from the growth medium, and cells were harvested for Western blot analysis at the indicated times.

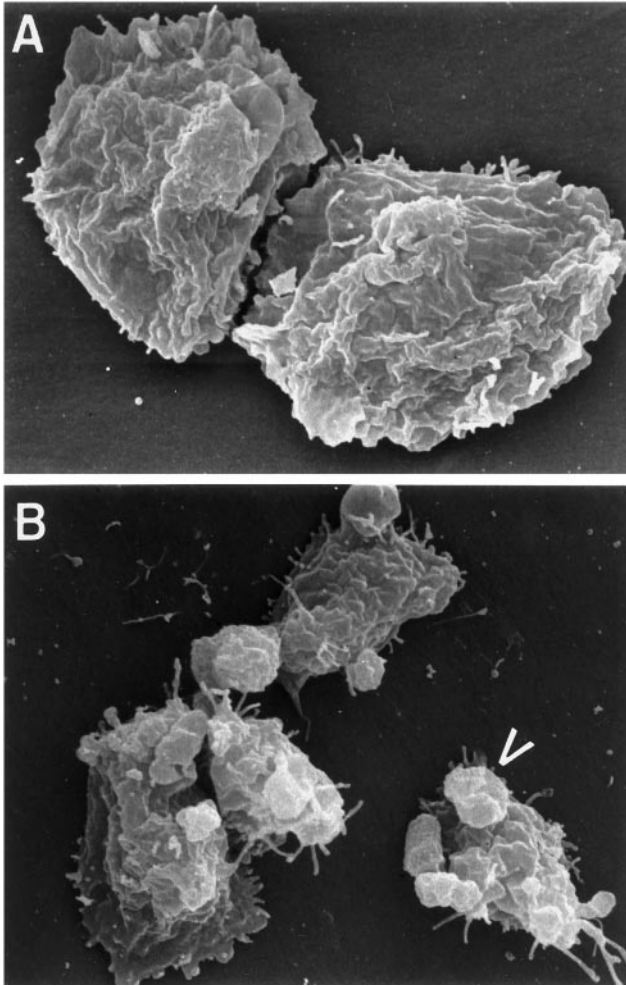
expressed the RacC protein (see MATERIALS AND METHODS). To allow us to differentiate between endogenously expressed and conditionally overexpressed forms of the protein, an HA epitope tag was added to the N terminus of RacC. As shown in Figure

1C, 24 h after folate was removed from the growth medium to induce expression from the *discoidein I* promoter, HA-RacC was expressed at a level two- to threefold higher than that of endogenous RacC. The same pattern and level of overexpression were consistently seen in four independent strains. After 48 h of induction, RacC was maximally expressed, at a level three- to fourfold higher than endogenous RacC. Interestingly, concurrent with RacC overexpression was the appearance of cells that contained phase-dark areas (membrane blebs) around their periphery (unpublished results). When RacC was maximally expressed (48 h after induction), up to 85% of the cells in a field displayed the “membrane blebs.”

To examine these bleb-like structures in greater detail, scanning electron microscopy was performed. Control Ax2 cells displayed a fairly uniform, ruffled appearance (Figure 2A), with an occasional “crown-like” structure observed. In contrast, RacC WT(+) cells displayed many protrusions that reached out from the dorsal and lateral surfaces of the cell (Figure 2B; see arrowhead). None of the bleb-like structures contained cup-like distal structures frequently found on “crowns.” Because Rho family GTPases have previously been shown to regulate the cellular F-actin cytoskeleton (Ridley and Hall, 1992; Ridley *et al.*, 1992), we hypothesized that RacC overexpression was altering cell morphology by mediating changes in the F-actin cytoskeleton of *Dictyostelium*.

To examine the organization of the F-actin cytoskeleton, control Ax2 and RacC WT(+) cells were fixed and incubated with FITC-phalloidin, and serial sections were visualized using confocal microscopy. We observed that the F-actin cytoskeleton of RacC WT(+) cells was strikingly different from that of control Ax2 cells (Figure 3). The F-actin of RacC WT(+) was organized into irregular structures that resembled the petals of a flower; therefore we termed these novel structures “petalopodia” (Figure 3, F–H). Interestingly, these petalopodia structures were distributed over the entire dorsal surface of the RacC WT(+) cells. In contrast, the F-actin of Ax2 cells was organized in a more cortical manner (Figure 3, B–D), with enriched staining at the pseudopod region and reduced amounts of F-actin at the dorsal surface of the cells.

Previous studies using a double-null mutant cell line,  $\Delta$ *ddpik1/ddpik2*, which contained only 25% of the wild-type level of PI 3,4,5-trisphosphate, indicated that PI 3-lipid kinases were important in regulating the organization of the F-actin cytoskeleton in *D. discoideum* (Buczynski *et al.*, 1997b; Zhou *et al.*, 1998). Furthermore, treatment of wild-type cells with the PI 3-kinase-specific inhibitors Wortmannin and LY294002 induced the same phenotypic effects observed in the  $\Delta$ *ddpik1/ddpik2* cell line (our unpublished result). This and other data suggest that DDPIK1 and DDPIK2 may be the major targets of LY294002 and



**Figure 2.** Scanning electron microscopy of Ax2 and RacC WT(+) cells. Control Ax2 cells (A) and HA-RacC WT(+) cells (B) were seeded on coverslips without folate, fixed with glutaraldehyde, critical point dried, sputter coated with gold, and prepared for scanning electron microscopy as described in MATERIALS AND METHODS. RacC WT(+) cells are characterized by protrusions that reach out from the surface of the cell (B, arrowhead), whereas the plasma membrane of control cells is characterized by a uniform, ruffled appearance.

wortmannin. To determine whether Wortmannin- and LY294002-sensitive PI 3-kinases were important for RacC-induced morphological and F-actin cytoskeleton changes, wild-type and RacC WT(+) cells were seeded on coverslips, treated with either LY294002 or wortmannin for 15 min, fixed, permeabilized, and stained with FITC-phalloidin. The cells were then examined using phase-contrast and fluorescence microscopy. As shown in Figure 4D, RacC WT(+) cells displayed a petalopodia-rich F-actin cytoskeleton; however, the addition of LY294002 (final concentration of 20  $\mu$ M) for 5 min caused the petalopodia to disappear from 95% of the cells, and the F-actin cytoskeleton of the

cells assumed a cortical distribution, similar to control Ax2 cells (Figure 4, compare F with B).

#### *Overexpression of RacC Stimulates Phagocytosis*

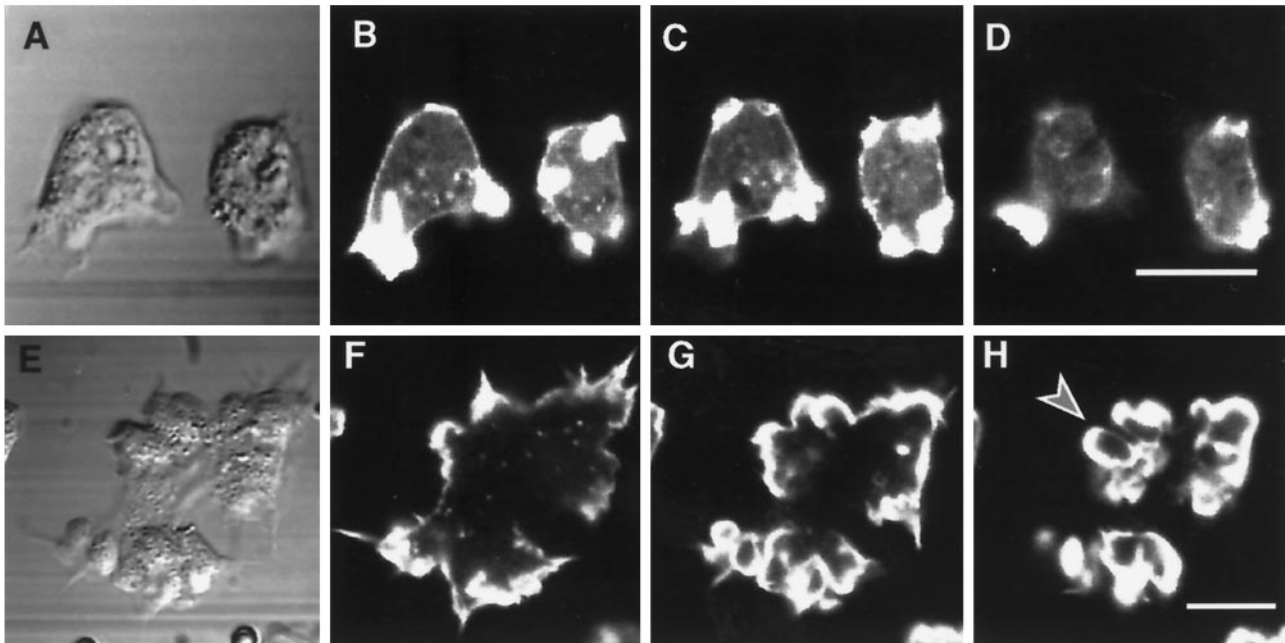
To determine whether RacC overexpression affected phagocytosis, a process dependent on the regulation of F-actin (Maniak *et al.* 1995; Temesvari *et al.*, 1996; Niewohner *et al.*, 1997), we examined the ability of control Ax2 and RacC WT(+) cells to internalize 1- $\mu$ m fluorescent latex beads. As shown in Figure 5A, RacC WT(+) cells internalized latex beads at a rate that was two- to threefold faster than control Ax2 cells. All four independent clones showed a comparable increase in phagocytosis, and we selected one clone for further analysis.

To ensure that the latex beads were actually being internalized into cells and were not just sticking to their surface, control experiments were carried out. Latex beads were added to cells at 4°C, a temperature at which beads will bind to the outside of cells but will not be internalized. In this case, we observed no difference in the number of beads attached to the outside of RacC WT(+) cells compared with control Ax2 cells, indicating that overexpression of RacC does not simply make cells more “sticky” for beads (unpublished results). Furthermore, we also performed the phagocytosis assay in the presence of both latex beads and the fluid-phase marker lucifer yellow (LY). At the end of the assay, the cells were washed in fresh HL5 media, seeded on coverslips, and viewed by phase-contrast and fluorescence microscopy. For both control Ax2 and RacC WT(+) cells, >90% of the latex beads associated with the cells were ringed with the fluid-phase marker LY, indicating that these beads were internalized and located on the inside of the cell (unpublished results). Finally, RacC WT(+) cells also internalized a natural food source, *E. coli*, faster than control cells, indicating that this response may be physiologically relevant (Figure 5F).

Surprisingly, although LY294002 treatment of RacC WT(+) cells appeared to reverse the formation of petalopodia, phagocytosis rates in these cells were not affected significantly (Figure 5B), indicating that PI 3-kinase activity and petalopodia structures might not be important for most of the RacC-induced stimulation of phagocytosis seen in RacC WT(+) cells.

#### *A Diacylglycerol-binding Protein May Be Required for Phagocytosis*

It has been demonstrated that protein kinase C (PKC) can transduce signals that lead to a rearrangement of the actin cytoskeleton and increases in phagocytosis in macrophages (Hartwig *et al.*, 1992; Allen and Aderem, 1996). Because RacC overexpression appeared to stimulate phagocytosis in *Dictyostelium*, it seemed plausi-



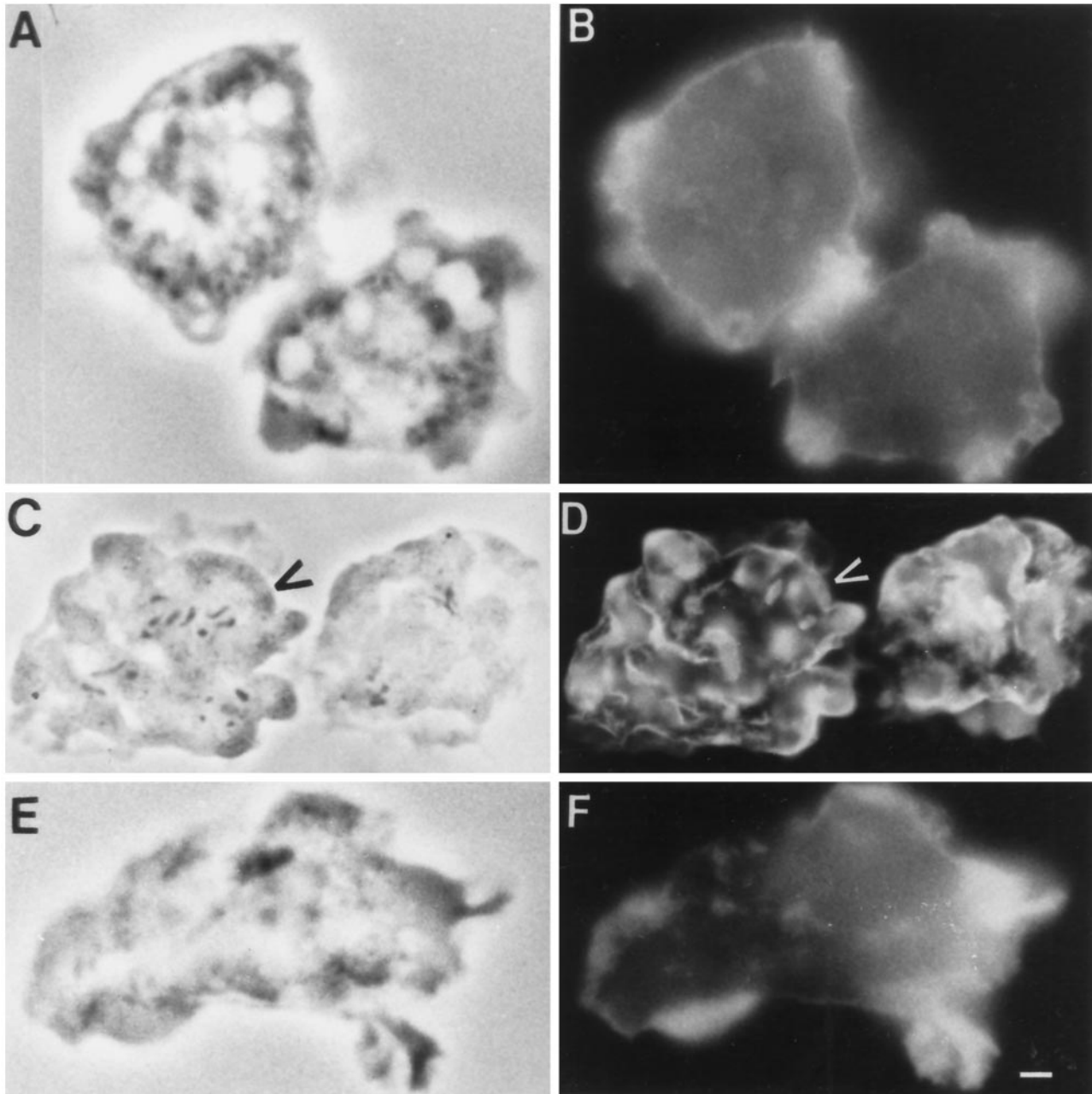
**Figure 3.** The F-actin cytoskeleton is altered in RacC WT(+) cells. Control Ax2 (A–D) and RacC WT(+) (E–H) cells were fixed and stained with rhodamine–phalloidin, and confocal z-sections were acquired at 0.72- $\mu\text{m}$  intervals. The fluorescence montage shows every third image in the series (sections from left to right are progressively more dorsal). DIC (A) was collected simultaneously with fluorescence image B, and DIC (E) was collected with image G. RacC WT(+) cells contain rounded F-actin protrusions (for example, petalopodia, arrowhead) over much of the dorsal surface, whereas control Ax2 cells display a normal F-actin cortex and enriched pseudopod staining. Bar, 10  $\mu\text{m}$ .

ble that PKC might be signaling upstream or downstream of RacC to mediate one or more of its effects. To test whether PKC or other proteins containing diacylglycerol (DAG)-binding motifs were important for RacC-mediated phagocytosis, we measured phagocytosis in RacC WT(+) cells in the presence of several PKC inhibitors. When wild-type or RacC WT(+) cells were preincubated with calphostin C (0.75  $\mu\text{M}$ ) for 20 min before latex beads were added to growing cells, phagocytosis appeared to be almost completely abrogated (Figure 5C). Because calphostin C competes with phorbol esters for binding to PKC enzymes (Kobayashi *et al.*, 1989), this result indicated that proteins containing DAG-binding motifs might be necessary for phagocytosis in *Dictyostelium*. To examine whether PKC was one of these proteins, we performed additional experiments using two specific inhibitors of PKC activity: bis-indolylmaleimide I and chelerythrine, both of which compete with ATP for binding to the active site of PKC. As shown in Figure 5, D and E, pretreatment with bis-indolylmaleimide I or chelerythrine had no effect on the rates of phagocytosis in control Ax2 or RacC WT(+) cells, suggesting that a DAG-binding protein, perhaps lacking PKC activity, was mediating RacC-induced phagocytosis in *Dictyostelium*.

#### *Macropinocytosis and Exocytosis of Fluid and Hydrolases Are Inhibited by RacC Overexpression*

RacC WT(+) cells internalized latex beads and bacteria at a faster rate than control Ax2 cells, perhaps because of a general stimulation of plasma membrane internalization, which might also result in an increase in pinocytosis. Therefore, we determined the rate of fluid-phase pinocytosis by measuring the ability of control Ax2 or RacC WT(+) cells to internalize the fluid-phase marker FITC–dextran (Figure 6A). Unexpectedly, RacC WT(+) cells internalized FITC–dextran fluid phase at a rate three times slower than that of control cells. This observation indicated that 1) the RacC-mediated positive effect on phagocytosis was specific for particles, and not the result of a general stimulation of all endocytic processes, and 2) fluid-phase pinocytosis and phagocytosis may independently regulate processes in *D. discoideum*.

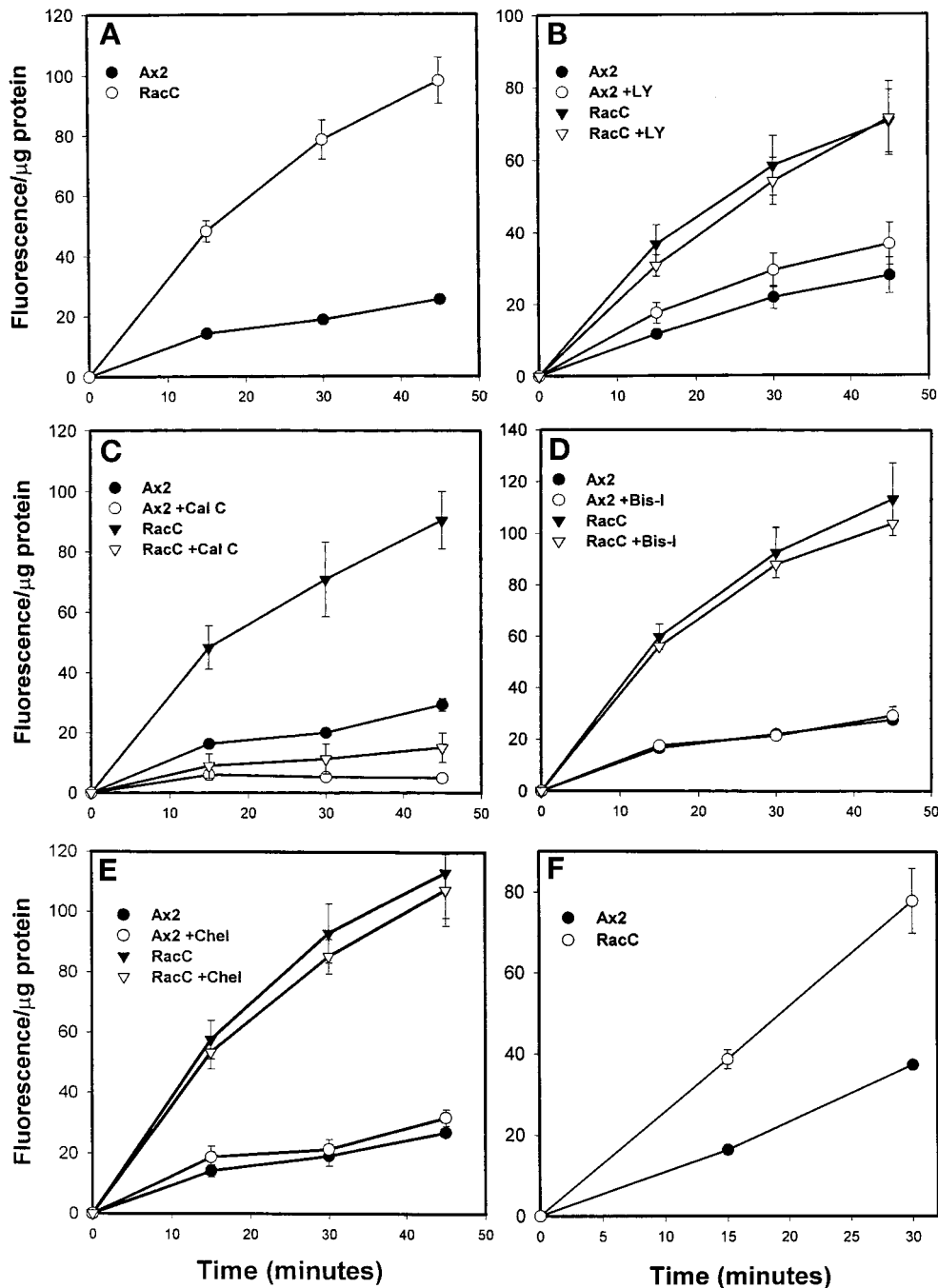
To determine whether RacC overexpression decreased macropinocytosis, one of the major pathways for fluid-phase internalization in *Dictyostelium* (Hacker *et al.*, 1997), cells were allowed to adhere to coverslips and were then incubated with the fluid-phase marker LY for 5 min in growth medium. After the cells were washed in fresh medium, they were viewed using phase-contrast and fluorescence microscopy. The results of this experi-



**Figure 4.** RacC-induced morphology is inhibited by PI 3-kinase inactivation. (A and B) Control Ax2 cells; (C and D) RacC WT(+) cells. RacC WT(+) cells were also treated with the PI 3-kinase inhibitor wortmannin ( $2 \mu\text{M}$ ) for 20 min (E and F). Cells were seeded on coverslips, pretreated with LY294002 or DMSO, fixed, permeabilized, stained with FITC-phalloidin, and viewed using phase-contrast (A, C, and E) or fluorescence (B, D, and F) microscopy. RacC WT(+) cells display the novel petalopodia actin-based structures on their surface (C and D; see arrowheads), whereas control Ax2 cells display actin in a cortical distribution (A and B). LY294002 treatment of RacC WT(+) cells reversed the formation of petalopodia (E and F) and caused the F-actin to redistribute to a more cortical pattern.

ment are shown in Figure 7. Few (<10%) of the RacC WT(+) cells internalized LY into large vesicles (Figure 7, C and D). In contrast, in a single field, 70% of control Ax2 cells formed at least one large vesicle that we consider to be a macropinosome (Figure 7, A and B).

In *D. discoideum*, fluid-phase markers are normally ingested, transferred to acidic lysosomal vesicles, and then transported to large nonacidic organelles termed postlysosomes before their release from the cell (Aubry *et al.*, 1993b; Padh *et al.*, 1993); no rapid recy-



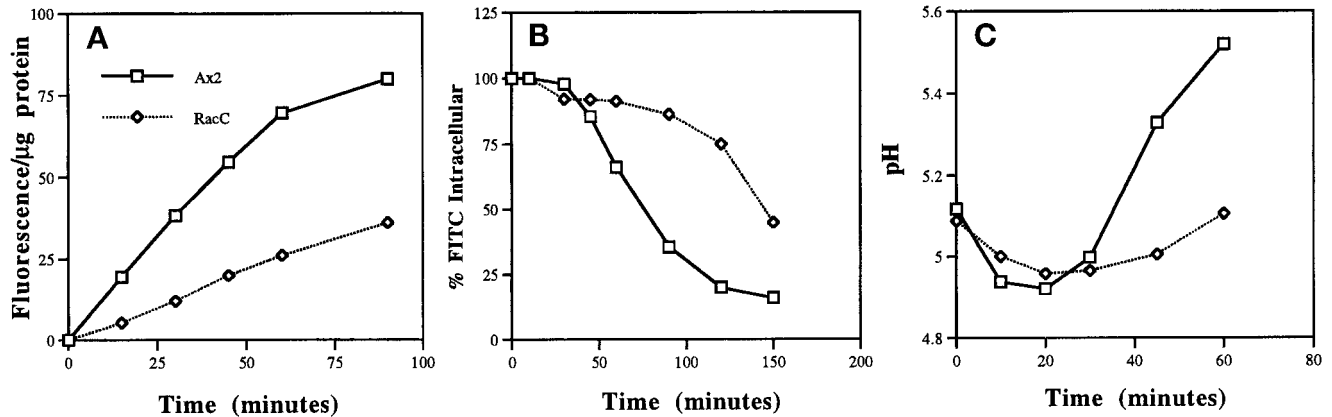
IBM, Armonk, NY). (A) No treatment. Ax2,  $14.2 \pm 1.1$  ( $n = 12$ ); RacC WT(+),  $48.2 \pm 3.5$  ( $n = 12$ ). (B) LY294002. Untreated: Ax2,  $11.6 \pm 1.3$  ( $n = 4$ ); RacC WT(+),  $36.7 \pm 5.4$  ( $n = 4$ ). LY294002-treated: Ax2,  $17.5 \pm 2.9$  ( $n = 4$ ); RacC WT(+),  $30.7 \pm 3.0$  ( $n = 4$ ). (C) Calphostin C. Untreated: Ax2,  $16.2 \pm 0.8$  ( $n = 3$ ); RacC WT(+),  $48.4 \pm 7.2$  ( $n = 3$ ). Calphostin C-treated: Ax2,  $6.0 \pm 1.8$  ( $n = 3$ ); RacC WT(+),  $8.9 \pm 4.0$  ( $n = 3$ ). (D) Bis-indolylmaleimide. Untreated: Ax2,  $16.5 \pm 1.0$  ( $n = 3$ ); RacC WT(+),  $59.9 \pm 5.0$  ( $n = 3$ ). Bis-indolylmaleimide I-treated: Ax2,  $17.5 \pm 1.4$ ; RacC WT(+),  $56.1 \pm 2.0$  ( $n = 3$ ). (E) Chelerythrine. Untreated: Ax2,  $14.2 \pm 2.0$  ( $n = 3$ ); RacC WT(+),  $57.6 \pm 6.4$  ( $n = 3$ ). Chelerythrine-treated: Ax2,  $18.7 \pm 3.7$ ; RacC WT(+),  $53.4 \pm 5.5$  ( $n = 3$ ). (F) FITC-*E. coli*: Ax2,  $16.4 \pm 0.5$  ( $n = 3$ ); RacC WT(+),  $38.7 \pm 2.4$  ( $n = 3$ ).

cling fluid-phase compartment has been observed. Interestingly, transport from acidic lysosomes to postlysosomes is blocked by cytochalasin A treatment,

which disrupts the actin cytoskeleton. To determine whether RacC overexpression, demonstrated above to regulate actin cytoskeleton distribution, also affected

**Figure 5.** RacC overexpression stimulates phagocytosis. For A–E, cells were incubated with 1- $\mu$ m fluorescent latex beads for the indicated time after a 20-min pretreatment with the appropriate drug or DMSO (the diluent). Cells were collected by centrifugation, washed, and prepared for fluorimetry. Shown are the rates obtained by averaging the indicated time points from independent experiments that were performed. The data are reported as fluorescence normalized to total protein/sample to account for potential differences in cell size between the two strains. (A) Untreated cells. RacC overexpression stimulates the rate of 1- $\mu$ m latex bead uptake three- to fourfold over control Ax2. (B) Cells treated with the PI 3-kinase inhibitor LY294002 (20  $\mu$ M). Inhibition of PI 3-kinase only partially represses the RacC-induced stimulation of phagocytosis. Control cells treated with PI 3-kinase inhibitors actually display a slight stimulation of bead uptake. (C) Cells treated with calphostin C (0.75  $\mu$ M), an inhibitor of enzymes containing DAG-binding motifs. Calphostin C treatment completely prevented the uptake of latex beads in control and RacC WT(+) cells. (D and E) Cells treated with bis-indolylmaleimide I (10  $\mu$ M; D) or chelerythrine (10  $\mu$ M; E), specific inhibitors of PKC. Phagocytosis was unaffected by treating cells with drugs that specifically inhibit PKC. (F) Cells were incubated with FITC-labeled *E. coli*, and internalization was measured in the same manner as for latex beads. All values are reported as fluorescence per micrograms of protein  $\pm$  SEM. Statistical analysis was performed on the data obtained for the 15-min (shown below) and 30-min time points to test the significance of the reported increases and decreases using the Student's two-tailed *t* test (Instat,



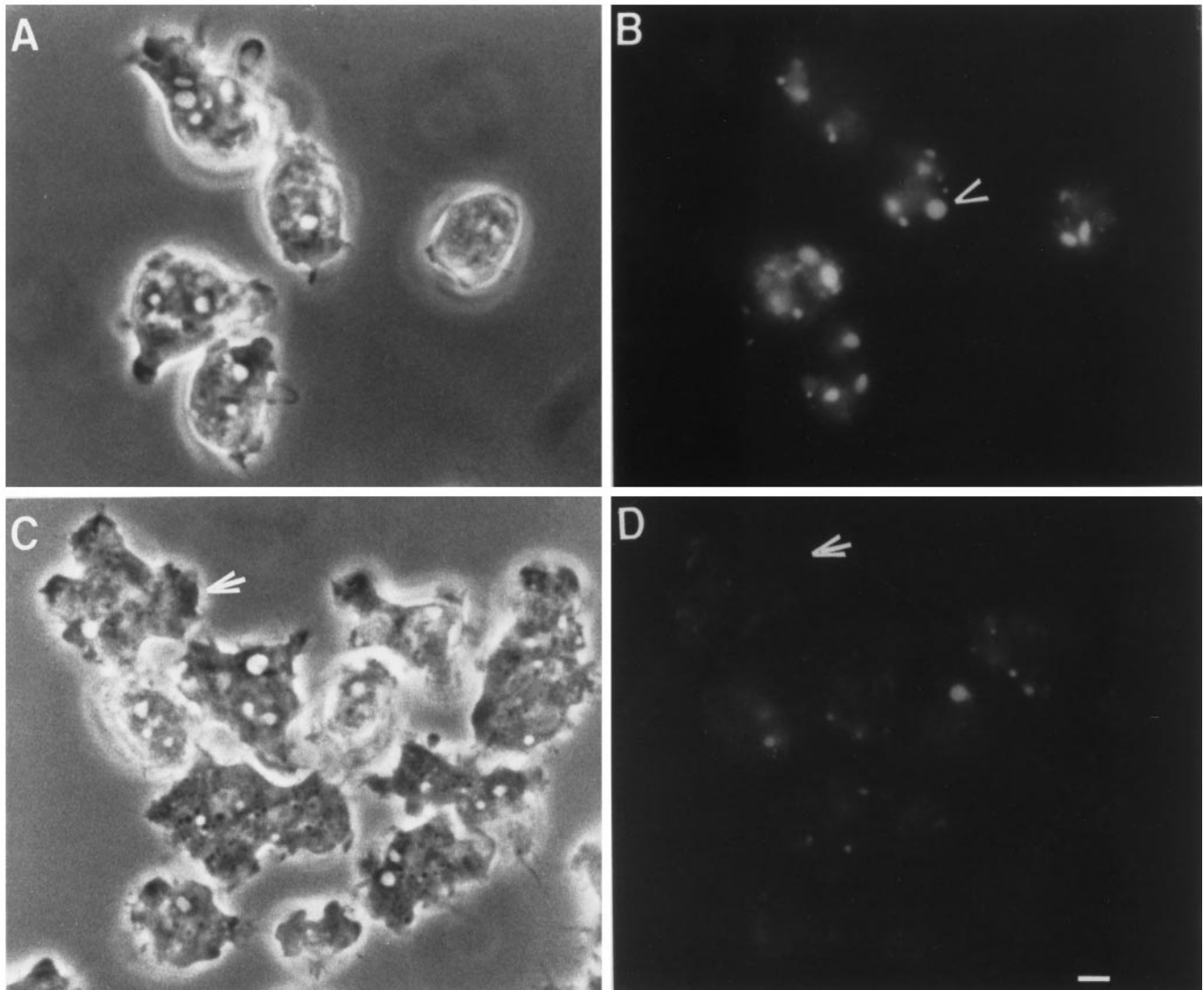


**Figure 6.** RacC WT(+) cells are defective in both fluid-phase endocytosis and exocytosis. (A) Control and RacC WT(+) cells were incubated with FITC-dextran for the times indicated, collected by centrifugation, washed, and prepared for spectrofluorimetry (see MATERIALS AND METHODS). The rates are reported as fluorescence per micrograms of protein to correct for any differences in cell size among the strains examined. RacC WT(+) cells internalized FITC-dextran at a rate two to three times slower than that of control Ax2 cells. Data from the 30-min time point: Ax2,  $37.0 \pm 1.5$  ( $n = 3$ ); RacC WT(+),  $11.6 \pm 1.3$  ( $n = 3$ ). (B) Cells were pulsed with FITC-dextran for 10 min to label the early compartments of the endolysosomal system, washed, and resuspended at the original titer in fresh medium. The cells were then collected by centrifugation at the indicated times, and the percentage of FITC-dextran that remained inside the cell was calculated as the ratio of the fluorescence at the indicated time compared with the fluorescence at time = 0. Shown is a representative experiment of three independent ones performed that indicated that RacC WT(+) cells displayed a delay in the release of fluid phase from the cell. In control Ax2 cells, fluid phase began to be released from the endolysosomal system after 45 min, whereas RacC WT(+) cells did not begin to release fluid phase until after 90 min. (C) Cells were pulsed with FITC-dextran for 10 min, chased in fresh media, and treated as described in B. The cells were then collected by centrifugation at the indicated times, and the intracellular pH was calculated as described in MATERIALS AND METHODS. There appeared to be a delay in the transport of fluid phase from acidic to nonacidic compartments, because FITC-dextran was released from an acidic compartment much more slowly in RacC WT(+) cells than in control Ax2 cells. This experiment was repeated two more times with the same results.

fluid-phase exocytosis, we performed fluid-phase flux experiments. Control Ax2 cells, pulsed for 10 min with FITC-dextran in growth medium and then resuspended in fresh media, began to efflux their fluid phase after 45–60 min (Figure 6B); in contrast, RacC WT(+) cells did not start exocytosing FITC-dextran until after 60–90 min. Furthermore, after 2.5 h control Ax2 cells had exocytosed almost all of the FITC-dextran into the supernatant, whereas RacC WT(+) cells still retained 40% of the FITC-dextran internalized during the pulse period. These data suggest that the movement of fluid phase through the endolysosomal system was being delayed in RacC WT(+) cells. To determine the step at which the movement of fluid phase was being delayed, the endosomal pH of RacC WT(+) cells was measured by a dual excitation ratio method using FITC-dextran as a pH probe (see MATERIALS AND METHODS). As indicated in Figure 6C, in wild-type cells, FITC-dextran moved from a slightly acidic compartment to more acidic lysosomes within 20 min of entering the cell. From these acidic compartments, fluid phase then moved to less acidic postlysosomes within 60 min; however, in RacC WT(+) cells, the fluid phase reached acidic compartments at a slightly reduced rate and only slowly entered less acidic compartments, presumably postlysosomes. These data indicate that RacC overexpression

leads to a delay at two different steps along the endolysosomal pathway: 1) in the internalization of fluid phase, via macropinocytosis, into the cell, and 2) in the movement of fluid phase from acidic to less acidic compartments.

Next, we examined the rate of secretion of the lysosomal hydrolase  $\alpha$ -mannosidase in growth medium to determine whether RacC overexpression affected lysosomal hydrolase secretion as well as the movement of internalized fluid phase markers through the endolysosomal system of *D. discoideum*. Not surprisingly, our experiments indicated that RacC WT(+) cells secreted  $\alpha$ -mannosidase at a much slower rate than the control Ax2 cell line when suspended in growth medium (Figure 8, bottom panel). In response to hypo-osmotic shock or starvation, *D. discoideum* cells more rapidly secrete lysosomal hydrolases into the extracellular environment (Cardelli, 1993). Because RacC WT(+) cells were defective in the secretion of  $\alpha$ -mannosidase in HL5 growth media, we predicted that they might be defective in the secretion of lysosomal enzymes induced by starvation conditions. To test this, secretion experiments were performed in the same manner as described above, except that cells were suspended in phosphate buffer to induce secretion. As predicted, the starved control Ax2 cells secreted  $\alpha$ -mannosidase at a higher rate than cells suspended in growth medium

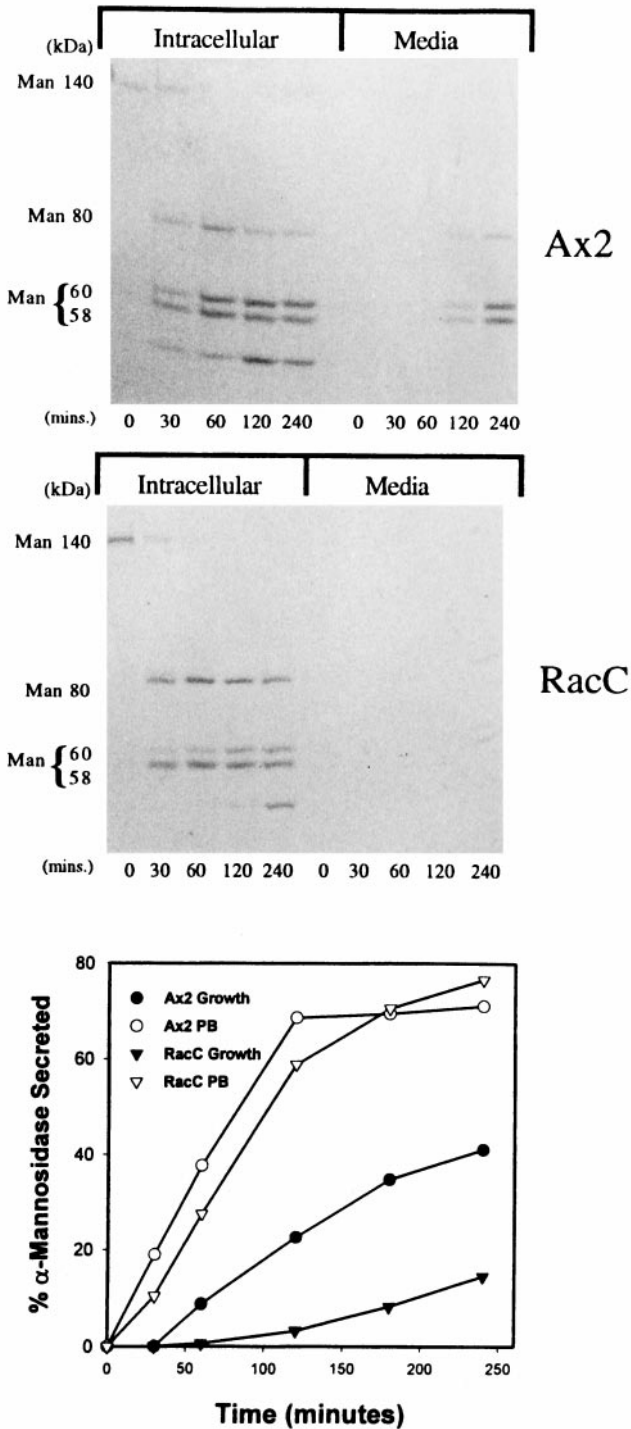


**Figure 7.** Macropinocytosis is inhibited in RacC WT(+) cells. Control Ax2 cells (A and B) and RacC WT(+) cells (C and D) were incubated with the fluid-phase marker lucifer yellow for 5 min to load up macropinosomes and then washed in fresh media, placed on a coverslip, and examined using phase-contrast (A and C) and fluorescence (B and D) microscopy. Because the size of micropinosomes is below the level of detection, only larger macropinosomes can be visualized by fluorescence microscopy. RacC WT(+) cells fail to form macropinosomes [arrow points to a RacC WT(+) cell], whereas control Ax2 cells contain three to four macropinosomes per cell (arrowhead). Bar, 2  $\mu$ m.

(Figure 8, bottom panel); however, in contrast to secretion rates observed in growth medium, the starved RacC WT(+) cell lines secreted  $\alpha$ -mannosidase at the same rate as control cells. This indicates that RacC may play a negative role in the secretion of  $\alpha$ -mannosidase under growth conditions but does not appear to play a significant role in regulating the secretion of lysosomal hydrolases from cells responding to starvation.

Because RacC overexpression negatively affected the secretion of  $\alpha$ -mannosidase and fluid phase from cells suspended in growth medium, we suspected that overexpression of this G protein might also affect

other lysosomal hydrolase trafficking events, such as proteolytic processing and sorting. To examine the kinetics of processing and the efficiency of sorting of lysosomal  $\alpha$ -mannosidase, cells were subjected to a radiolabel pulse-chase protocol followed by immunoprecipitation of cellular and extracellular radiolabeled  $\alpha$ -mannosidase. The immunoprecipitated protein was subjected to SDS-PAGE followed by autoradiography. The top two panels of Figure 8 indicate that RacC overexpression did not appear to have an effect on the processing rates of the 140-kDa precursor to the mature 58- and 60-kDa forms or the targeting efficiency of  $\alpha$ -mannosidase to lysosomes, as evidenced by the low



**Figure 8.** RacC overexpression does not affect the processing, targeting, or secretion of lysosomal  $\alpha$ -mannosidase. (Top two panels) Cells were pulsed with [ $^{35}$ S]methionine in HL5 growth medium for 20 min, washed, and placed in fresh medium. At the indicated times, cells and supernatants were collected by centrifugation, and  $\alpha$ -mannosidase was immunoprecipitated from the samples. The resultant immunoprecipitates were fractionated using SDS-PAGE and then subjected to autoradiography. The processing and sorting

level of secretion of radiolabeled 140-kDa precursor polypeptides; however, consistent with the secretion assays described above, there was an apparent defect in the rate of secretion of radiolabeled  $\alpha$ -mannosidase from RacC WT(+) *D. discoideum* cells. In control cells, the mature form of  $\alpha$ -mannosidase (and some of the 80-kDa intermediate) began to be secreted into the extracellular milieu after a 120-min chase; however, RacC WT(+) cells did not begin to secrete  $\alpha$ -mannosidase until after a 240-min chase.

**DISCUSSION**

In studies reported here, we demonstrate that modest overexpression of a Rho family GTPase, RacC, results in changes in cell morphology and actin cytoskeleton organization in the simple eukaryote *D. discoideum* and leads to a significant increase in phagocytosis rates. Cells overexpressing HA-tagged RacC at a level three- to fourfold higher than endogenous RacC contained F-actin-rich "membrane blebs" that were organized into dynamic dorsal and lateral surface structures that we term petalopodia, because they resemble the petals of a flower. The petalopodia structures described here are not to be confused with earlier defined structures termed "petaloid coelomocytes," from the sea urchin *Strongylocentrotus droebachiensis* (Edds, 1977), which spontaneously change into filopodia to induce the clotting process in the celom of this organism. Although the petalopodia appearance is reminiscent of coronin-containing "crown-like" structures described previously by Hacker *et al.* (1997), we believe that they are distinct from one another. First of all, crown-like structures are predominantly located on the dorsal surface of the cell, whereas we observed petalopodia forming on both the lateral edges and the dorsal surfaces of RacC WT(+) cells. In addition, crown-like structures were only infrequently observed in RacC WT(+) cells. Finally, crown-like structures are

**Figure 8 (cont).** of  $\alpha$ -mannosidase is normal in both control Ax2 and RacC WT(+) cells.  $\alpha$ -Mannosidase is synthesized as a 140-kDa precursor protein, which is processed to a mature 58- and 60-kDa form through an 80-kDa intermediate protein. There is a defect in the secretion of mature  $\alpha$ -mannosidase from the postlysosomal compartment of RacC WT(+) cells, however. In control Ax2 cells, the mature form of  $\alpha$ -mannosidase (and some of the 80-kDa intermediate) is secreted into the extracellular milieu after a 120-min chase. RacC WT(+) cells did not begin to secrete  $\alpha$ -mannosidase until 240 min after chase. (Bottom panel) Control Ax2 and RacC WT(+) cells were incubated in HL5 growth medium or phosphate buffer and were harvested at the indicated times. The samples were separated into cell pellets and supernatants by centrifugation, and the amount of  $\alpha$ -mannosidase activity in these samples was calculated. RacC WT(+) cells were defective in the secretion of  $\alpha$ -mannosidase compared with control cells in growth medium; however, under starvation conditions, control Ax2 and RacC WT(+) cells secrete the lysosomal hydrolase  $\alpha$ -mannosidase at equivalent rates. This experiment was repeated two more times with identical results.

associated with the engulfment of both fluid-phase and particulate matter and open up as a cup-like structure to bring material into the cell. Using video microscopy to examine GFP-labeled ABP-120 in the RacC WT(+) cells, we observed that the cellular F-actin never formed into open crown-like structures (unpublished results). Because the petalopodia are also morphologically different from other previously identified actin-based structures such as lamellipodia or filopodia, they may represent a new form of actin-based structure.

The biochemical mechanisms regulating petalopodia formation are not known, although data presented here suggest that their formation involves RacC and one or more wortmannin- or LY294002-sensitive PI 3-kinase(s). The fact that PI 3-kinase activity appeared necessary for the formation of petalopodia was not unexpected because previous studies have indicated that these enzymes were also necessary for PDGF-induced membrane ruffling in porcine aortic endothelial cells (Wennstrom *et al.*, 1994) and the Ras-mediated signal transduction pathway that leads to actin cytoskeleton changes (Rodriguez-Viciano *et al.*, 1997). Rac can also interact with PI 3-kinases, and growth factors can activate Rac through activation of PI 3-kinases (Hawkins *et al.*, 1995; Bokoch *et al.*, 1996); however, our results do not distinguish between PI 3-kinase and RacC operating in the same or parallel pathways to regulate the formation of petalopodia, although we favor the first possibility.

RacC WT(+) cells internalized both latex beads and bacteria at three times the rate of control cells. This same phenotype was observed for cells overexpressing non-epitope-tagged RacC, suggesting that the HA epitope does not influence phagocytosis (unpublished results). Interestingly, treating cells with the PI 3-kinase-specific inhibitors Wortmannin and LY294002, which inhibited the formation of petalopodia, only slightly and insignificantly impaired RacC WT(+) cells in their ability to internalize latex beads and bacteria (Figure 6B). These drugs also did not inhibit phagocytosis in control cells and, in fact, caused a slight stimulation in bead uptake. These results indicate that, although PI 3-kinases may be necessary for RacC-induced morphological and actin cytoskeletal changes, they do not play a major role in RacC-induced phagocytosis, and that RacC-induced actin cytoskeleton changes (petalopodia formation) may not play an important role in the stimulation of phagocytosis. These results are in contrast to other studies that suggested PI 3-kinases were necessary for phagocytosis in macrophages (Araki *et al.*, 1996). Although our results were unexpected, they are consistent with previous observations indicating that membrane ruffling (dependent on PI 3-kinase activity) could be functionally uncoupled from a stimulation of pinocytosis in mammalian cells. (Kotani *et al.*, 1994; Li *et al.*, 1997).

Interestingly, RacC WT(+) cells were inhibited in their ability to internalize FITC-dextran markers via macropinocytosis, although they internalized particles at rates higher than control cells. This is consistent with previous experiments that indicated that Rac1 and Rho may negatively regulate receptor-mediated endocytosis (Lamaze *et al.*, 1996), although Rho proteins have been implicated in positively regulating bulk fluid-phase pinocytosis (Schmalzing *et al.*, 1995). This is an important observation, because it has been proposed previously that phagocytosis and macropinocytosis are biochemically similar processes in macrophages (Araki *et al.*, 1996) and *D. discoideum* (Hacker *et al.*, 1997). In contrast, our data suggest that these processes are mechanistically distinct in *D. discoideum*. Because the formation of crown-like structures is associated with macropinocytosis (Hacker *et al.*, 1997), this evidence also supports our hypothesis that the actin-based petalopodia structures are functionally distinct from crown-like structures; however, our results presented here do not distinguish between RacC acting as a negative regulator of macropinocytosis or as an inducer of the formation of F-actin structures that are incompatible with macropinocytosis.

Several lines of evidence indicate that PKC may play a role in the regulation of the actin cytoskeleton and/or phagocytosis in mammalian cells such as macrophages (Allen and Aderem, 1996). For instance, PKC is activated in response to ligation of the Fc receptor in human monocytes (Zheleznyak and Brown, 1992), suggesting that it may be involved in signal transduction leading to phagocytosis; however, our studies presented here indicated that PKC per se might not play a major role in regulating phagocytosis in *Dictyostelium*, because treatment of cells with the PKC inhibitors bis-indolylmaleimide I and chelerythrine did not reduce phagocytosis. Importantly, it has been shown previously that bis-indolylmaleimide I-sensitive forms of PKC do exist in *D. discoideum* (Phillips *et al.*, 1997); however, bacteria and latex bead uptake were significantly inhibited when cells were treated with the inhibitor calphostin C (a drug that competes with phorbol esters for binding to DAG-binding domains), suggesting that a non-PKC protein containing a DAG-binding motif was mediating phagocytosis in *Dictyostelium*. Also, calphostin C treatment effectively reversed the formation of petalopodia in RacC WT(+) cells. Whether this unidentified protein is signaling upstream or downstream of RacC remains to be determined at this time and will require the generation of a *Dictyostelium* cell line that overexpresses a constitutively active form of RacC. Interestingly, many Rho family GEFs contain DAG-binding domains (Cerione and Zheng, 1996), suggesting that the target of calphostin C may be an upstream activator of RacC.

In addition to inhibiting macropinocytosis, RacC overexpression also led to a decrease in the rate of

efflux of fluid-phase and lysosomal enzymes from the endolysosomal system of *D. discoideum*. This block appeared to be imposed at the level of fluid-phase movement from acidic to nonacidic compartments. Although yet to be demonstrated conclusively, this result suggests that RacC overexpression may reduce the transport of fluid phase from lysosomes to postlysosomes, a process that may depend on proper F-actin regulation and the function of other GTPases and lipid kinases. For instance, in addition to RacC, we (and others) have demonstrated that Rab7, a Rab4-like GTPase (RabD), the PI 3-kinases DdPIK1 and DdPIK2, vacuolin B, and actin regulate this step (Bush *et al.*, 1996; Temesvari *et al.*, 1996; Buczynski *et al.*, 1997a,b; Hacker *et al.*, 1997; Jenne *et al.*, 1998). It will be important to determine whether (and how) these proteins functionally interact and, if so, the order of interaction required for the formation of postlysosomes. Why RacC overexpression mediates disparate effects on phagocytosis and fluid-phase pinocytosis and trafficking is unclear at this time, but it raises the question that we are currently experimentally attempting to answer: are the effects mediated by RacC on the movement of fluid-phase matter throughout the endolysosomal system direct, or are they indirect effects caused by a recruitment of F-actin that leads to a stimulation of phagocytosis?

We and others have demonstrated recently that other GTPases, not belonging to the Rho subfamily, can also regulate the distribution of the cellular actin cytoskeleton and endolysosomal processes. For example, overexpressing wild-type or constitutively activated (GTP-bound) forms of a Rap1-like GTPase induced the formation of lamellipodia (Rebstein *et al.*, 1997) and stimulated phagocytosis in *D. discoideum*, whereas overexpressing dominant negative (GDP-bound) forms of Rap1 inhibited phagocytosis (unpublished results). Taken together, these experiments suggest a possible model in which small GTPases may interact in the signal transduction pathway regulating phagocytosis in *Dictyostelium*. Defining the biochemical nature of these interactions is a subject of future investigations.

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