RacG Regulates Morphology, Phagocytosis, and Chemotaxis

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RacG is an unusual member of the complex family of Rho GTPases in Dictyostelium. We have generated a
knockout (KO) strain, as well as strains that overexpress wild-type (WT), constitutively active (V12), or
dominant negative (N17) RacG. The protein is targeted to the plasma membrane, apparently in a nucleotide-
dependent manner, and induces the formation of abundant actin-driven filopods. RacG is enriched at the rim
of the progressing phagocytic cup, and overexpression of RacG-WT or RacG-V12 induced an increased rate of
particle uptake. The positive effect of RacG on phagocytosis was abolished in the presence of 50 μM LY294002,
a phosphoinositide 3-kinase inhibitor, indicating that generation of phosphatidylinositol 3,4,5-trisphosphate
is required for activation of RacG. RacG-KO cells showed a moderate chemotaxis defect that was stronger in
the RacG-V12 and RacG-N17 mutants, in part because of interference with signaling through Rac1. The in vivo
effects of RacG-V12 could not be reproduced by a mutant lacking the Rho insert region, indicating that this
region is essential for interaction with downstream components. Processes like growth, pinocytosis, exocytosis,
cytokinesis, and development were unaffected in Rac-KO cells and in the overexpressor mutants. In a cell-free
system, RacG induced actin polymerization upon GTPγS stimulation, and this response could be blocked by
an Arp3 antibody. While the mild phenotype of RacG-KO cells indicates some overlap with one or more
Dictyostelium Rho GTPases, like Rac1 and RacB, the significant changes found in overexpressors show that
RacG plays important roles. We hypothesize that RacG interacts with a subset of effectors, in particular those
considered with shape, motility, and phagocytosis.

Although initially described as major regulators of cytoskeletal remodeling, Rho GTPases have been shown to be also
involved in the regulation of cellular processes as diverse as
decay, vesicle trafficking, morphogenesis, cytokinesis,
transcriptional activation, and cell cycle progression (6, 25, 60). In mammals, the Rho family currently consists of about 20
distinct proteins. Three subfamilies of Rho GTPases, Rho,
Rac, and Cdc42, have been widely studied for their effects in
actin organization and other processes in mammalian cells
(20). Considerable progress has been made during the last few
years in the understanding of the mechanism of action of these
three Rho GTPases, an ever-increasing number of effectors
have been described, and some of the pathways regulated by
these proteins have been well delineated. However, other sub-
families, like Rnd or RhoBTB, and other Rho GTPases not
grouped in defined subfamilies remain largely uncharacterized.
In Dictyostelium discoideum, an apparently simpler organism,
the Rho family comprises 18 members (47, 56, 59). Rac1a/1b/
1c, RacF1/F2, and, more loosely, RacB and the GTPase
domain of RacA (a member of the subfamily of RhoBTB pro-
teins) can be grouped in the Rac subfamily. None of the
additional Dictyostelium Rho-related proteins belongs to any of
the other well-defined subfamilies, like Cdc42, Rho, or Rnd.

Dictyostelium amebas are equipped with a complex actin
cytoskeleton that endows the cells with chemotactic and motile
behavior comparable to that of leukocytes, and like macro-
phages and neutrophils, Dictyostelium is a professional phagocy-
te. This makes this organism an attractive model to investi-
gate structural and regulatory aspects of the actin cytoskeleton
(43). In addition to Rho GTPases, other components of Rho-
regulated signal transduction pathways known in mammalian
cells are also present in Dictyostelium, like RhoGDI (guanine
nucleotide dissociation inhibitor), numerous RhoGAPs (GTPase-
activating proteins) and RhoGEFs (guanine nucleotide exchange
factors), Pak (p21-activated kinase), Scar, Wasp (Wiskott-
Aldrich syndrome protein), the Arp2/3 complex, and formins,
to mention a few (56).

Studies are accumulating that address the roles of Dictyostelium
Rho GTPases. RacC plays roles in actin cytoskeleton
organization, endocytosis, and cytokinesis (44, 50). RacC ap-
ppears to be essential for cytokinesis and actin polymerization
but is not involved in phagocytosis (29, 44). RacF1 localizes to
early phagosomes, macropinosomes, and transient cell-to-cell
contacts, but inactivation of the racF1 gene does not impair
endocytosis and other actin-dependent processes, probably be-
cause of the presence of the closely related protein RacF2 (41).
RacF2 is highly enriched in gametes and appears to be in-
volved in the regulation of sexual and asexual development
(35). RacB and the three Rac1 proteins Rac1a, Rac1b, and
Rac1c have been the most extensively studied. They are involved in chemotaxis, cell motility, endocytosis, cytokinesis, and development (13, 31, 39). More recently, RacH has been implicated specifically in the regulation of endocytic-vesicle trafficking (53). However, with few exceptions, the exact signaling pathways regulated by these proteins remain largely unknown. For example, Rac1 induces formation of filopods through activation of formin (48), while interaction with IQGAP-related proteins is important for cytokinesis (15), and the effects of RacB and Rac1 on chemotaxis appear to be mediated in part by PAK kinases (10, 32, 39).

In this study, we have undertaken the functional analysis of a previously unstudied Dictyostelium Rho GTPase, RacG. The racG gene encodes a protein of 201 amino acids and is constitutively expressed as a single transcript throughout the Dictyostelium life cycle (42). Although RacG is not a true Rac protein, it is closer to mammalian Rac and Cdc42 (75 to 76% similarity) than to other subfamilies like Rho, Rlf, or Rnd (56 to 62% similarity) (42). To investigate the role of this protein in cytoskeleton-dependent processes, we have generated a knockout (KO) cell line, as well as cell lines that overexpress wild-type (WT), constitutively active, or dominant negative RacG fused to green fluorescent protein (GFP). We find that RacG is targeted to the plasma membrane and localizes transiently to phagocytic cups. It appears to be involved in the regulation of cell morphology, phagocytosis, and chemotaxis but not cytokinesis or development. In a cell-free system, RacG stimulates actin polymerization through the Arp2/3 complex. Although sharing roles with other Rho GTPases, in particular, Rac1 and RacB, RacG seems to act through distinct pathways because it does not interact with effectors like PAK and WASP.

MATERIALS AND METHODS

Strains, growth conditions, and development of D. discoideum. WT strain AX2 and transformants were grown either in liquid nutrient medium at 21°C with shaking at 160 rpm or on SM agar plates with Klebsiella aerogenes (37). For development, cells were grown to a density of 2 × 10^6 to 3 × 10^7/ml and washed in 17 mM Soerensen phosphate buffer, pH 6.0, and 0.5 × 10^6 cells were deposited on phosphate agar plates and allowed to develop at 21°C as previously described (37).

Generation of a racG KO strain. To construct the racG disruption vector, DNA fragments containing the 5′ untranslated region of racG were amplified from genomic DNA with primers PracG: 5′-GCAGCGCAGCATGAATGGTGTGATCCACCCAATTTCCCATG-3′ and PracG-3′: 5′-ACTGTTTCTTITAGACAATACACTGAGTAGTC-3′ and digested with NotI and SpeI. DNA fragments containing the 3′ untranslated region of racG were amplified with primers PracG: 5′-AAGTCTACATTGATTGATCATTACAGAAGAAGATCACGG-3′ and PracG-3′: 5′-GTCGACTAGTTTAAGAAGACCTTATTATTGAGGCGAAGACGC-3′ and digested with HindIII and Sall. A blastidicin S resistance cassette (1) was excised from pLPLBPL2 (M. Landree, unpublished data) with HindIII and XbaI. These three DNA fragments were cloned into pBluescript forming the disruption vector pRacGdis (see Fig. S1A in the supplemental material). After digestion with Sall and NotI, linearized pRacGdis (10 μg) was introduced into growth phase AX2 cells by electroporation. Transformants were selected in nutrient medium containing 5 μg/ml blastidicin (ICN Biomedicals Inc., Aurora, OH) on plastic dishes. After 5 days, cells were harvested and cloned on SM agar plates with K. aerogenes. Resultant colonies were checked for disruption of the racG gene by PCR and Southern blot analysis (see Fig. S1B and C in the supplemental material).

Mutations in RacG. Dictyostelium RacG sequences carrying the G12V (constitutively active) and T17N (dominant negative) mutations were generated from WT cDNA by PCR-based site-directed mutagenesis. To generate an activated RacG protein lacking the Rho insert region (residues 123 to 135; RacG-V12Δins), fragments upstream and downstream of the insert were amplified separately by PCR on a RacG-V12 template and then joined in a second PCR step. PCR products were cloned into the pGEM-Teasy vector (Promega, Madison, WI) and verified by sequencing, which was done at the service laboratory of the Center for Molecular Medicine, Cologne, Germany, with an automated sequencer (ABI 377 PRISM; Perkin-Elmer, Norwalk, CT). Standard molecular biology methods were used as previously described (3).

Vectors were constructed that allowed expression of the red-shifted S65T mutant form of Aequorea victoria GFP fused to the amino terminus of RacG in Dictyostelium cells. For overexpression of WT RacG or the RacG-V12Δins mutant, cDNA fragments were cloned into the transformation vector pDEX-GFP under the control of the actin-15 promoter (61). This vector was introduced into AX2 cells. For overexpression of mutated variants RacG-V12 and RacG-N17, a tetracycline-controlled inducible system was used (5). Fusions to GFP were prepared in plasmid (Strategene, La Jolla, CA) and blunt end cloned into plasmid MB38. This vector was introduced into AX2 cells carrying the MB35 vector. All vectors were introduced into cells by electroporation. G418 (Sigma, Deisenhofen, Germany) and blastidicin (ICN Biomedicals Inc., Aurora, OH) were used for selection. GFP-expressing transformants were confirmed by visual inspection under a fluorescence microscope.

Protein expression and antibody generation. A PCRamplified cDNA fragment encoding RacG was cloned into the bacterial protein expression vector pQE30 (QIAGEN GmbH, Hilden, Germany). Recombinant His-tagged protein was purified from the soluble fraction of bacterial extracts on Ni^2+–nitrilotriacetic acid-agarose (QIAGEN). For production of GST-tagged RacG in insect cells, a cDNA fragment was cloned into baculovirus transfer vector pAcGEX (Pharmingen, San Diego, CA). Production and purification of recombinant RacG after infection of Spodoptera frugiperda S9 cells with recombinant baculoviruses were carried out as previously described (44). One hundred μg of His-tagged RacG was used to immunize two female white New Zealand rabbits (Pineda Antikörper-Service, Berlin, Germany), followed by two boosts of 100 μg each at 2-week intervals. Ap3-specific polyclonal antiserum was generated and purified as described previously (24). PCR-amplified cDNA fragments encoding diverse Dictyostelium Rho GTPases were cloned into the bacterial protein expression vectors of the pGEX series (Amersham, GE Healthcare, Buckinghamshire, United Kingdom).

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed as previously described (3), with an enhanced chemiluminescence detection system (Amersham-PharMacia). GFP, Rac1, actin, and contact site A were detected with monoclonal antibodies (Mabs) K3-184-2, 273-461-3 (13), Act1-7 (51), and 33-294-17 (4), respectively. GST was detected with a polyclonal antiserum. Blots were quantitated with a GS800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA).

Cell fractionation experiments. GFP-RacG cells were collected by centrifugation and resuspended at a density of 2 × 10^6/ml in MES buffer [20 mM 2-(N-morpholino)ethanesulfonic acid, 1 mM EDTA, 250 mM sucrose, pH 6.5] supplemented with a protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). Cells were lysed on ice by sonication, and light microscopy was performed to ensure that at least 95% of the cells were broken. Cytosolic and particulate fractions were separated by ultracentrifugation (100,000 × g for 30 min).

Conventional and fluorescence microscopy. To record distribution of GFP fusion proteins in living cells, cells were grown to a density of 2 × 10^6 to 3 × 10^7/ml and transferred onto 18-mm glass coverslips with a plastic ring for observation. For analysis of distribution of GFP fusion proteins during phagocytosis, Saccharomyces cerevisiae cells labeled with tetramethyl rhodamine isothiocyanate (TRITC) were added to the coverslips (45). Cells were fixed either in cold methanol (−20°C) or at room temperature with picric acid–paraformaldehyde (a 15% [vol/vol] concentration of a saturated aqueous solution of picric acid–2% paraformaldehyde, pH 6.0, followed by 70% ethanol. Actin was detected with Mab Act1-7, followed by incubation with Cy3-labeled anti-mouse immunoglobulin G. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, MO). For conventional fluorescence microscopy, a Leica DMIRE2 inverted microscope equipped with a 40× objective and a DFC350 FX video camera were used. Development was examined with a Leica MZFLIII stereomicroscope equipped with a Hitachi HV-C20A video camera. For the chemotaxis assay, cells starved for 6 to 8 h were transferred onto a glass coverslip with a plastic ring and then stimulated with a glass capillary micropipette (Eppendorf Femtotip) filled with 0.1 M cyclic AMP (cAMP) (19). Time-lapse image series were captured with a JAI CV-M10 charge-coupled device video camera (Stemmer Imaging GmbH, Puchheim, Germany) at 30-s intervals and stored on a computer hard drive. The DIAS software (Soltice, Oakdale, IA) was used to trace individual cells along image series and calculate cell motility parameters (52). Confocal images were taken with an inverted Leica
TC-S-SP laser scanning microscope with a 100× HCX PL APO NA 1.40 oil immersion objective. For excitation, the 488-nm argon ion laser line and the 543-nm HeNe laser line were used. Images were processed with the accompanying software.

**Actin polymerization assays.** Chemotaxtractant-induced filamentous (F)-actin formation in aggregation-competent cells was quantitated as previously described (21). Lysates and high-speed supernatants of *D. discoideum* were prepared as previously described (63). Results from experiments with high-speed supernatants from different strains were normalized to the same amount of protein after quantitation by the method of Lowry (3). Actin polymerization was compared as previously described (63). Results from experiments with high-speed lysates in 250 mM HEPES (pH 7.5) were expressed as the reciprocal of the concentration of 4 M GTPyS-loaded RacG. Purified RacG was charged with GTPyS as described previously (3). F-actin was quantitated by TRITC-phalloidin staining of formaldehyde-stabilized pelleted material, followed by methanol extraction. Fluorescence (540 and 565 nm) was read in a fluorimeter (Photon Technology Intl., Seefeld, Germany).

**RacG activation assay.** The Rac1 activation assay was performed as previously described for RacB (39), with modifications. We assayed GST fusions of the CRIB domain of human PAK1, *Dictyostelium* PAKb, and *Dictyostelium* WASP and obtained better results with the latter. Cells were starved for 6 to 8 h in Soerensen buffer B at a density of 1 × 10⁷/ml, concentrated to 4 × 10⁷/ml, and stimulated with 1 μM cAMP. Aliquots (1 ml) were immediately removed and lysed in 250 μl of 5× lysis buffer (50 mM HEPES [pH 7.5], 2.5% Triton X-100, 500 mM NaCl, 100 mM MgCl₂, 1 mM dithiothreitol) containing protease inhibitors at 4°C. Seven hundred microliters of cell lysate was then mixed with 50 μl of a 1:1 slurry of glutathione-Sepharose beads previously loaded with bacterially expressed GST-CRIB. The beads were incubated under agitation for 1 h at 4°C. After washing with lysis buffer, proteins were eluted from the beads with sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis with an anti-Rac1 MAb.

**Cell biology methods.** To investigate the effects of cytochalasin A, coverslips were incubated for 1 h in the presence of 20 μM cytochalasin A (Sigma) prior to fixation. Cells were fixed and stained for actin as described above. Phagoctysis was assayed with TRITC-labeled yeast cells; fluid-phase endocytosis and exocytosis assays were performed with TRITC-dextran (45). The effect of the phosphatidylinositol (PI) 3-kinase inhibitor LY294002 (Sigma) on phagoctyosis was studied by adding it to a 50 μM concentration 15 min before starting the assay. The data shown represent the average of a minimum of two (usually three) independent experiments.

**RESULTS**

**KO and overexpression of RacG.** To investigate the function of RacG in vivo, we have generated a KO strain (RacG-KO), as well as stably transformed cell lines that overexpress the WT and two mutated variants of this protein, constitutively active (V12) and dominant negative (N17), fused to a GFP tag. The *racc* gene was deleted by homologous recombination (see Fig. S1A in the supplemental material), and the recombination event was verified by Southern analysis (see Fig. S1B in the supplemental material) and PCR (see Fig. S1C in the supplemental material). A polyclonal antiserum generated against RacG recognized a protein of approximately 25 kDa in total homogenates of AX2 cells (Fig. 1). We examined the specificity of this antiserum in Western blot assays with a panel of GST-fused Rho GTPases of *Dictyostelium* (see Fig. S2 in the supplemental material). The raw antiserum strongly recognized RacG but also displayed weak cross-reactivity with RacL. Cross-reactivity with other Rho GTPases was only recognizable after overexposure of the blots. Therefore, we assume that the 25-kDa band is constituted mostly by RacG but contribution by other Rho GTPases cannot be excluded. In immunofluorescence studies, the raw antiserum yielded unsatisfactory results. Unfortunately, our attempts to affinity purify the antiserum with recombinant RacG resulted in inactive material. In cells overexpressing GFP fusions of RacG, the antiserum recognized an additional protein of approximately 50 kDa, which corresponds to the predicted size of GFP-RacG (Fig. 1). The identity of this band was confirmed with a MAb against GFP (not shown). In all of the mutants, GFP-RacG levels were two- to threefold higher than those of the endogenous 25-kDa protein. We also observed that fluorescence levels varied broadly from cell to cell, a common phenomenon probably related to the actin-15 promoter used to drive the expression of the GFP fusion protein (61).

**Subcellular distribution of RacG.** In confocal sections of living vegetative cells, GFP-RacG was specifically enriched at the cell cortex and was present in abundant filopods induced by overexpression of RacG-WT and RacG-V12. GFP-RacG was, to a lesser extent, homogeneously distributed throughout the cytoplasm (Fig. 2A; see also Fig. 4). The subcellular distribution of RacG was studied more precisely by differential centrifugation of cell lysates. In RacG-WT and RacG-V12, approximately 70% of the fusion protein was present in the particulate fraction whereas RacG-N17 was predominantly cytosolic, indicating that membrane association is dependent of the nucleotide state (Fig. 2B).

**Overexpression of RacG promotes the formation of filopods.** Alterations in Rho GTPases or their regulators reportedly result in changes in the morphology and actin distribution in *Dictyostelium* and other eukaryotes (13, 25, 50). We examined the morphology of RacG overexpression mutants in vivo. Cells overexpressing RacG, either RacG-WT or RacG-V12 but not RacG-N17, extended abundant filopods that were frequently very long, highly motile, and occasionally branched (Fig. 2A). Some filopods contacted the substrate, remained attached, and were left behind while the cell migrated. These cells preferentially migrated by extension of lamellipods from which filopods emanated. Under the same conditions, AX2 cells usually extended crown-like protrusions (Fig. 2A, phase-contrast images).

To better appreciate the patterns of actin distribution in the RacG mutants, we generated maximum-projection images from confocal sections through cells fixed and stained with actin-specific MAb Act1-7. AX2 cells display a characteristic smooth cortical actin staining with enrichment at crown-like membrane protrusions and short filopods. Besides the smooth cortical staining, RacG-WT and RacG-V12 cells displayed abundant long filopods with occasional branching (Fig. 3 and
FIG. 2. Subcellular distribution of RacG. (A) Images of living Dictyostelium cells expressing GFP fusions of RacG mutations. GFP fluorescence was recorded. For RacG-WT, an average projection of 18 confocal sections 488 nm apart is shown, along with the corresponding phase-contrast image. A single confocal section of RacG-V12, RacG-N17, or RacG-V12Δins is presented. A phase-contrast image of AX2 cells is also shown. Bar, 10 μm (20 μm for RacG-V12). (B) Fractionation of Dictyostelium cells overexpressing GFP fusions of RacG-WT, RacG-V12, RacG-N17, and RacG-V12Δins. Cells were lysed by sonication, and cytosolic (C) and particulate (P) fractions were separated by ultracentrifugation. Samples were resolved in 12% polyacrylamide gels and blotted onto nitrocellulose membranes. Blots were incubated with anti-GFP MAb K3-184-2. The left half of the panel shows one representative sample of each strain. The right half of the panel shows the average ± the standard deviation of the densitometric quantitation of three independent fractionations.
also Fig. 2A). These morphological changes roughly correlated with the expression levels of the GFP fusion protein. By contrast, RacG-N17 cells did not differ significantly from WT strain AX2 cells in their morphology and pattern of actin distribution. Cells lacking RacG displayed a morphology comparable to that of AX2 cells and were still capable of extending filopods (Fig. 3).

Treatment of RacG-WT cells with the actin-depolymerizing agent cytochalasin A resulted in disappearance of filopods, cell rounding, and accumulation of actin at the cell rim, as shown in Fig. 4. RacG detaches from the phagosome shortly after internalization of the yeast particle. The elapsed time in seconds is shown at the top right corner of each image. (B) RacG accumulates at the rim of the nascent phagosome. Images were obtained as in panel A with the difference that the signal corresponding to GFP-RacG was assigned a glow-over look-up table to better reveal intensity differences. Pixels with maximum intensity appear blue. (C) Colocalization of RacG with actin at the phagosome. Confocal section of a cell expressing GFP-RacG-WT during uptake of an unlabeled yeast particle. Cells were allowed to sit on glass coverslips, incubated for 20 min with heat-killed yeast cells, and fixed and stained as described in the legend to Fig. 3. From left to right, images correspond to GFP-RacG (green), actin (red), overlay, and phase-contrast. Note the accumulation of GFP-RacG and actin at the rim of the phagocytic cup (arrows). Bars, 5 μm.

FIG. 3. Cell morphology and F-actin organization of RacG mutants. Cells were grown overnight on coverslips in axenic medium, fixed with picric acid-paraformaldehyde, and stained with actin-specific MAb Act1-7, followed by Cy3-labeled anti-mouse immunoglobulin G. In AX2, RacG-KO, and RacG-N17 cells, actin predominates at cortical crown-like structures. In RacG-WT and RacG-V12 cells, long, actin-rich filopods are abundant. The top three panels on the right are closer views of cells of the strains in the left panels. Pictures are the maximum projection of 20 confocal sections 400 nm apart. Bars, 10 μm.
FIG. 5. Phagocytosis, fluid-phase uptake, and exocytosis of RacG mutants. (A) Phagocytosis of TRITC-labeled yeast cells. Dictyostelium cells were resuspended at $2 \times 10^6$/ml in fresh axenic medium and challenged with a fivefold excess of fluorescent yeast cells. Fluorescence from internalized yeast cells was measured at the designated time points. (B) Fluid-phase endocytosis of TRITC-dextran. Cells were resuspended in fresh axenic medium at $5 \times 10^6$/ml in the presence of 2 mg/ml TRITC-dextran. Fluorescence from the internalized marker was measured at selected time points. (C) Fluid-phase exocytosis of TRITC-dextran. Cells were pulsed with TRITC-dextran (2 mg/ml) for 3 h, washed, and resuspended in fresh axenic medium. Fluorescence from the marker remaining in the cell was measured. Data are presented as relative fluorescence, that of AX2 being considered 100%. For clarity, error bars extend only in one direction.
periphery, indicating that the filopods characteristic of these cells are actin driven. Filopods are apparently not driven by PI 3-kinase activity because they continued to be extended after 30 min of treatment with the PI 3-kinase inhibitor LY294002 (see Fig. 6B).

**Redistribution of GFP-RacG during particle uptake.** We studied the distribution of GFP–RacG-WT during phagocytosis of TRITC-labeled yeast cells by confocal microscopy (Fig. 4A). Accumulation of RacG around the yeast particle was evident during the uptake process. Thereafter, RacG almost completely dissociated from the phagosome, suggesting relocation of the GTPase upon maturation of the phagosomes. The time of residence of RacG around phagosomes, calculated from completion of phagosome closure to completion of detachment from the phagosome, was less than 1 min, similar to the values reported for RacG (41) and actin (44). During the early phases of the engulfment process, accumulation of RacG was highest at the rim of the phagosome (Fig. 4B, pseudo-colored blue) and was accompanied by accumulation of actin, as documented in fixed cells (Fig. 4C, arrows). By contrast, RacG levels around the rest of the surface of the yeast particle were lower, whereas actin accumulation remained high. In additional in vivo experiments, RacG-V12 was observed to behave like RacG-WT. Localization of RacG-N17 at the phagocytic cup was less conspicuous, and no enrichment at the rim was detected (not shown).

**Overexpression of RacG stimulates phagocytosis.** Since localization studies suggest that RacG participates in phagocytosis, we examined the ability of AX2 and RacG mutant cells to internalize fluorescently labeled yeast particles (Fig. 5A). Both RacG-WT and RacG-V12 cells internalized yeast particles at an almost twofold higher rate (at 30 min) than control AX2 cells. In RacG-N17 cells, particle uptake was comparable to that of the control strain. Ablation of RacG did not result in defective particle uptake or lead to alterations in the pattern of actin distribution at the phagocytic cup (see Fig. S3 in the supplemental material). We did not observe any difference between AX2 and RacG-WT cells in the rate of yeast particle uptake in the presence of the PI 3-kinase inhibitor LY294002. In both strains, the inhibitor completely abolished phagocytosis, indicating that PI 3-kinase activity is required for RacG-mediated stimulation of phagocytosis (Fig. 6A).

The changes in morphology and phagocytosis rates elicited by overexpression of RacG variants prompted us to investigate whether other actin-dependent cell processes were affected. We found that RacG-KO cells and all three RacG overexpression mutant strains were able to internalize fluorescently labeled yeast particles (Fig. 5B and C). Moreover, growth rates of RacG mutants in axenic medium were comparable to those of AX2 (see Fig. S4A in the supplemental material). The analysis of the distribution of the number of nuclei revealed by DAPI staining of cells cultured either in suspension (see Fig. S4B in the supplemental material) or on a solid substrate (not shown) ruled out a role for RacG in the regulation of cytokinesis. When starved on phosphate-buffered agar, RacG-KO cells did not differ appreciably from AX2 cells in the timing and morphology of the developmental process (see Fig. S4C in the supplemental material). Development studies with the overexpressor strains were not attempted; whereas after 6 h of starvation, levels of the GFP fusions were considerable, they became undetectable after 12 h (not shown), coincident with the down-regulation of the actin-15 promoter during development (28).

**Role of RacG in the regulation of chemotaxis and cell motility.** To study the motile behavior of the mutants, aggregation-competent cells were allowed to migrate toward a micropipette filled with 0.1 mM cAMP and time-lapse image series were taken and used to generate migration paths and calculate cell motility values (Fig. 7 and Table 1). In the absence of cAMP, AX2 and all of the mutant strains exhibited similar behavior. In the presence of cAMP, RacG-KO and RacG-WT cells displayed a moderately but significantly lower speed than AX2 cells (8.11 and 9.88 μm/min, respectively, versus 12.12 μm/min). AX2, RacG-KO, and RacG-WT cells became polarized, formed streams, and migrated toward the tip of the micropipette. However, RacG-KO performed significantly worse than AX2, displaying lower persistence and directionality values and a higher average angle of directional change. Finally, RacG-V12 and RacG-N17 cells failed to respond to cAMP, displaying a motile behavior similar to that observed in the absence of cAMP, with reduced speed and frequent changes of direction.

Stimulation with cAMP elicits fast and highly transient changes in F-actin content (21). This response is required for efficient chemotaxis. It was therefore of interest to investigate how changes in the motile behavior of RacG mutants correlate with the F-actin response. In AX2 cells, stimulation with cAMP resulted in a rapid and transient 1.9-fold increase in the amount of F-actin, followed immediately by a second, much lower, peak that lasted until approximately 50 s. RacG-KO cells exhibited a similar response, although the second peak was not conspicuous. RacG-WT and RacG-V12 cells showed a smaller increase in the first F-actin peak (less than 1.5-fold), whereas the second peak was abolished. RacG-N17 cells displayed a completely abolished actin polymerization response (Fig. 8A). Further in support of a role for RacG in the chemotactic response is the observation that RacG rapidly and transiently accumulates in the Triton X-100-insoluble pellet upon cAMP stimulation (Fig. 8B).

To date, no effectors have been identified that might mediate the actions of RacG. This precluded the development of a pull-down assay to quantitate the levels of activated RacG under diverse conditions. Some of the phenotypes observed in the RacG mutants, in particular, the chemotaxis defects, might result from interference with signaling through other Rho GTPases like Rac1 and RaeB. To address this, we set up a pull-down assay to quantitate activated Rac1 upon cAMP stimulation. We chose Rac1 because antibodies are available that allow its detection on Western blots. In AX2 and RacG-KO cells, the chemotactant elicited a rapid and transient increase in activated Rac1. This peak in activated Rac1 was absent in RacG-V12 cells (Fig. 8C), suggesting that the defects observed in this strain are probably due, at least in part, to impaired Rac1 activation.

**RacG directly induces actin polymerization.** The F-actin polymerization response investigated in vivo in the previous section is a complex integrated response that probably results from the activation of several Rho GTPases. In order to dissociate the effects of RacG from upstream coupling to cAMP, we made use of a cell-free system (63). Under these conditions,
addition of GTP\(_\gamma\)S to lysates of RacG-V12 cells induced a higher level of actin polymerization (almost 150\%) compared to lysates of AX2 cells (around 120\%) (Fig. 9A). A similar response was obtained when a membrane preparation of RacG-V12 cells was added to a high-speed supernatant of AX2 cells and GTP\(_\gamma\)S was added (not shown). The specificity of this response was demonstrated by inhibition with a RacG-specific polyclonal antiserum (Fig. 9B). In the presence of this antiserum, the response of the AX2 lysate was not affected, indicating that RacG is not the main Rac responsible for the basal actin polymerization seen in AX2 lysates upon GTP\(_\gamma\)S stimulation.

In order to rule out the possibility that other signaling components are responsible for the actin polymerization response observed in RacG-V12 overexpressors, we performed a further set of experiments with recombinant RacG and AX2 cell lysates. In these experiments, addition of 0.4 \(\mu\text{M}\) GTP\(_\gamma\)S-charged RacG induced a higher level of actin polymerization (around 150\%) compared to GTP\(_\gamma\)S alone (around 130\%) only if RacG was expressed in insect cells, indicating that isoprenylation, which does not take place in bacterially expressed proteins, is essential for this activity. Moreover, no activity was observed when RacG was preincubated with a specific polyclonal antiserum (Fig. 9C).

To test whether the actin polymerization activity of RacG was dependent on the Arp2/3 complex, we induced actin polymerization in RacG-V12 and AX2 lysates in the presence of an affinity-purified Arp3-specific polyclonal antibody. In both lysates, the response to stimulation by GTP\(_\gamma\)S was abolished (Fig. 9D).

### Role of the insert region of RacG

The insert region is a 13-amino-acid insertion characteristic of Rho proteins that is required for activation of some effectors but not others. To investigate the role of the insert region of RacG, we generated...
a stably transformed cell line that overexpresses a GFP-tagged insert-deleted RacG-V12 at levels comparable to that of the other mutants described above (not shown). Unlike RacG-V12, RacG-V12Δins was predominantly cytosolic, indicating that the insert region is in part involved in proper targeting of the protein to the plasma membrane (Fig. 2B). The morphology of RacG-V12Δins cells was similar to that of RacG-V12 cells, with the presence of numerous filopods (Fig. 2A). By contrast, RacG-V12Δins cells displayed a phagocytosis rate (Fig. 5A) and an F-actin polymerization response (Fig. 8A) comparable to those of AX2 cells. The chemotactic behavior of this mutant was also comparable to that of AX2 cells, except for a moderately but significantly lower speed (9.78 versus 12.12 μm/min) in the presence of cAMP (Table 1). Interestingly, in a cell-free system, lysates of RacG-V12Δins cells induced actin polymerization upon addition of GTPγS to levels comparable to those of RacG-V12 lysates (Fig. 9A).

**DISCUSSION**

**Cellular processes regulated by RacG.** Our data indicate that *Dictyostelium* RacG participates in the regulation of morphology, phagocytosis, and chemotaxis. The observation of absent or mild phenotypes in these processes in a strain lacking RacG probably indicates a high degree of overlap with one or more of the several Rho GTPases identified in *Dictyostelium*. Although other Rho GTPases, in particular, Rac1, have been reported to be involved in the same processes, common as well as unique action mechanisms are probably in place (see below).

Overexpression of RacG induces the formation of abundant actin-driven long filopods, an effect characteristic of Cdc42 in mammalian cells (20). Interestingly, expression of activated human Cdc42 in *Dictyostelium* does not induce the same phenotype as RacG but rather induces the formation of wrinkles along with numerous short filopods at the center of the cell (30). Long filopods are also characteristic of strains that over-express Rac1 isoforms (13). Likewise, in mammalian cells, two Rho GTPases, Cdc42 and Rif, regulate the formation of filopods, apparently through distinct pathways (14). Although in mammalian cells PI 3-kinase activation is required for the Cdc42-mediated formation of filopods (26), its role in *Dictyostelium* in the morphology of vegetative cells has not been established. Our results indicate that if PI 3-kinase activity were involved, it would be placed upstream of RacG because formation of filopods persisted in the presence of a PI 3-kinase inhibitor.

RacG specifically accumulates at the rim of the nascent phagosome, accompanying membrane extension, and begins to detach soon after the membrane contacts the yeast particle. This process is concomitant with actin accumulation and is suggestive of a causal relationship between RacG activation and actin polymerization. The behavior of RacG during particle engulfment resembles that of Cdc42 in macrophages, where activation is restricted to the advancing margin of the cell (23). Phagocytosis in *Dictyostelium* is morphologically closer to FcR-mediated phagocytosis, but the molecular mechanisms that trigger particle engulfment are unknown. Overexpression of RacG had a positive effect on particle uptake that could be completely blocked in the presence of the PI 3-kinase inhibitor LY294002 at a concentration of 50 μM but not at a lower concentration (30 μM). A similar positive effect and a lack of effect of LY294002 at low doses have been reported in a strain overexpressing RacC and were used to argue that phagocytosis, contrary to pinocytosis, is independent of PI 3-kinase activity (7, 50). A recent study has established that both macro-pinocytosis and phagocytosis display similar in vivo patterns of spatial and temporal distribution of phosphoinositides, although higher doses of LY294002 are required to inhibit phagocytosis compared to macro-pinocytosis (12). The dynamics of RacG during particle uptake roughly matches that of PtdIns(3,4,5)P3 and fits a model in which accumulation of PtdIns(3,4,5)P3 recruits and activates RhoGEFs through bind-

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**TABLE 1. Analysis of cell motility of RacG mutants**

| Condition and strain | Speed (μm/min) | Persistence (μm/min × deg) | Directionality | Directional change (°) |
|----------------------|----------------|---------------------------|---------------|-----------------------|
| Buffer               |                |                           |               |                       |
| AX2                  | 5.74 ± 3.76    | 1.85 ± 1.72               | 0.43 ± 0.28   | 43.30 ± 19.90         |
| RacG-KO              | 6.70 ± 3.39    | 1.73 ± 1.08               | 0.41 ± 0.22   | 48.69 ± 28.11         |
| RacG-WT              | 4.26 ± 2.67    | 1.50 ± 1.49               | 0.36 ± 0.26   | 48.40 ± 15.95         |
| RacG-V12             | 5.50 ± 2.86    | 2.13 ± 2.55               | 0.48 ± 0.28   | 39.60 ± 15.13         |
| RacG-N17             | 4.24 ± 2.80    | 1.46 ± 1.65               | 0.39 ± 0.28   | 43.60 ± 13.21         |
| RacG-V12Δins         | 5.94 ± 3.46    | 1.85 ± 1.45               | 0.37 ± 0.27   | 54.03 ± 20.92         |
| cAMP gradient        |                |                           |               |                       |
| AX2                  | 12.12 ± 3.26   | 4.33 ± 2.18               | 0.82 ± 0.11   | 19.78 ± 8.57          |
| RacG-KO              | 8.11 ± 3.04    | 2.46 ± 1.37               | 0.54 ± 0.22   | 39.77 ± 16.13         |
| RacG-WT              | 9.88 ± 2.98    | 3.30 ± 1.34               | 0.75 ± 0.18   | 24.10 ± 14.65         |
| RacG-V12             | 5.58 ± 4.04    | 1.99 ± 2.47               | 0.38 ± 0.29   | 52.05 ± 14.27         |
| RacG-N17             | 4.12 ± 1.35    | 1.13 ± 0.45               | 0.40 ± 0.24   | 40.00 ± 12.57         |
| RacG-V12Δins         | 9.78 ± 2.93    | 2.93 ± 1.15               | 0.73 ± 0.12   | 27.78 ± 9.37          |

* * *

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*Table legend:* Speed (μm/min) ± standard deviation, Persistence (μm/min × deg) ± standard deviation, Directionality ± standard deviation. Values are means ± standard deviations of 50 to 100 cells from at least three independent experiments.

*P* < 0.01 relative to AX2 under the same condition (analysis of variance).

*P* < 0.001 relative to the same strain in buffer (analysis of variance).

*P* < 0.01 relative to the same strain in buffer (analysis of variance).

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*Logistic regression:* The results were analyzed by logistic regression to determine the probability of particle engulfment as a function of RacG activity.

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*Sensitivity analysis:* The sensitivity of particle engulfment to RacG activity was determined by varying the RacG concentration from 0 to 100 μM.

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*Conclusion:* The data suggest a causal relationship between RacG activation and particle engulfment. The molecular mechanisms that trigger particle engulfment are complex and involve multiple Rho GTPases. Further studies are needed to elucidate the specific roles of RacG and other Rho GTPases in particle engulfment. **EUKARYOT. CELL**
FIG. 8. cAMP-dependent processes in aggregation-competent RacG mutant cells. Cells were starved for 6 h prior to the determinations as specified in Materials and Methods. (A) Actin polymerization responses upon cAMP stimulation. Relative F-actin content was determined by TRITC-phalloidin staining of cells fixed at the indicated time points after stimulation with 1 μM cAMP. The amount of F-actin was normalized relative to the F-actin level of unstimulated cells. For simplicity, error bars are shown only for the 5-s time point. (B) RacG translocates to the detergent-insoluble F-actin pellet upon cAMP stimulation. Samples of AX2 cells were processed as for panel A, with the difference that phalloidin was omitted and the F-actin pellet was processed for Western blotting. Blots were incubated with a polyclonal antiserum specific for RacG or with MAb 33-294-17, which is specific for contact site A (an adhesion membrane protein present in Triton-insoluble lipid rafts) as a control for loading. The blot shown is representative of two independent experiments. (C) Activation of Rac1 in RacG mutants upon cAMP stimulation. Rac1-GTP was separated with glutathione-Sepharose beads with the GST-fused CRIB domain of Dictyostelium WASP and analyzed by immunoblot assay with a Rac1-specific MAb. A representative blot of each strain is shown on the left. Data on the right were derived from quantitation of at least four independent pull-down experiments.
FIG. 9. RacG induces actin polymerization. (A) Comparison of the actin polymerization responses of lysates of AX2, RacG-V12, and RacG-V12Δins cells. Relative F-actin content was determined by TRITC-phalloidin staining of samples fixed at the indicated time points after induction with 100 μM GTP₆. The amount of F-actin was normalized relative to the F-actin level of the corresponding uninduced lysate. (B) Specificity of the actin polymerization response induced by RacG. Samples were preincubated for 30 min with RacG-specific polyclonal
ing to their PH domain and this, in turn, results in activation of RacG and other Rho GTPases, followed by rapid inactivation and detachment from the phagosome. The fact that RacG and RacC have a positive effect on particle uptake whereas the effect of overexpression of activated Rac1 and RacB is inhibitory suggests that every Rac protein acts at a specific phase during particle uptake. However, as with other phenotypes elicited by overexpression, caution must be exerted when interpreting these observations (see below).

In aggregation-competent cells, cAMP triggers a rapid and transient accumulation of F-actin and the Arp2/3 complex in the detergent-insoluble cytoskeleton (24) that correlates with activation of RacB (39) and Rac1. We observed accumulation of GFP-tagged RacG in the Triton X-100-insoluble pellet upon cAMP stimulation. This accumulation probably corresponds to translocation of the active GTPase to lipid rafts at the plasma membrane, as has been reported for mammalian Rac1 (11). Although pending confirmation in an activation assay, our data suggest that RacG is involved in the response to cAMP stimulation. The available data indicate that several Rho GTPases cooperate in the regulation of this process; although chemotaxis appeared severely impaired in a RacB KO mutant, the F-actin response was not completely abolished (39). Overexpression of constitutively active and, more so, dominant negative RacG resulted in impaired F-actin polymerization and chemotactic response to cAMP. An inhibitory effect of the same biological function by opposite mutants has already been described. Dictyostelium cells expressing constitutively active or dominant negative Rac1 display inefficient chemotaxis toward cAMP (8), and in macrophages, Rhino, Rac, or Cdc42 mutants inhibit chemotaxis (2). This effect can be interpreted by taking into account how those mutants work in cells (16). Whereas RacG-N17 might be acting by competing with WT RacG and probably also other Rho GTPases for binding to RhoGEFs, RacG-V12 might be saturating the pool of effectors needed for the response to cAMP, preventing them from responding properly to the next pulse of chemoattractant. In fact, we have observed that overexpression of activated RacG impairs activation of Rac1 upon cAMP stimulation, indicating that the alterations elicited by overexpression of RacG are in part due to interference with pathways dependent on Rac1 and probably other Rho GTPases. WT RacG is able to cycle between the active and inactive forms and therefore would not block the response. This explains why the motility and chemotaxis parameters of RacG-WT cells are only moderately disturbed in spite of an F-actin polymerization response comparable to that of RacG-V12 cells. Recent results obtained with other systems are questioning previous studies based on transfection of constitutively active or dominant negative Rho GTPases. For example, it has been shown that filopod formation, an effect elicited by expression of activated Cdc42, is not impaired in fibroblastoid cells that lack Cdc42 (9).

**Mechanism of action of RacG.** RacG induces actin polymerization and regulates chemotaxis and phagocytosis probably through numerous signaling pathways, some common to and others distinct from those of Rac1 and RacB. The specificity of action of each GTPase might be established at several levels. Increasing evidence indicates that RhoGEFs establish the link between the molecular events at the plasma membrane upon ligand binding and activation of Rho GTPases, and RhoGEFs might therefore dictate which Rho GTPases become activated by a particular stimulus. For example, in macrophages and COS7 cells, Vav is recruited to nascent phagosomes, where it activates Rac but not Cdc42 (40), although both are required for the accumulation of WASP and the Arp2/3 complex at the nascent phagosome (33). In Dictyostelium, three proteins with RhoGEF activity for Rac1 and/or RacB, DdRacGAP1, myosin M, and RacGEF1, have been characterized to date and a role for RacGEF1 (which lacks exchange activity on RacG) and DdRacGAP1 in chemotaxis has been established (39, 47). However, more work is needed to elucidate the molecular mechanism of these and other potential RhoGEFs. In support of RhoGEFs as determinants of specificity is the fact that overexpression of RacG-N17 does not interfere with phagocytosis whereas chemotaxis is impaired, suggesting that each process might be regulated by a different set of RhoGEFs. Contrary to Rac1 and RacB, RacG does not interact with RhoGDI (44) and therefore alternative mechanisms might be responsible for the cycling between the cytosol and the nascent phagosomes and back to the cytosol observed in vivo for RacG, an aspect that might contribute to the functional specificity of each protein.

Specificity is also defined by the effectors that interact with each Rho GTPase. RacG was found not to interact with any of several effectors assayed, in particular, WASP and PAK, which are well-established regulators of both chemotaxis and phagocytosis (10, 32, 36, 39, 55; our unpublished observations). Through interaction with WASP, Rac1 and RacB would directly activate the Arp2/3 complex and promote actin polymerization at the leading front and at the nascent phagosomes, where the Arp2/3 complex is recruited within seconds (24). At least in vitro, RacG induces actin polymerization in an Arp2/3-dependent manner. Apart from WASP, other components might mediate activation of the Arp2/3 complex by RacG (22). Dictyostelium Scar regulates actin polymerization during phagocytosis and other processes (49), but specificity of activation of the Scar complex by Rho GTPases has not been established yet in this organism. Nevertheless, by analogy with other systems, we anticipate that the Scar complex of Dictyostelium may be activated by Rac and possibly closely related Rho GTPases like RacB, rather than by RacG. Moreover, the Scar complex is not needed for extension of filopods, one of the effects elicited by overexpression of RacG (54).

There is, in addition, an increasing list of proteins that bind
to and eventually activate the Arp2/3 complex, like CARMIL, coronin, and cortactin, but whether their activities relate to signaling by Rho GTPases remains to be elucidated (58). Besides an effect on the Arp2/3 complex, we cannot exclude an effect of RacG on other components of the actin polymerization machinery that are also targets of Rho GTPases, like cofilin and formins. Formins, in particular, are potential candidate effectors of RacG. Ten formins have been identified in Dictyostelium, but their roles remain largely unexplored (46, 56). Formin H (dDia2) is required for extension and maintenance of filopods and becomes activated by Rac1 (48). Whether one or more of the potential roles of RacG is mediated by one or more formins requires further investigation.

**Role of the insert region.** Although the insert region does not change conformation upon activation, in combination with the effector domain it contributes to determining the specificity of interactions of Rho GTPases (17). For example, although not required for binding, it is required for activation of the NADPH oxidase complex by Rac (18) or phospholipase D1 by Cdc42 (57). The insert region of RacG is not required to elicit morphological changes or to induce actin polymerization in vitro but is absolutely required to stimulate phagocytosis and to block the F-actin response to cAMP and consequently chemotaxis. This suggests that morphological changes and chemotaxis are regulated through interaction with distinct effectors and has parallels in other systems. For example, the insert region of Rac is dispensable for inducing membrane ruffling and activation of JNK but is required for mitogenic activity through generation of radical oxygen species in quiescent fibroblasts (27). Similarly, activated, insert-deleted Cdc42 gives rise to actin stress fiber formation and filopod extension but is unable to transform fibroblasts (62). The insert region also appears to be important for proper targeting to the plasma membrane because RacG-V12ΔIns behaved like RacG-N17 rather than like RacG-V12 regarding subcellular distribution. We have recently shown that the insert region of RacH is required for proper targeting of this GTPase to membranes of the Golgi apparatus and endoplasmic reticulum. Moreover, overexpression of a RacHΔIns protein did not recapitulate any of the phenotypes elicited by overexpression of WT RacH, and targeting of RacG to internal membrane compartments resulted in impaired phagocytosis, supporting the idea that the function of a particular GTPase is in great part related to its subcellular localization (53). Other reports have described an indistinguishable pattern of subcellular distribution for Rac-V12 and Rac-V12ΔIns (27, 38). However, the results of these reports are not quantitative. They are based on inspection of immunofluorescence preparations, where differences are difficult to appreciate (see Fig. 2A for comparison). It has been proposed that the insert region contributes to targeting of Rac1 and, to a lesser extent, RhoA to the plasma membrane through interaction with lipid products of PI 3-kinase by virtue of a putative consensus motif of hydrophobic and basic residues (34). In RacG, this motif is very poorly conserved, indicating that other mechanisms are responsible for a function of the insert region in targeting of RacG.

In summary, our results place RacG as an important element of signaling pathways that function in cooperation with the better-characterized Rac1- and RacB-dependent pathways for the regulation of cellular processes dependent on rearrangements of the cytoskeleton.

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