Rac1 GTPases control filopodia formation, cell motility, endocytosis, cytokinesis and development in *Dictyostelium*

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SUMMARY

The function of the highly homologous Rac1A, Rac1B, and Rac1C GTPases of the *Dictyostelium* Rac1 group was investigated. All three GTPases bound with an equal capacity to the IQGAP-related protein DGAP1, with a preference for the activated GTP-bound form. Strong overexpression of wild-type Rac1 GTPases N-terminally tagged with green fluorescent protein (GFP), predominantly induced the formation of numerous long filopodia. Remarkably, expression of the constitutively-activated GTPases resulted in dominant-negative phenotypes: these Rac1-V12 mutants completely lacked filopodia but formed numerous crown shaped structures resembling macropinosomes. Moreover, these mutants were severely impaired in cell motility, colony growth, phagocytosis, pinocytosis, cytokinesis and development. Transformants expressing constitutively-inactivated Rac1-N17 proteins were similar to wild-type cells, but displayed abundant and short filopodia and exhibited a moderate defect in cytokinesis. Taken together, our results indicate that the three GTPases play an identical role in signaling pathways and are key regulators of cellular activities that depend on the re-organization of the actin cytoskeleton in *Dictyostelium*.

Movies available on-line:
http://www.biologists.com/JCS/movies/jcs4711.html

Key words: Actin cytoskeleton, *Dictyostelium*, DGAP1, GFP, Rac1

INTRODUCTION

Rho family members of small GTP-binding proteins regulate a broad diversity of cellular functions including cytoskeletal organization, membrane trafficking, transcriptional regulation, cytokinesis, cell proliferation, cell motility, and aspects of development (reviewed by Van Aelst and D'Souza-Schorey, 1997). These G-proteins function as molecular switches in signal transduction pathways by cycling between an active GTP-bound and an inactive GDP-bound state. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of bound GDP for GTP, whereas GTP activating proteins (GAPs) increase their intrinsic GTPase activity, while GDP dissociation inhibitors (GDIs) prevent release of bound GDP. The activated forms of three members of the mammalian Rho family, Cdc42, Rac, and Rho, are believed to regulate actin filament dynamics during filopodia formation, membrane ruffling and the formation of stress fibers and focal contacts, in addition to mediating other cellular activities (Ridley, 1996; Hall, 1998; Johnson, 1999).

In fibroblasts, GTPase signaling is thought to occur through a linear, hierarchical cascade in which one GTPase controls the action of the other GTPase in a process mediated by bifunctional linker molecules and effector proteins (Chant and Stowers, 1995; Nobes and Hall, 1995). Although a variety of Rac effectors and target proteins such as IQGAP1/2, PAK,

POR1, tubulin, lipid kinases, gelsolin, DdLim, and others have been identified (Hartwig et al., 1995; Best et al., 1996; Azuma et al., 1998; Prassler et al., 1998; reviewed by Aspenstrom, 1999), considerable effort will be required to better understand the regulatory pathways that lead to re-arrangements of the cytoskeleton. This task becomes even more challenging since Rho-GTPases may crosstalk and simultaneously operate in different signaling pathways (Westwick et al., 1997; Burridge, 1999).

Although no Rho or Cdc42 homologues are known in *Dictyostelium*, cladistic analysis has identified 14 *rac*-like genes (Bush et al., 1993; Larochelle et al., 1996; Rivero et al., 1999). Of these, only the genes encoding RacC, RacE, and RacF1 have been characterized. RacC overexpression causes an increase in phagocytosis and induces unusual F-actin based structures (Seastone et al., 1998), whereas GTP-bound RacE is required for cytokinesis of cells grown in suspension (Larochelle et al., 1997). RacF1 associates with dynamic structures that are formed during pinocytosis and phagocytosis, but is not essential for these processes (Rivero et al., 1999).

We have previously reported that the IQGAP1-related protein, DGAP1, is involved in the regulation of the F-actin/G-actin equilibrium, cytoskeletal organization, cell motility, cytokinesis and development in *Dictyostelium* (Faix and Dittrich, 1996; Faix et al., 1998). DGAP1-null cells project numerous filopodia, show increased cell motility during

growth and form multi-tipped aggregates during development. Unlike DGAP1, the mammalian IQGAPs, and the yeast homologue Iqg1, harbor an actin-binding calponin homology domain in their N-terminal region (Brill et al., 1996; Bashour et al., 1997; Epp and Chant, 1997). DGAP1 does not contain this domain and consequently, it does not directly bind to actin. However, similarly to the IQGAPs (Hart et al., 1996; Brill et al., 1996; Kuroda et al., 1996), DGAP1 lacks RhoGAP activity and preferentially interacts with the GTP-bound form of human Rac1 and *Dictyostelium* Rac1A (Faix et al., 1998). DGAP1 is considered a potential effector since it binds directly to activated Rac1A (Faix et al., 1998). *DGAP1* mRNA is uniformly expressed throughout the first 12 hours of development (Faix and Dittrich, 1996) while the expression of the *Rac1* group mRNAs is developmentally regulated. The transcripts levels present during growth increase 3-fold during the aggregation-phase and drop thereafter to the similar levels observed during growth (Bush et al., 1993).

In the present study, we explore the function of the *Dictyostelium* GTPases Rac1A, 1B, and 1C, which show more than 90% identity to each other and more than 85% identity to human Rac1 (Bush et al., 1993). Each activated Rac1 group member bound strongly to DGAP1. Overexpression of the wild-type forms of these small G-proteins induced the formation of numerous filopodia and subsequently of membrane ruffles. Furthermore, we provide evidence that these GTPases play identical roles in a variety of major cellular processes including growth, motility, endocytosis, cytokinesis and development. Unexpectedly, constitutive activation of each of these Rac1 GTPases led to dominant-negative effects in each of these processes while the constitutively-inactivated mutants had virtually no effect. A plausible mechanism leading to these responses will be discussed.

MATERIALS AND METHODS

cDNA cloning

To obtain the complete cDNA of the *rac1C* gene, a *D. discoideum* λgt11 cDNA-expression library (Clontech) was screened by polymerase chain reaction (PCR) with an oligonucleotide derived from the 3′ end of the known cDNA fragment of the *rac1C* gene (Bush et al., 1993) and with a complementary primer to the left arm of the cDNA vector λgt11. The primers carried *Eco*RI sites to facilitate cloning. The two largest *rac1C* PCR fragments, 6r and 16r, were digested with *Eco*RI, purified in agarose gels, and cloned into the *Eco*RI site of pIC20R. Sequencing of these two clones showed that they contained the identical coding sequence of the *rac1C* cDNA and differed only in the length of the 5′ non-coding region. The sequence of longer clone, 16r, was deposited in the GenBank database under accession number AF153328. The nucleotide and derived amino acid sequences were analyzed with the University of Wisconsin Genetics Computer Group software package (Devereux et al., 1984).

Protein expression and GST-fusion protein binding assays

The cDNA fragments encoding full length Rac1B and Rac1C were amplified from a *D. discoideum* λgt11 cDNA library (Clontech), the sequences verified, and cloned into the *Eco*RI site of expression vector pGEX-5X-1 (Pharmacia). GST (glutathione S-transferase)-fusion proteins were expressed in *E. coli* strain JM83 and purified from the soluble fraction of bacterial extracts using glutathione-Sepharose affinity columns according to the instructions of the manufacturer. After purification, all GST-fusion proteins were dialyzed against PBS containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.3, and supplemented with 1 mM $MgCl_2$, 1 mM benzamidine, 2 mM DTT, and 0.1% NaN_3. Expression of GST-Rac1A and subsequent binding assays with the purified GST-fusion protein were performed as previously described (Faix et al., 1998). DGAP1 interaction was determined in western blots using anti-DGAP1 antibody 216-394-1.

Monoclonal antibodies and immunoblotting

Monoclonal antibodies against Rac1A were obtained by immunizing BALB/c mice with recombinant Rac1A together with Alugel S (Serva) and pertussis toxin (Sigma) as adjuvants. Spleen cells were fused with PAIB3Ag8I myeloma cells. Specificity of the antibodies was determined in solid phase assays and in western blots. mAb 273-461-3, specific for Rac1A, Rac1B, and Rac1C was used in this study.

Proteins were resolved by SDS-PAGE in 10% or 12% gels, and immunoblotting was performed by standard procedures using DGAP1-specific mAb 216-394-1 (Faix and Dittrich, 1996) or Rac1-group specific mAb 273-461-3. GFP was labeled with mAb 264-449-2 (Weber et al., 1999) and csA was detected with mAb 33-294-17 (Berthold et al., 1985). Primary antibodies were detected with either iodinated sheep anti-mouse IgG (Amersham) or phosphatase coupled anti-mouse IgG (Dianova). Quantification of labeled bands was determined by scanning densitometry or phosphoimaging. For fluorescence studies, mAb 176-3-1 specific for coronin was used (de Hostos et al., 1991).

Transformation vectors

The pDGFP-MCS-Neo vector was constructed to express, under control of the actin 15 promoter, fusion proteins in *D. discoideum* that contain GFP at the N terminus and Rac1 sequences at the C terminus. This vector is derived from vector pDGFP-MCS (Weber et al., 1999). A 700 bp sequence encoding the red-shifted GFP S65T (Heim and Tsien, 1996) together with an in-frame sequence encoding the linker $(GGS)_2$ and a multiple cloning site containing *Sal*I, *Hinc*II, *Xho*I and *Eco*RI restriction sites behind the GFP sequence was excised with *Hin*dIII from vector pDGFP-MCS and inserted in sense orientation in the expression vector pDEXH (Faix et al., 1992). The resulting vector was termed pDGFP-MCS-Neo. The wild-type (WT) forms of Rac1A, Rac1B, and Rac1C, as well as the constitutively-activated (G12V) and constitutively-inactivated (T17N) mutants of Rac1A and Rac1C were amplified from Rac1A, Rac1B, and Rac1C cDNAs with sequence specific primers and cloned in the sense orientation into the *Eco*RI site of pDGFP-MCS-Neo. The sequences of all PCR generated fragments were verified by DNA sequencing.

Cell culture and transformation of *D. discoideum* cells

If not indicated otherwise cells of the AX2 wild-type strain, of DGAP1-null strain G10-, and of transformants expressing GFP-fusion proteins were cultivated in axenic medium at 23°C as described by Claviez et al. (1982) on polystyrene culture dishes. Analysis of cytokinesis was performed with cells grown in shaken suspension at 150 rpm for 60 hours. For the initiation of development, the cells were washed and adjusted to a density of 1×10^7 cells/ml in 17 mM K/Na-phosphate buffer (PB), pH 6.0, and shaken for 12 hours at 150 rpm. For development on a substratum, the cells were washed twice in PB, and 2×10^8 cells were plated onto non-nutrient agar plates (∅ 10 cm) containing 0.8% Bacto-agar (Life Technologies) in PB. Development was monitored after 27 hours.

D. discoideum cells were transformed by electroporation and strains expressing GFP-fusion proteins were selected on plates in nutrient medium containing 20 μg/ml of G418 (Sigma), and cloned on SM agar plates with *Klebsiella aerogenes*. For transfected cells, G418 was added to a final concentration of 20 μg/ml. Transformants, here referred to as control, were obtained after transformation of AX2 wild-type cells with pDGFP-MCS-Neo alone. AX2 derived transformants expressing the wild-type forms of Rac1A, 1B, and 1C

are referred to as Rac1A-WT, Rac1B-WT, and Rac1C-WT, respectively. AX2 derived transformants with an amino acid substitution in the expressed Rac1A or Rac1C proteins were designated Rac1A-V12, Rac1A-N17, Rac1C-V12, and Rac1C-N17. DGAP1-null derived transformants expressing wild-type and mutant forms of Rac1A were termed DGAP1-Rac1A-WT, DGAP1-Rac1A-V12, and DGAP1-Rac1A-N17. Independent transformants that expressed GFP-fusion proteins were isolated. Since they showed the same phenotype, one clonal cell line for each construct was chosen and used for all experiments.

Confocal and fluorescence microscopy

For localization of GFP-fusion proteins, cells cultivated in nutrient medium were washed in PB and transferred to a glass surface. Confocal fluorescence images were obtained at 10 second intervals from a Zeiss LSM 410, equipped with a 100×/1.3 Plan-Neofluar objective. The 488 nm band of an argon-ion laser was used for excitation and a 515-565 nm band-pass filter for emission. Data were processed with the Adobe PhotoShop and CorelDraw software packages. Three-dimensional reconstructions were generated from fluorescence intensities using the AVS software package (Advanced Visual Systems) as described by Weber et al. (1999).

For immunofluorescence labeling, growth-phase cells were washed twice with PB and allowed to adhere on glass coverslips. The cells were fixed with picric acid/formaldehyde according to the method of Humbel and Biegelmann (1992) and labeled for coronin with mAb 176-3-6 followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (Sigma). F-actin was visualized with TRITC-conjugated phalloidin (Sigma). For the counting of nuclei, cells cultivated in shaken suspension for two days were fixed as described, and stained with 4′,6-diamidino-2-phenylindole (DAPI). The specimens were analyzed using a Zeiss Axiophot microscope equipped with a 100× Plan-Neofluar objective.

Determination of growth rates, cell motility, phagocytosis, and pinocytosis

Growth rates were determined by seeding cells with a toothpick onto a lawn of *Klebsiella aerogenes* on SM nutrient agar plates. The plates were incubated at 23°C and the radius of colonies was measured every 24 hours. Quantitative analysis of cell motility of growth-phase cells was performed as described previously (Faix et al., 1998).

Phagocytosis assays using heat-killed yeast in shaken suspension were carried out as described by Maniak et al. (1995), and quantitative fluid-phase uptake was performed with TRITC-labeled dextran according to Hacker et al. (1997). The results from quantitative phagocytosis and pinocytosis assays were normalized to total protein content determined by the BCA method (Pierce).

RESULTS

DGAP1 preferentially interacts with the activated forms of Rac1A, 1B, and 1C in vitro

We previously reported that the IQGAP-related protein DGAP1 binds directly and preferentially to activated, GTP-bound *Dictyostelium* Rac1A and human Rac1 (Faix et al., 1998). Rac1A shows more than 90% identity to Rac1B and the published fragment of Rac1C (Bush et al., 1993). In addition, all three *Dictyostelium* Rac1 GTPases share 100% identity throughout the core and putative extended effector domains to each other and with human Rac1 (Fig. 1A). To explore the possibility that DGAP1 also interacts with Rac1B and Rac1C, we first isolated the complete cDNA sequence of the *rac1C* gene using polymerase chain reaction (for details see Materials

and Methods). A comparison of the complete polypeptide sequences of Rac1A, 1B, and 1C is shown in Fig. 1B.

For binding experiments, Rac1A, 1B, and 1C were expressed as GST-fusion proteins in *E. coli*. The glutathione Sepharose-bound GTPases were charged with either GDP or GTPγS (guanosine 5′-O-(3-thio)triphosphate), and incubated with *Dictyostelium* lysates prepared from AX2 wild-type cells. After repeated washing of the beads, the presence of bound DGAP1 was analyzed in western blots using DGAP1-specific monoclonal antibody mAb 216-394-1 (Faix and Dittrich, 1996). As shown in Fig. 1C, DGAP1 did not only strongly bind to the Rac1A positive control, but also bound in an equal capacity to Rac1B and Rac1C. No binding of DGAP1 was detected to GST alone. The association of DGAP1 with all three Rac1 GTPases was most prominent when GTPγS was bound to the GTPases, indicating that DGAP1 binds preferentially to their activated, GTP-bound forms. These results define DGAP1 as a Rac1 group interacting protein.

Overexpression of Rac1 GTPases promotes the formation of filopodia and membrane ruffles

To define the function of Rac1 GTPases in vivo, we expressed the wild-type (WT) forms of Rac1A, 1B, and 1C tagged N-terminally with GFP in AX2 wild-type cells, and compared them with control cells expressing GFP alone. The N-terminal fusion was chosen so as to avoid interference with the C-terminal structural elements responsible for membrane association (Schafer and Rine, 1992). Strong expression levels were seen in all three cell lines, most likely due to the high copy number generated by G418 selection. Expression levels were on the average 2.5-fold higher for Rac1A-WT than for either Rac1B-WT or Rac1C-WT (Fig. 2A), and we noted moderate differences in fluorescence levels between the cells.

In control cells, the fluorescence was uniformly distributed throughout the cytoplasm with the exception of an accumulation at the nuclear region (Fig. 2B, left lower panel). In contrast, fluorescence of Rac1A-WT, Rac1B-WT, and Rac1C-WT cells was localized not only to the cytoplasm, but was also markedly enriched at the plasma membrane and emanating cell protrusions (Fig. 2B). The strong Rac1 overexpression in Rac1A-WT, Rac1B-WT, and Rac1C-WT cells induced the formation of numerous long filopodia (Fig. 2B). These filopodia extended between 2-6 μm in length and often showed branching. Being highly dynamic, they rapidly dissociated from and reattached to the underlying glass surface. Rac1A-WT developed more abundant filopodia than either Rac1B or Rac1C, which correlated with the level of overexpression. These cells also exhibited periods of intense membrane ruffling as shown for a Rac1A-WT cell (Fig. 2B, right upper panel). From these observations, it appears that all three Rac1 members are equally involved in triggering the formation of filopodia and membrane ruffles. Thus, *Dictyostelium* Rac1 GTPases carry out functions known from mammalian Rac1 and, most interestingly, also from Cdc42.

Constitutively-activated and constitutively-inactivated Rac1 mutants exhibit unexpected phenotypes

Since all three Rac1 GTPases mediate similar responses in the cell, the two most divergent members, Rac1A and Rac1C, were chosen for further studies. The objective of these experiments
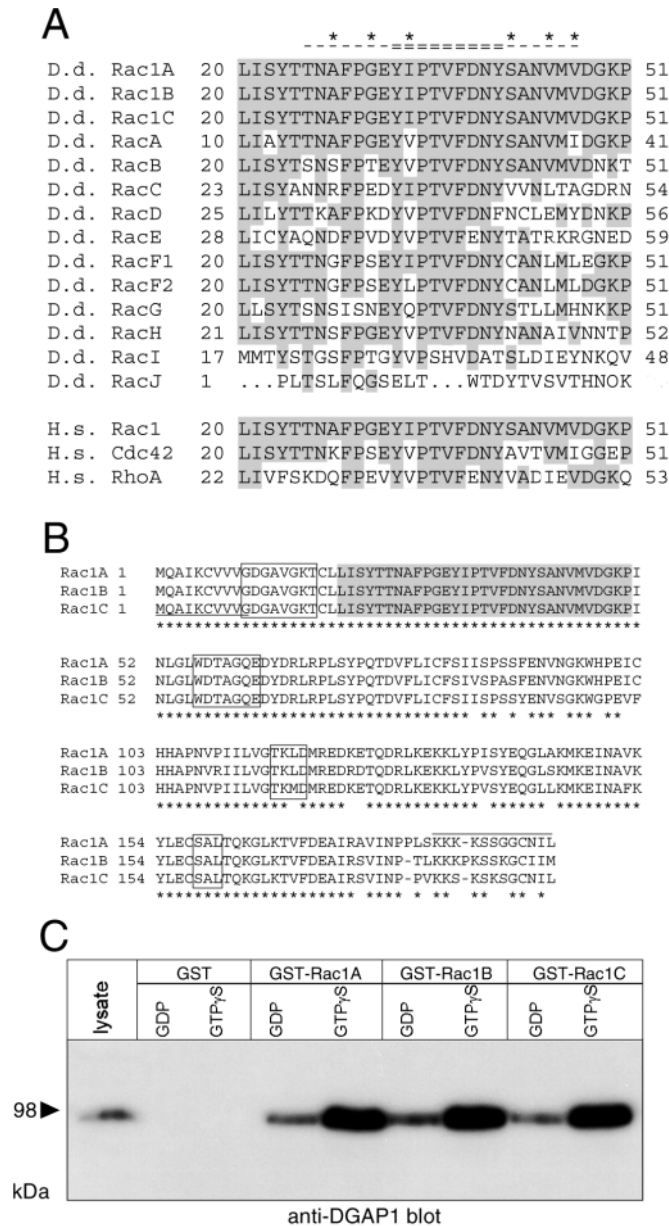
A

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                                   *  *  *  *==========  *  *  *
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D.d. Rac1A   20 LISYTTNAFPGEYIPTVFDNYSANVMVDGKP 51
D.d. Rac1B   20 LISYTTNAFPGEYIPTVFDNYSANVMVDGKP 51
D.d. Rac1C   20 LISYTTNAFPGEYIPTVFDNYSANVMVDGKP 51
D.d. RacA    10 LIAYTTNAFPGEYVPTVFDNYSANVMIDGKP 41
D.d. RacB    20 LISYTSNSFPTEYVPTVFDNYSANVMVDNKT 51
D.d. RacC    23 LISYANNRFPEDYIPTVFDNYVVNLTAGDRN 54
D.d. RacD    25 LILYTTKAFPKDYVPTVFDNFNCLEMYDNKP 56
D.d. RacE    28 LICYAQNDFPVDYVYVTVFENYTATRKRGNED 59
D.d. RacF1   20 LISYTTNGFPSEYIPTVFDNYCANLMLEGKP 51
D.d. RacF2   20 LISYTTNGFPSEYLPTVFDNYCANLMLDGKP 51
D.d. RacG    20 LLSYTSNSISNEYQPTVFDNYSTLLMHNKKP 51
D.d. RacH    21 LISYTTNSFPGEYVPTVFDNYNANAIVNNTP 52
D.d. RacI    17 MMTYSTGSFPTGYVPSHVDATSLDIEYNKQV 48
D.d. RacJ     1 ...PLTSLFQGSELT...WTDYTVSVTHNOK

H.s. Rac1    20 LISYTTNAFPGEYIPTVFDNYSANVMVDGKP 51
H.s. Cdc42   20 LISYTTNKFPSEYVPTVFDNYAVTVMIGGEP 51
H.s. RhoA    22 LIVFSKDQFPEVYVPTVFENYVADIEVDGKQ 53
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B

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Rac1A 1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPI
Rac1B 1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPI
Rac1C 1   MQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPI
          **************************************************

Rac1A 52  NLGLWDTAGQEDYDRLRPLSYPQTDVFLICFSIISPSSFENVNGKWHPEIC
Rac1B 52  NLGLWDTAGQEDYDRLRPLSYPQTDVFLICFSIVSPASFENVNGKWHPEIC
Rac1C 52  NLGLWDTAGQEDYDRLRPLSYPQTDVFLICFSIISPSSYENVSGKWGPEVF
          ******************************* ** * *** *** **

Rac1A 103 HHAPNVPIILVGTKLDMREDKETQDRLKEKKLYPISYEQGLAKMKEINAVK
Rac1B 103 HHAPNVPIILVGTKLDMREDRDTQDRLKEKKLYPVSYEQGLSKMKEINAVK
Rac1C 103 HHAPNVPIILVGTKMDMREDKETQDRLKEKKLYPVSYEQGLLKMKEINAFK
          ***************** ***** ************* ****** *****

Rac1A 154 YLECSALTQKGLKTVFDEAIRAVINPPLSKKK-KSSGGCNIL
Rac1B 154 YLECSALTQKGLKTVFDEAIRSVINP-TLKKKPKSSKGCIIM
Rac1C 154 YLECSALTQKGLKTVFDEAIRSVINP-PVKKS-KSKSGCNIL
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C

anti-DGAP1 blot

Fig. 1. DGAP1 binds to the three highly related Rac1 GTPases. (A) The effector domain region of the Rac1 GTPases is identical to each other and to that of human Rac1. Multiple sequence alignment of the effector domain regions of the known Rac-like proteins from *D. discoideum* with human Rho GTPases Rac1, Cdc42, and RhoA. Highlighted residues indicate the sequence identity. Double overlines and dashed overlines denote core and putative extended effector domains, and asterisks above indicate invariant residues of Rac1 group members which show highest degree of divergence with other Rac family members. D.d., *Dictyostelium discoideum*; H.s., *Homo sapiens*. GenBank accession numbers: Rac1A, L11588; Rac1B, L11589; Rac1C, AF153328; RacA, L11591; RacB, L11592; RacC, L11593; RacD, L11594; RacE, U41222; RacF1, AF037042; RacF2, C94218; RacG, C92890; RacH, C92764; RacI, C84883; RacJ, C91054; Hs Rac1, TVHUC1; Hs Cdc42, NP_001782; Hs RhoA, TVHU12. Sequences corresponding to RacG, RacH, RacI, and RacJ, as well as the amino termini of RacD, were found by screening the databank of the Japanese *Dictyostelium* cDNA sequencing project (University of Tsukuba, Japan). (B) Multiple alignment of the complete cDNA-derived polypeptide sequences of the Rac1 group members Rac1A, 1B, and 1C. Included are the Rho-related small GTPase functional domains: nucleotide interacting sites (boxed), effector domains (shaded in gray), Rho-insert domains (stippled line) and membrane association domains (overlined). The N-terminal residues unveiled by PCR to complete the entire Rac1C protein sequence are underlined. Dashes indicate gaps introduced to optimize for best alignment. The asterisks below the sequences indicate identical residues. (C) The glutathione-Sepharose bound GST-fusion proteins indicated were loaded with either GDP or GTPγS and were incubated for 1 hour with lysates prepared from AX2 wild-type cells. Proteins remaining on the washed beads were eluted with SDS sample buffer, and aliquots subjected to SDS-PAGE. DGAP1 interaction was detected by western blotting using anti-DGAP1 antibody mAb 216-394-1 followed by iodinated anti-mouse IgG.

was to determine whether filopodia formation required the cycling of the GTPases between the active (GTP-bound) and inactive (GDP-bound) forms. AX2 transformants were generated that expressed constitutively-activated (Rac1A-V12 and Rac1C-V12) and constitutively-inactivated (Rac1A-N17 and Rac1C-N17) mutants for Rac1A and Rac1C linked to GFP. For multiple independent transformants expressing these mutant forms, the average fluorescence intensity of the GFP signal was homogeneous but weak, and significantly lower than in the wild-type forms. To determine the ratio of GFP-tagged wild-type and mutant forms of Rac1A and Rac1C to total endogenous Rac1 proteins in AX2 cells, immunoblots of total cell lysates were labeled with the monoclonal antibody 274-461-3 which is specific for the Rac1A, 1B, and 1C group (Fig. 3). The results of this quantitative analysis was 13-fold for Rac1A-WT, 1-fold for Rac1A-V12, 2.6 fold for Rac1A-N17 and 5.2-fold for Rac1C-WT, 0.7-fold for Rac1C-V12, and 1-fold for Rac1AC-N17. Interestingly, the expression of

endogenous Rac1 in transformant Rac1A-WT cells was reduced to only 25% of that found in wild-type cells (Fig. 3), hinting these GTPases are feedback regulated. Taken together, these findings suggest that locking the Rac1 GTPases in either constitutively-activated or constitutively-inactivated forms has deleterious effects when expressed at high levels and, furthermore, strong overexpression is permissive so long as the GTPase is able to cycle.

The low GFP fluorescence intensity of Rac1 mutants precluded a similar analysis of live cells using confocal laser scanning microscopy. Thus, cell morphology and the organization of F-actin was examined in fixed cells using phase contrast and epifluorescence microscopy. In AX2 wild-type cells, F-actin was enriched at the cell cortex, crowns, filopodia and accumulated at the leading edge of cells (Fig. 4). Rac1A-WT and Rac1C-WT cells showed high levels of F-actin at the cortex, as well as in the very long and frequently branched filopodia. Surprisingly, both of the V12 mutants did not project filopodia. Instead, they displayed prominent crown-shaped extensions from the cell surface that were highly enriched with F-actin (Fig. 4). To examine the spatial organization of actin in these extensions, we reconstructed the three-dimensional distribution of F-actin from a confocal image series. Interestingly, these cup-shaped structures shown in Fig. 5B were also strongly labeled with mAb 176-3-1 for the cytoskeletal protein coronin (de Hostos et al., 1991), normally found in macropinosomes (de Hostos et al., 1991; Maniak et al., 1995). On the other hand, both N17 mutants displayed
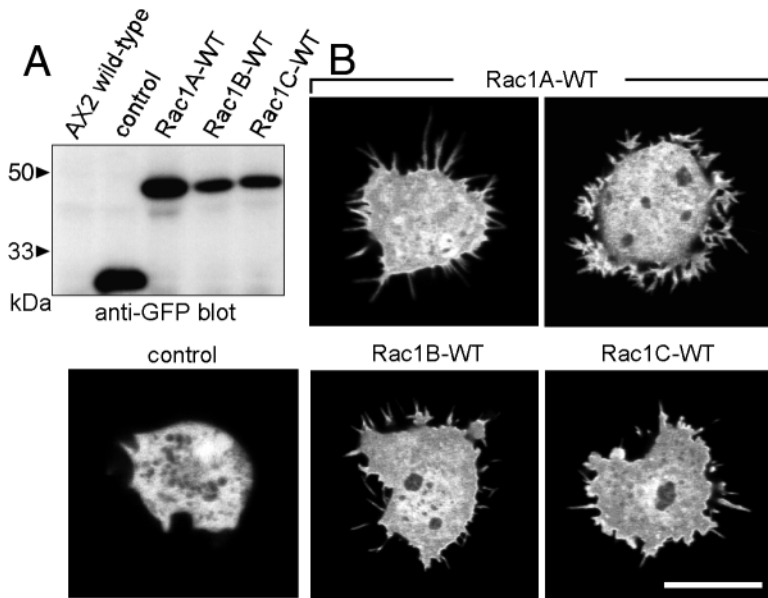
Fig. 2. Overexpression of GFP-tagged Rac1 GTPases promotes the formation of filopodia and membrane ruffles. (A) Equal amounts of total cellular proteins from the AX2 wild-type, from control cells expressing GFP alone, and from transformants Rac1A-WT, Rac1B-WT, and Rac1C-WT were loaded per lane, subjected to SDS-PAGE, blotted onto nitrocellulose, and labeled with anti-GFP mAb 264-449-2 followed by iodinated anti-mouse IgG. Quantification of labeled bands was determined by phosphoimaging. (B) Confocal sections of control cells and of the transformants indicated are shown to visualize GFP expression and distribution in living cells. Control cells showed an uniform cytoplasmic distribution with an accumulation in the nucleus and exclusion from vacuoles. Cells of the transformants Rac1A-WT, Rac1B-WT, and Rac1C-WT showed a cytoplasmic localization with an enrichment at the cell cortex. In contrast to the control, these cells predominantly formed numerous, long and bifurcated filopodia followed by intense membrane ruffling periods as indicated by the second sequence of a Rac1A-WT cell (upper right panel). Bar, 10 µm. Time lapse movies of the shown cells can be downloaded from the Web (www.physiology.wisc.edu/~faix/rac1.html)
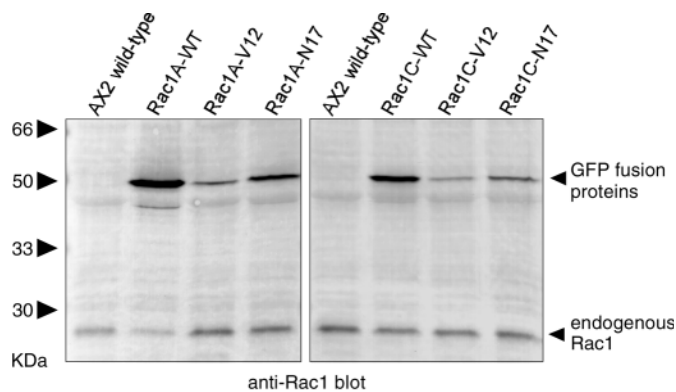
Fig. 3. Expression levels of wild-type (WT), constitutively-activated (V12), and of constitutively-inactivated (N17) forms of Rac1A and Rac1C tagged with GFP in AX2 wild-type cells. Total cellular proteins of the cell lines indicated were loaded per lane, subjected to SDS-PAGE, blotted onto nitrocellulose, and labeled with anti-Rac1 mAb 273-461-3 followed by phosphatase conjugated anti-mouse IgG. A parallel blot was incubated with Rac1 mAb 273-461-3 followed with iodinated anti-mouse IgG. Quantification of labeled bands in these blots was determined by densitometry or phosphoimaging.

numerous shorter filopodia compared to Rac1A-WT and Rac1C-WT cells. The indistinguishable cytoskeletal organization of the Rac1A and Rac1C transformants provide further evidence that members of the Rac1 group are equally involved in re-modeling of the actin cytoskeleton.

We previously reported that mutant cells deficient for the Rac1A-binding protein DGAP1 develop numerous long filopodia (Faix et al., 1998). To evaluate a potential role for DGAP1 as a Rac1 effector in vivo, the wild-type and mutant forms of Rac1A were expressed in DGAP1-null cells (Fig. 6A). The expression levels of the recombinant proteins were similar to those in the AX2 derived transformants depicted in Fig. 3. DGAP1-Rac1A-WT cells extended many filopodia that emanated from the cell periphery which were longer and thicker than those seen in either the DGAP1-null or Rac1A-WT cells (Fig. 6B). DGAP1-Rac1A-V12 cells were spread out to a greater extent giving a larger appearance than their AX2 counterparts and also formed numerous crown-shaped structures on their dorsal cell surface. DGAP1-Rac1A N17 cells showed shorter, but significantly more filopodia than the parent strain. These results demonstrate that cytoskeletal re-arrangements induced by constitutive expression of wild-type and mutant forms of Rac1 were augmented in DGAP1-null transformants.

Constitutively-activated Rac1 mutants are severely impaired in growth, motility, and endocytosis

The cytoskeletal changes induced by the expression of wild-type and mutant forms of Rac1 prompted us to investigate whether other cell processes that require a dynamic actin cytoskeleton, such as cell growth, cell migration, and endocytosis were affected. The colony growth rates of AX2 wild-type, DGAP1-null, and Rac1 transformants were determined by measuring the increase in colony size in bacterial lawns with time (Fig. 7A,C). In Rac1A-WT cells, colony growth was enhanced by 33% over the wild-type, whereas growth of Rac1A-V12 cells was decreased by 20%. Rac1A-N17 colonies grew at a rate comparable to that of AX2 (Fig. 7A). Similar, but less pronounced results were obtained for cells expressing wild-type and mutant forms of Rac1C (Fig. 7B), in accord with their lower expression levels (see Fig. 3). DGAP1-Rac1A-WT cells showed equivalent colony growth as the cells of DGAP1-null and DGAP1-Rac1A-N17 strains, while the growth rate of DGAP1-Rac1A-V12 cells decreased by 40% (Fig. 7C). Since the formation of a colony is a cumulative function of both growth rate and cell motility, we also compared the motility of these cells in nutrient medium using a quantitative assay. The measured velocity of cell motility on a glass surface for Rac1A-WT cells was 26% higher than those of AX2 wild-type and Rac1A-N17 cells, whereas the cell velocity of Rac1A-V12 cells was reduced by 30% compared to AX2 (Fig. 7D). Similar values were measured for cells expressing wild-type and mutant forms of Rac1C (Fig. 7E). DGAP1-Rac1A-WT cells showed a similar cell velocity as DGAP1-null and DGAP1-Rac1A-N17 cells,

which suggests the DGAP1-null cells had already obtained their maximal rate of motility. DGAP1-Rac1A-V12 cells showed a decreased cell migration by 37% when compared to the parent strain (Fig. 7F).

Endocytosis, the internalization of particles and fluid by *Dictyostelium* cells depends on the function of actin cytoskeleton (Maniak et al., 1995), and a large fraction, if not

the entire uptake of solutes in these cells occurs through macropinocytosis (Hacker et al., 1997). Therefore, we determined the internalization rate of the fluid-phase marker TRITC-dextran. Compared to wild-type cells the rate of fluid-phase uptake was increased 22% in Rac1A-WT cells, while it decreased in Rac1A-V12 cells by 50%, and was unchanged in Rac1A-N17 cells (Fig. 8A). In Rac1C-V12 cells, the rate
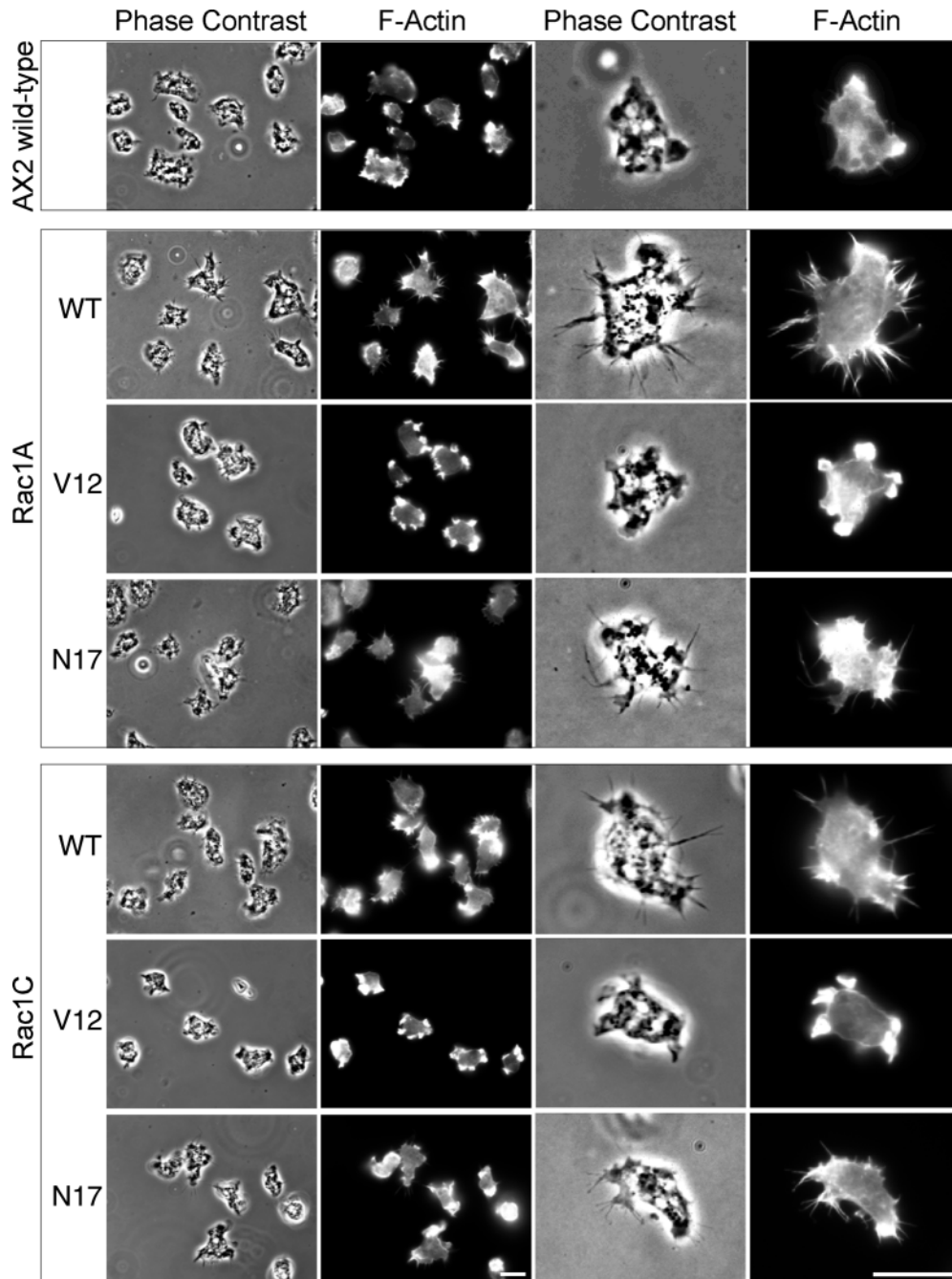
Fig. 4. Cell morphology and F-actin organization of AX2 wild-type cells and in cells of this strain expressing wild-type (WT), constitutively-activated (V12), and constitutively-inactivated (N17) forms of Rac1A and Rac1C. The cells were fixed and labeled with TRITC-phalloidin to visualize F-actin. Low magnification overview (2 left columns) shows general homogeneity in the cell populations. High magnification of individual cells (2 right columns) shows detailed morphology and F-actin organization. In AX2 cells, F-actin was detected within a variety of structures including crowns and small filopodia, whereas F-actin label in Rac1A-WT and Rac1C-WT cells was accumulated predominantly in numerous long filopodia. V12 transformants altogether lacked filopodia and F-actin was seen in cortical crown-like structures. N17 mutants displayed more, but shorter filopodia. Bars, 10 μm.

Fig. 5. Distribution and organization of F-actin in two transformants that expressed the wild-type (A) or the constitutively-activated (B) forms of the small GTPase Rac1A. The cells were fixed and labeled with TRITC-phalloidin to visualize F-actin. Starting at the base of the cells, confocal sections of the TRITC-phalloidin fluorescence were recorded at intervals of 0.2 μm and processed into a three-dimensional image using a ray-tracing algorithm. The obtained images were color-coded according to the emitted fluorescence intensity: blue represents areas with low and red with high F-actin concentrations. Rac1-WT cells formed numerous long filopodia, while Rac1A-V12 were devoid of filopodia. Instead, these cells formed crown-shaped structures resembling macropinosomes. Bar, 5 μm.
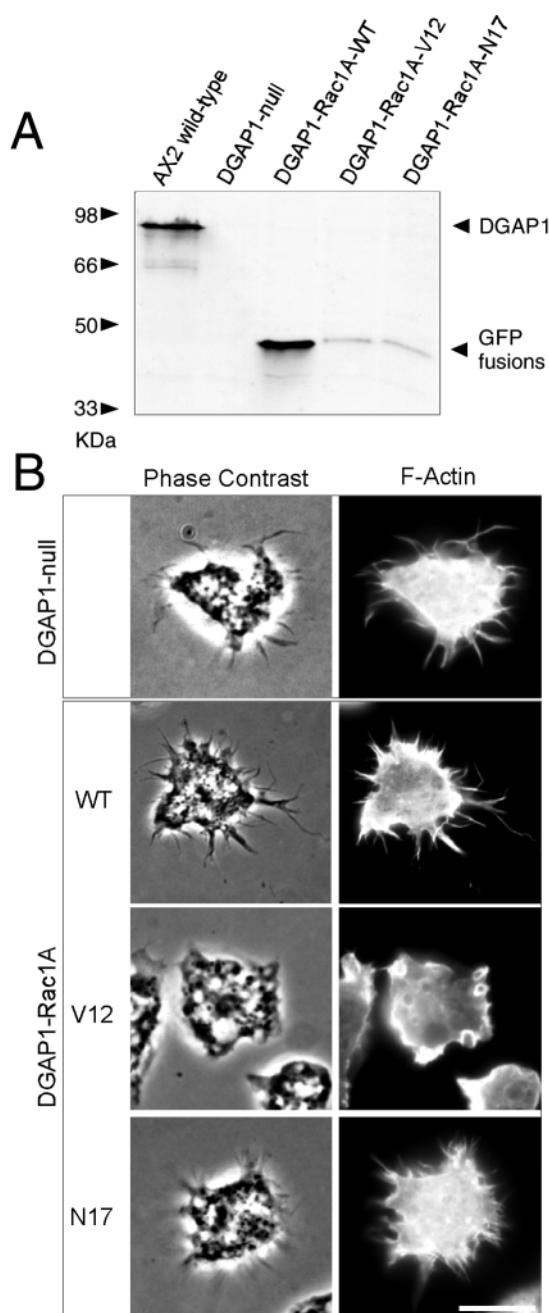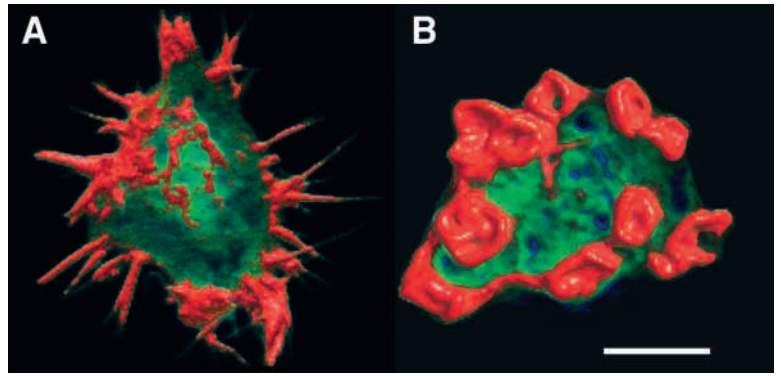
decreased to approximately 50% relative of the wild-type rate. The significant reduction in fluid-phase uptake by V12 mutants of Rac1A and Rac1C paralleled their slow growth rate in liquid culture, with generation times of 13 hours for Rac1A-V12 cells and 11.5 hours for Rac1C-V12 cells compared to 8 hours for the AX2 wild type.

We also investigated a signaling pathway in which local rearrangements of the F-actin cytoskeleton are triggered by an external stimulus. In *Dictyostelium*, phagocytosis is induced by adhesion of a particle to the cell surface, as opposed to macropinocytosis, which is a constitutive process (Maniak et al., 1995). Therefore, we determined the rate of phagocytosis of yeast particles in wild-type and Rac1-expressing cells. Particle uptake was reduced to approximately 20% of the wild-type rate in Rac1A-V12 and Rac1C-V12 cells, while the uptake rate for Rac1A-WT and Rac1A-N17 cells was slightly lower, but in a range comparable to that of the wild-type (Fig. 8B). Taken together, these results establish an important function of Rac1 GTPases in the growth, motility, and in fluid-phase and solid-phase endocytosis of *D. discoideum* cells.

Impaired cytokinesis in constitutively-activated and constitutively-inactivated Rac1 mutants

Light microscopic analysis showed many large cells in shaken suspension cultures of constitutively-activated Rac1-V12 transformants. Thus quantitative fluorescence microscopy of 4′,6-diamidino-2-phenylindole (DAPI) labeled nuclei of wild-type and mutant cells was used to establish the role of Rac1 GTPases in cytokinesis. More that 95% of the nuclei in AX2

Fig. 6. Cell morphology and F-actin organization in DGAP1-null cells and derived transformants expressing wild-type and mutant forms of Rac1A. (A) Expression levels of wild-type (WT), constitutively-activated (V12), and of constitutively-inactivated (N17) forms of Rac1A tagged with GFP in DGAP1-null cells. Total cellular proteins of the cell lines indicated were loaded per lane, subjected to SDS-PAGE, blotted onto nitrocellulose, and labeled with anti-DGAP1 mAb 216-394-1 and anti-GFP mAb 264-449-2 followed by phosphatase conjugated anti-mouse IgG. (B) F-actin in fixed cells was visualized with TRITC-phalloidin. Cells of the DGAP1-null parent strain showed prominent F-actin organization in numerous long filopodia. This labeling pattern was similar but even more pronounced in DGAP1-Rac1A-WT cells that formed extremely long and thick filopodia. DGAP1-Rac1A-V12 cells showed multiple crown-like structures but were lacking filopodia. DGAP1-Rac1A-N17 cells formed more, but shorter filopodia when compared to DGAP1-null and to DGAP1-Rac1A-WT cells. Bar, 10 μm.

wild-type and Rac1A-WT strains were found in cells that were mono- or bi-nucleated. Under the same conditions, 24% of the nuclei in Rac1A-N17 cells and 57% of the nuclei in the Rac1A-V12 cells were found in cells that contained more than 3 nuclei per cell (Fig. 9A,B). Similar results were obtained for Rac1C

transformants (Fig. 9C,D). The cytokinesis defect induced by the expression of constitutively-activated Rac1A was even more pronounced in the DGAP1-null background, where 80% of all nuclei were found in cells that contained more than 3 nuclei (Fig. 9E,F). Similar to the localization of RacE during
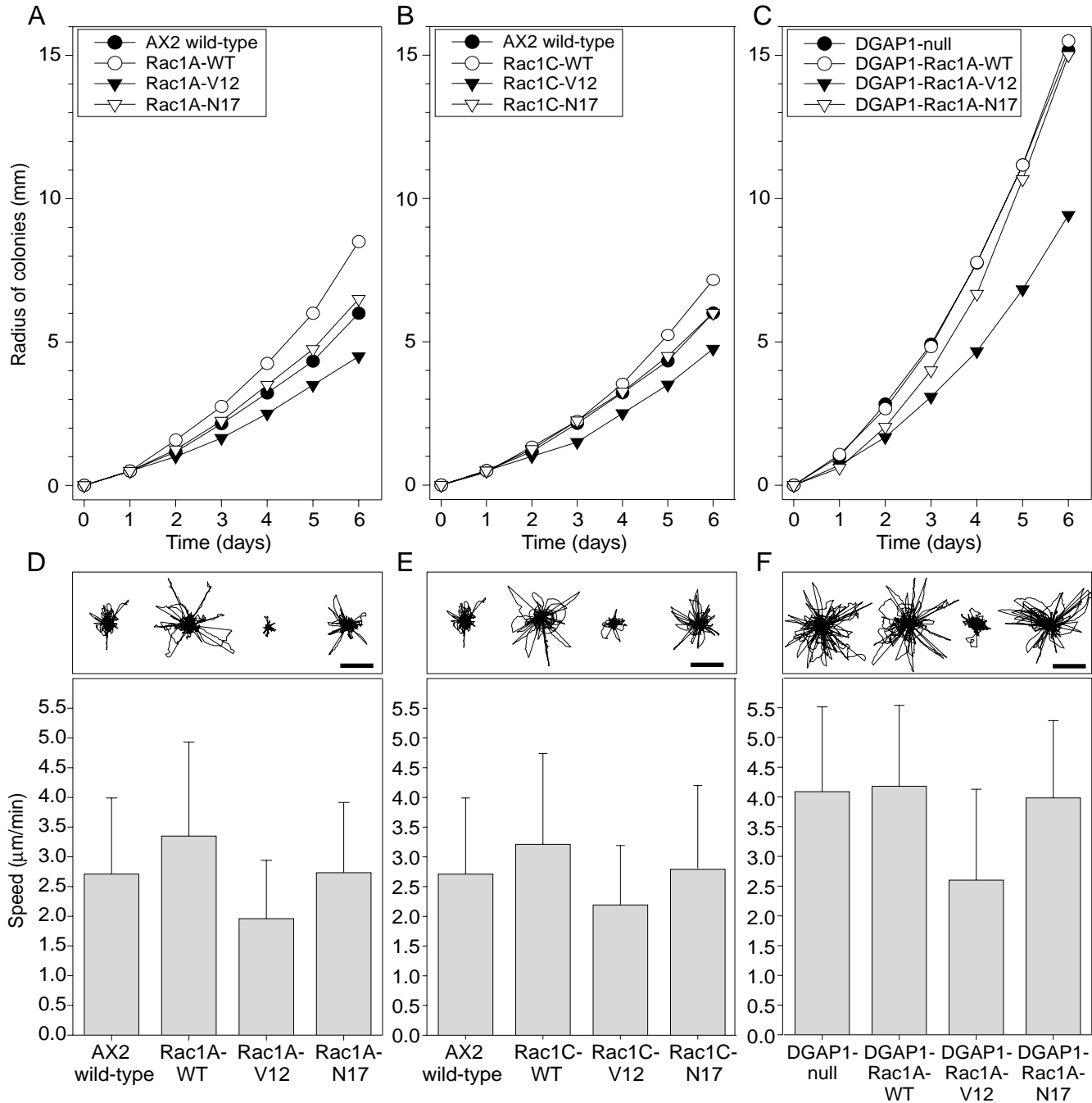
Fig. 7. Reduced colony growth and cell motility in cells expressing the activated forms of Rac1A and Rac1C. Smaller colonies were formed in bacterial lawns by Rac1A-V12 (A), by Rac1C-V12 (B), and also by DGAP1-Rac1A-V12 (C). The mean values of three separate experiments are shown. (D-F) Cell motility for same strains determined in nutrient medium. Individual tracks of migrating cells were recorded over a 30 minute period, superimposed, and plotted to a common origin using an image processing system (top). For each experiment a field containing approximately 100 cells was monitored. Motility rates were similar for both parent strains and derived transformants expressing N17, slightly faster for WT, but reduced for V12. Bars, 100 μm. Quantification of cell motility for the same cell lines (bottom). The bars show the mean values for the cell velocities from 5 experiments for each cell line. The error bars indicate the standard deviation of the mean. The differences between V12 and the parent strains were highly significant ($P<10^{-4}$, two-sided t-test). The differences between AX2 and derived transformants expressing wild-type forms of Rac1A and Rac1C were significant ($P<10^{-3}$, two-sided t-test).
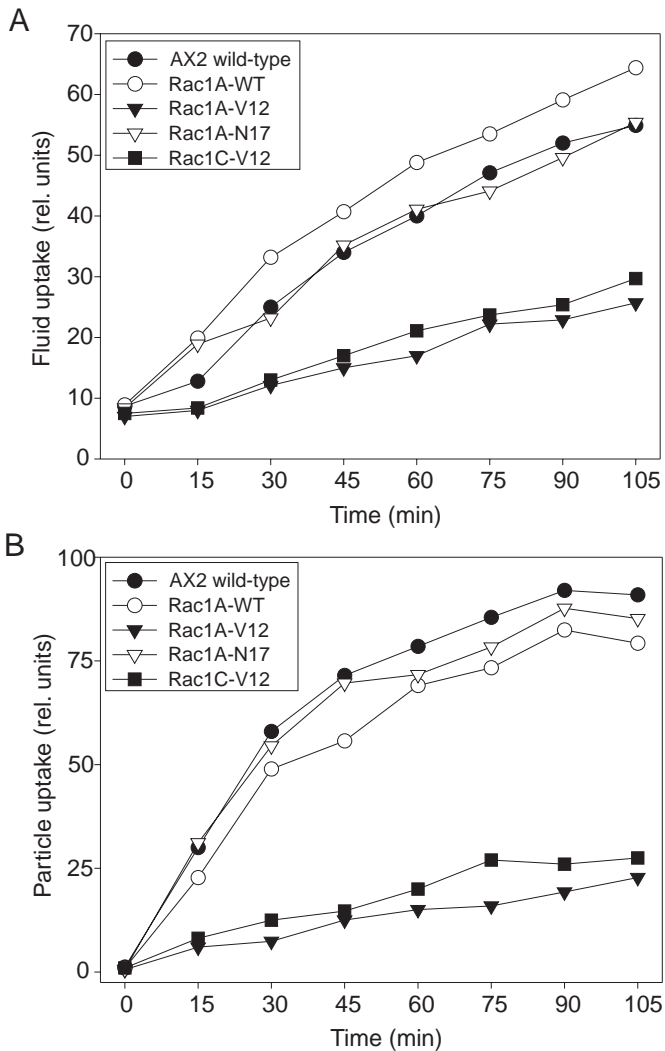
developmentally regulated genes followed by the aggregation of single cells into a multicellular organism that culminates with the formation of fruiting bodies (Gerisch, 1987). To determine the function of Rac1 GTPases in early development, we monitored accumulation of the contact site A glycoprotein (csA) in wild-type and transfected cells. CsA is a strictly developmentally regulated cell adhesion molecule that is not detectable in growth-phase cells and is maximally expressed during early development between 6 and 9 hours of starvation in suspension (Faix et al., 1992). Immunoblots of total cellular proteins labeled with anti-csA mAb 33-294-17 showed a somewhat premature accumulation of csA in Rac1A-WT and Rac1A-N17 cells when compared with AX2 cells. In contrast, a markedly delayed csA expression of approximately 3 hours occurred in Rac1A-V12 cells. In addition, Rac1A-V12 cells did not reach the maximal csA expression levels seen in the other cell lines (Fig. 10A, left panel). A comparable pattern of delayed csA accumulation was observed for Rac1C-V12 cells (Fig. 10A, right panel). These results suggest that Rac1 GTPases are also involved in the regulation of gene expression in *D. discoideum* development.

When multicellular development on a solid surface was compared after 27 hours of starvation, both V12 transformants differed consistently from the AX2 parent strain, while the Rac1-WT and Rac1-N17 transformants were indistinguishable from the parent strains. AX2 cells had formed mature fruiting bodies with a rounded sorocarp at the tip of a tapered stalk, whereas both the Rac1A-V12 and Rac1C-V12 cells were significantly delayed in terminal differentiation (Fig. 10B). After 27 hours of development a large proportion of these cells was still found in the migrating slug stage, which roughly corresponds to the 12 hours developmental stage of the wild-type (Faix et al., 1992). In addition, many cells were left in clusters on the substratum. The fruiting bodies that did form were on the average smaller and less numerous when compared to the wild type. But although Rac1A-V12 and Rac1C-V12 did not form normal looking fruiting bodies even after 60 hours, they produced viable spores.

During development, DGAP1-null cells form aggregates with multiple tips that develop into abnormally branched multicellular structures with short thick stalks and often irregularly shaped clusters of spores (Faix and Dittrich, 1996). These structures were different from DGAP1-Rac1A-V12 cells, which formed notably smaller and less branched multicellular structures (Fig. 10B). The smaller size and more regular distribution of these structures on the plates indicate that these cells were recruited from smaller aggregation areas. Taken together, these results demonstrate an important function of Rac1 GTPases in early and late *Dictyostelium* development.

DISCUSSION

In Swiss 3T3 fibroblasts, the activated forms of the Rho family GTPases Cdc42, Rac, and Rho have been implicated regulating the dynamics of specific F-actin structures. In their activated forms, Cdc42 induces the formation of microspikes and filopodia, Rac leads to the formation of membrane ruffles or lamellipodia, while Rho induces the formation of stress fibers linked to focal adhesions plaques (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). *Dictyostelium*
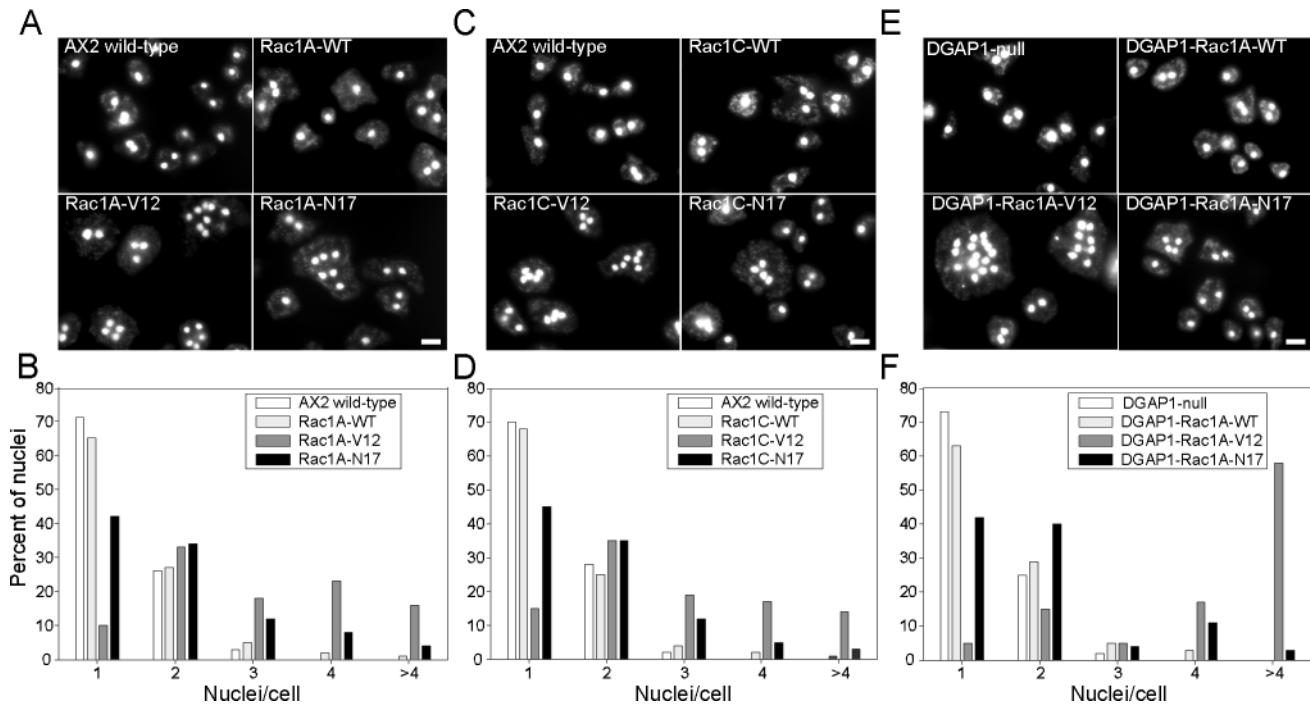

Fig. 8. Reduced fluid-phase uptake and uptake rate of yeast particles in Rac1A-V12 and Rac1C-V12 cells. AX2 wild-type, Rac1A-WT, Rac1A-V12, Rac1A-N17, and Rac1C-V12 cells were incubated in shaken suspension with TRITC-labeled dextran to monitor the time course of pinocytosis (A), or with heat-killed TRITC-labeled yeast particles to monitor the time course of phagocytosis (B). The mean values of two separate experiments for A and B are shown.

cytokinesis (Larochelle et al., 1997), the wild-type forms of all three Rac1 members did neither accumulate in the cleavage furrow nor were they enriched at the poles of dividing cells (data not shown). No noteworthy cytokinesis defect in the transformants was detectable in Petri dish cultures where the cells grow in contact with a solid surface (data not shown). Taken together, these data indicate that in suspension grown Rac1A-V12 cells, and to a lower extent in Rac1-N17 cells, undergoing mitosis cytokinesis is not reliably coupled to nuclear division.

Constitutively-activated Rac1 mutants are delayed in early and multicellular development

During their growth phase, cells of *D. discoideum* exist as individual amoebae. Depletion of nutrients initiates a developmental program beginning with the expression of

Fig. 9. Defect in cytokinesis induced by the expression of constitutively-activated and constitutively-inactivated Rac1A and Rac1C. Cells of AX2 wild-type (A and C), of DGAP1-null cells (E), and of derived transformants indicated were cultivated with nutrient medium in shaken suspension for 60 hours, allowed to attach to glass cover slips, and fixed. The nuclei were stained with DAPI to monitor the relation of mono- to multi-nucleate cells. Histograms showing distribution of the number of nuclei in AX2 wild-type cells (B and D), in DGAP1-null cells (F), and in derived transformants indicated. A strong defect in cytokinesis was detected in cells of transformant Rac1A-V12 and Rac1C-V12, and was most prominent in DGAP1-Rac1A-V12 cells. A moderate cytokinesis defect was also seen for these strains expressing constitutively-inactivated Rac1 proteins. 450-650 nuclei were counted for each strain. Bars, 10 μm.

appears to lack Cdc42 and Rho homologues, but a cladistic analysis has instead revealed 14 *rac*-like genes (Bush et al., 1993; Larochelle et al., 1996; Rivero et al., 1999). In this report, we describe the function of Rac1A, Rac1B, and Rac1C, three closely related GTPases that within all *Dictyostelium* Rac members show highest degree of homology to human Rac1. When the wild-type forms of the Rac1 GTPases were strongly overexpressed under transcriptional control of the actin 15 promoter, each induced the formation of prominent, long filopodia and subsequently of membrane ruffles (Fig. 3). Thus, the *Dictyostelium* Rac1 GTPases exert activities on the actin cytoskeleton, known for mammalian Rac1 but also in particular for Cdc42. Overexpression of wild-type forms of Rho family GTPases activate their respective pathways mentioned above (Moorman et al., 1999), and this is in line with our results that show a correlation of Rac1 expression with the number of filopodia in these cells (Figs 2 and 3). However, due to the delayed appearance of membrane ruffling, we can not at this point distinguish whether this process is directly induced by Rac1 GTPase activation, or by a subsequent event triggered by the activation of another downstream GTPase, as reported for the activation of Rac1 by Cdc42 in fibroblasts (Kozma et al., 1995).

Remarkable results in this study were obtained with transformants expressing constitutively-activated (V12) and constitutively-inactivated (N17) Rac1 proteins. Rac1-V12 mutants exhibited strong defects in all major cellular events associated with the regulation and organization of the actin

cytoskeleton including filopodia formation, membrane ruffling, growth, motility, endocytosis, cytokinesis, morphogenesis and in gene expression during early development. With the exception of more and shorter filopodia (Fig. 4), as well as a moderate defect in cytokinesis (Fig. 9), Rac1-N17 cells were similar to the wild type. These results were unforeseen since Rac1 signaling is usually induced with constitutively-activated and blocked by constitutively-inactivated proteins (Ridley et al., 1992; Ridley and Hall, 1992; Kozma et al., 1995). However, the inhibitory effects of constitutively-activated Rho family GTPases have also been reported from other organisms. Constitutitvely-activated RhoA, Rac1, or Cdc42 was shown to inhibit cell migration of murine macrophages (Allen et al., 1998). Similarly, in chick neurons constitutively-activated Rac1 impaired outgrowth, whereas neritogenesis was stimulated by wild-type Rac1 (Albertinazzi et al., 1998).

Our results may provide some insights in the mechanism of Rac1 downstream signaling in *Dictyostelium*. We propose that GTP-bound Rac1 forms tightly bound GTPase-effector complexes, which ultimately require the GTPase to dissociate in order to allow downstream signal transduction. This dissociation is most likely mediated by hydrolysis of the bound GTP, and this is largely blocked in Rac1-V12 cells. In addition, endogenous Rac1, and possibly other closely related GTPases, would be precluded from access to common effector molecules. Therefore, the inability to properly signal leads to the defects observed in these cells. The inactivated proteins
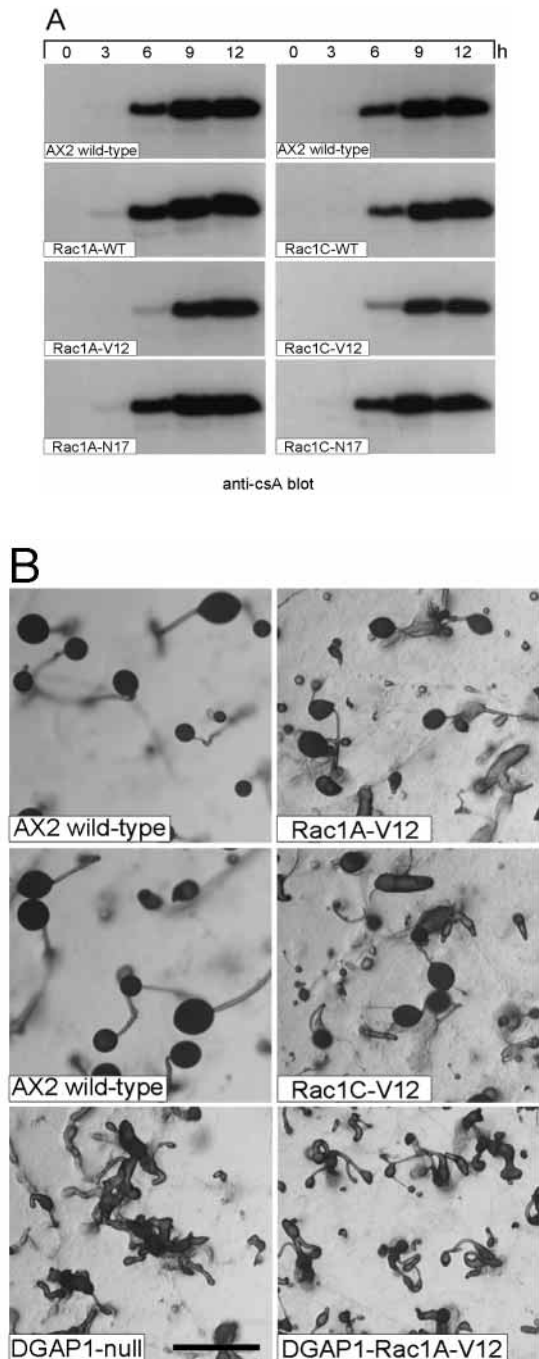
Fig. 10. Delay in early and late development in transformants expressing activated Rac1. (A) Time course of csA accumulation in the AX2 parent strain and in AX2 derived transformants expressing wild-type and mutant forms of Rac1A (left panel) or Rac1C (right panel) in non-nutrient buffer. Equal amounts of total cellular proteins from growth-phase cells (0 hours) or developing cells (3, 6, 9, 12 hours of starvation) were separated by SDS-PAGE, blotted onto nitrocellulose, and labeled for csA with iodinated mAb 33-294-17. (B) Late development of AX2 wild-type cells, of DGAP1-null mutant, and of derived transformants indicated. Cells of the parent strains and of the transformants were harvested from nutrient medium, washed in non-nutrient buffer and plated on nutrient-agar plates. Images were taken 27 hours after initiation of development. Bar, 1 mm.

cannot form the activated GTPase-effector complexes, but the subtle phenotype observed in Rac1-N17 transformants suggests that activation and downstream signaling still occurred through the activation of endogenous Rac1. Thus, it is likely that the ability to cycle between the GTP-bound and GDP-bound forms, as exemplified by the Rac1 wild-type forms, is crucial for normal signaling. Indeed, we were able to achieve a very strong, and apparently tolerable, 13-fold overexpression in Rac1A-WT cells. In contrast, lower levels of expression comparable to that of the total endogenous Rac1 were obtained in Rac1A-V12 and 2.6-fold higher in Rac1A-N17 cells (Fig. 3), indicating that expression of these mutant proteins was tightly regulated in order to avoid deleterious effects. Nevertheless, in Rac1A-V12 and Rac1C-V12 cells, the low levels were sufficient to result in a strong functional impairment whereas they were insufficient to cause the expected dominant-negative effects in Rac1-N17 cells. Taken together, these findings suggest that normal function of Rac1 GTPases is dependent on their ability to cycle between the activated and inactivated forms.

This situation contrasts with that for at least two other *Dictyostelium* Ras-related small GTPases, RacE and Rap1. RacE is essential for cytokinesis in suspension (Larochelle et al., 1996), and the cytokinesis defect of RacE-null cells was rescued by the expression of constitutively-activated RacE (Larochelle et al., 1997). Similarly, the expression of activated Rap1 led to an increased rate of phagocytosis (Seastone et al., 1999). Evidently, these GTPases act as permissive switches in their respective pathways and do not have to cycle in order to propagate their signals.

We have established a role for Rac1 GTPases in growth, motility, endocytosis, cytokinesis, and development of *Dictyostelium*. The re-organization of the cortical actin cytoskeleton is crucial for these events to occur (Bretscher, 1991). In pinocytosis and in phagocytosis, *Dictyostelium* cells protrude F-actin rich extensions from its periphery to trap and engulf endocytic cargo (Maniak et al., 1995; Hacker et al., 1997). A precisely regulated process also applies to cell migration, where re-organization of the cytoskeleton during protrusion of a leading edge must be spatially and temporally coordinated, in order to achieve persistent directional movement (Mitchison and Cramer, 1996). In cytokinesis, the orchestrated activities of the actin-rich cell cortex in conjunction with the microtubule-based spindles and asters guarantee that newly duplicated nuclei segregate properly into the daughter cells (Satterwhite and Pollard, 1992; Neujahr et al., 1997). Likewise, morphogenesis in *Dictyostelium* is a complex process that not only requires the expression of specific genes, but also needs extensive cell movement towards and within the aggregate in addition to differential cell-cell adhesion. The findings that these processes were strongly impaired in Rac1-V12 cells suggest, that these activities are dependent on an appropriate regulation of actin filament dynamics. Conversely, Rac1-WT cells readily formed filopodia and membrane ruffles (Figs 2B and 4), migrated faster (Fig. 7), and increased the rate of pinocytosis (Fig. 8) when compared to AX2 wild-type cells, indicating that overexpression of Rac1 wild-type proteins enhanced the dynamics of the re-organization of cortical actin.

Activated GTPases transmit signals to the actin cytoskeleton through a set of specific effector proteins (reviewed by Van

Aelst and D'Souza-Schorey, 1997). Rac1A, 1B, and 1C share identical effector domains between each other and with human Rac1, but not with any other known *Dictyostelium* Rac-like GTPase (Fig. 1A), indicating that Rac1 GTPases have a unique set of effector proteins in common (Bush et al., 1993). The most likely candidate for a Rac1 GTPase effector protein appeared to be the IQGAP-related protein DGAP1, since it bound directly to Rac1A and strongly interacted with each of the activated Rac1 GTPases (Fig. 1C). This assumption was further substantiated by the analysis of DGAP1 mutants, which displayed considerable changes in both actin cytoskeleton organization and actin-based activities (Faix and Dittrich, 1996; Faix et al., 1998). To assess a potential role for DGAP1 as a Rac1 GTPases effector in vivo, wild-type and mutant forms of Rac1A were expressed into DGAP1-null cells and compared with AX2 cells expressing the same constructs in order to identify a cellular pathway dependent on DGAP1. Although DGAP1 transformants showed an augmented phenotype, no specific process strictly dependent on DGAP1 was identified in this study. It is nevertheless conceivable that DGAP1 is a component of a multi-protein effector complex, which is able to propagate signals, even in the absence of DGAP1. It is also quite possible that DGAP1 is a negative regulator of Rac1. DGAP1 binding to and sequestering of activated Rac1 could prevent GTP-bound Rac1 GTPases from interacting with downstream targets. This latter assumption is in line with previous studies obtained with cells that either lacked or overexpressed DGAP1 (Faix et al., 1998). The F-actin cytoskeleton of DGAP1-null cells is rapidly re-arranged to form membrane ruffles and numerous filopodia whereas in DGAP1-overexpressing cells, the formation of cellular projections such as filopodia is largely suppressed. Furthermore, DGAP1-overexpressing cells also have strong defects in cell motility, in phagocytosis and in cytokinesis.

In conclusion, our results provide strong evidence that Rac1A, 1B, and 1C are key regulators of a wide array of cellular processes based on the re-organization of actin cytoskeleton in *Dictyostelium*. The objective of future studies will be the identification and characterization of additional components of the Rac1 signal transduction pathways in order to unravel the molecular mechanisms leading to filopodia formation, cell motility, endocytosis, cytokinesis, and morphogenesis in *Dictyostelium*.

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