

Dictyostelium RasG Is Required for Normal Motility and Cytokinesis, But Not Growth

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Abstract. RasG is the most abundant Ras protein in growing *Dictyostelium* cells and the closest relative of mammalian Ras proteins. We have generated null mutants in which expression of RasG is completely abolished. Unexpectedly, *RasG*⁻ cells are able to grow at nearly wild-type rates. However, they exhibit defective cell movement and a wide range of defects in the control of the actin cytoskeleton, including a loss of cell polarity, absence of normal lamellipodia, formation of un-

usual small, punctate polymerized actin structures, and a large number of abnormally long filopodia. Despite their lack of polarity and abnormal cytoskeleton, mutant cells perform normal chemotaxis. However, *rasG*⁻ cells are unable to perform normal cytokinesis, becoming multinucleate when grown in suspension culture. Taken together, these data suggest a principal role for RasG in coordination of cell movement and control of the cytoskeleton.

RAS proteins are a family of small GTPases that control a range of fundamental processes in all known eukaryotic cells. Ras was first discovered in an activated form as the product of a viral oncogene (Shih et al., 1979). It has since been found to be a central regulator of mammalian cell growth and division, differentiation, shape, and motility (for reviews see Sorcher et al., 1993; Valencia and Sander, 1995). Distinct roles for Ras have been found in several organisms. In particular, Ras proteins mediate signaling through receptors such as the Sevenless protein in *Drosophila* (Fortini et al., 1992), and a Ras homologue (the let-60 gene product) is required for the specification of the vulval cells of *Caenorhabditis elegans* (Han and Sternberg, 1990). The RAS1 and RAS2 genes of *Saccharomyces* control cell growth by regulating the activity of adenyl cyclase (Toda et al., 1985) and may also influence the cytoskeleton through the CAP protein (Wang et al., 1993). Relatives of Ras proteins in the Rac and Rho subfamilies act downstream of Ras; again, they appear to control both growth and motility (Ridley et al., 1992). Rac and Rho seem to control separate aspects of cell architecture, with Rac and its relative Cdc42 controlling actin-rich protrusions (ruffles and filopodia, respectively) and Rho regulating the formation of actomyosin bundles known as stress fibers (Ridley et al., 1992; Nobes and Hall, 1995).

Ras proteins are active when GTP is bound and become inactive by hydrolyzing this GTP to GDP. Two families of proteins regulate Ras activity by controlling the bound nucleotide. Guanine nucleotide exchange factors (GEFs)¹ such as CDC25 and Sos activate Ras by allowing GDP to dissociate and be replaced by GTP. GTPase-activating proteins (GAPs), on the other hand, inactivate Ras by binding to the active form and stimulating the hydrolysis of GTP to GDP (Boguski and McCormick, 1993). Both may be controlled by different stimuli. In mammalian cells, the binding of receptor tyrosine kinases to their ligands can cause recruitment of both GEFs and GAPs to the membrane through a family of adaptor proteins such as Grb2 and Shc (Lowenstein et al., 1992; Pelicci et al., 1992); the interaction between *Drosophila* Sevenless and Boss proteins is transmitted to Ras through the Grb2 homologue Drk (Olivier et al., 1993). In yeast, the signals that regulate Ras activity through CDC25 and IRA1&2 are not yet understood.

The work described in this paper suggests a connection between Ras proteins and cytokinesis. Correct cell division involves two distinct processes: nuclear division (karyokinesis) and then partitioning of the cytoplasm and organelles (cytokinesis; Rappaport, 1986). Karyokinesis is mainly accomplished by the microtubules that form the spindle, whereas cytokinesis is apparently based around actin and

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1. Abbreviations used in this paper: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factors.

myosin II (classical, double-headed myosin). Soon after karyokinesis, a concentration of polymerized actin (F-actin) is visible at the equator of the cell, and a cleavage furrow containing myosin II forms which pinches the daughter cell in two (Fishkind and Wang, 1995). Mutants in several species affected in myosin II function lose the ability to perform cytokinesis properly, despite apparently normal karyokinesis (De Lozanne and Spudich, 1987; Karess et al., 1991). The simple eukaryote *Dictyostelium discoideum* has recently proved to be an excellent subject for the study of cytokinesis, in particular because the cells possess an alternative method of partitioning cell contents when normal partition cannot take place (De Lozanne and Spudich, 1987). This process, which has been named "traction-mediated cytofission" (Fukui et al., 1990), allows the survival of mutants with strong cytokinesis phenotypes, which would be inviable in other systems. *Dictyostelium* also offers relatively simple gene disruption by homologous recombination, and is a much-studied target for analysis of cytoskeletal proteins.

The first cytokinesis mutants to be isolated in *Dictyostelium*, and consequently the most extensively studied mutants, are defective in cytoskeletal proteins, for example, myosin II heavy chain (DeLozanne and Spudich, 1987) and coronin (de Hostos et al., 1993). However, several recent reports have identified two GAP proteins of unknown substrate specificity (Faix and Dittrich, 1996; Lee et al., 1997) and the small GTP-binding protein RacE (Larochelle, 1996) as putative regulators of cytokinesis. Neither GAP proteins nor RacE are likely to be directly involved in the physical separation of the daughter cells; instead they must lie on the signaling pathways controlling the process.

Dictyostelium possesses an unusual, extended family of *ras* genes (Daniel et al., 1995). Two of the products (RasG and RasD; Reymond et al., 1984; Robbins et al., 1989) are closely related to mammalian Ras proteins (68% and 65% overall identity to human H-ras, respectively), whereas the RasB, RasC, and RasS gene products (Daniel et al., 1993, 1994) are more divergent (though still clearly members of the Ras subfamily). *Dictyostelium* cells grow unicellularly but aggregate and form a multicellular fruiting body upon starvation. The *rasD* and *rasS* genes are only expressed during multicellular development (Reymond et al., 1984; Daniel et al., 1994). *RasD*, furthermore, is expressed at higher levels in the stalk cell precursors than the prespore cells. The gene products are therefore presumed to have a function specifically connected with aggregation or cell-type differentiation. RasG, RasB, and RasC are expressed during growth (Robbins et al., 1989; Daniel et al., 1993). *RasG* mRNA expression ceases as soon as multicellular development begins, and RasG protein is lost from the cells during development, suggesting a specific requirement during growth (Khosla et al., 1990, 1996).

Aside from their different patterns of expression, little is known about the functions of *Dictyostelium* Ras proteins. Overexpression of an activated form of RasD causes aberrant signaling and developmental arrest in aggregates (Reymond et al., 1986). Similar activating mutants of RasG cause a block in aggregation (Khosla et al., 1996) and cytoskeletal changes (Rebstein et al., 1997). Neither of these lines has allowed elucidation of the function of the Ras proteins in normal *Dictyostelium* cells. In this work we report the

disruption of the gene that encodes RasG; disruptants show a range of phenotypes based around the control of the actin cytoskeleton, in particular during cytokinesis. This suggests that one major role of RasG is to control cell architecture, rather than growth or differentiation.

Materials and Methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and all enzymes from New England Biolabs (Beverly, MA).

Cell Strains, Growth, and Transformation

Dictyostelium discoideum AX2 cells were maintained at 22°C in HL-5 medium (Sussman, 1987). For bacterially grown cells, SM plates were inoculated with 10^5 – 10^6 *Dictyostelium* cells plus 200 μ l of a suspension of *Klebsiella aerogenes* in L-broth. Transformation was performed by a modification of Howard et al. (1988); briefly, cells in log phase growth were mixed with 25 μ g of linearized DNA and electroporated at 1.0 or 1.1 mV, 3 μ F with a 5 Ω resistance in series. After 10 min incubation on ice, cells were warmed for 15 min in the presence of 2 μ l healing solution (100 mM MgCl₂, 100 mM CaCl₂), and then HL-5 was added. Blasticidin (ICN, Irvine, CA) or G418 (GIBCO BRL, Gaithersburg, MD) at 10 μ g/ml final concentration were added 24 h after electroporation. Transformants appearing after 4–5 d were cloned on lawns of *Klebsiella* growing on SM agar plates.

Molecular Biology

The *rasG* knockout vector was constructed by inserting a version of the pBsr Δ Bam marker (Adachi et al., 1994) with BamHI linkers into the single BglIII site of pRASG (Robbins et al., 1992). Transformants in which the marker was oriented in the same direction as the *rasG* gene were selected to ensure the presence of a terminator between the actin promoter and the *rasG* coding sequence. From the resulting vector a 3.2-kb fragment was digested with EcoRI and HindIII restriction enzymes and used to transform *Dictyostelium* strain AX2 as described above. To analyze clones, AX2 or *rasG*⁻ genomic DNA prepared by the method of Sun et al. (1990) was digested with HindIII and EcoRI, blotted onto nylon membrane (Qiagen Inc., Chatsworth, CA), and probed with the [α -³²P]dATP labeled BglIII–HindIII fragment from the *rasG*-coding sequence (Robbins et al., 1989) as described in Sambrook et al. (1989).

Western Blotting

rasG⁻ + AX2 cell lysates were separated by SDS-PAGE and blotted onto 0.45 μ m PVDF membrane (Amersham Life Science, Pittsburgh, PA) by standard procedures (Sambrook et al., 1989). The membrane was probed with rat α -Ras mAb Y13-259 (Oncogene Science, Mineola, NY) at 10 μ g/ml final concentration, or with a polyclonal antibody to RasG as described in Khosla et al. (1994). Secondary HRP-conjugated anti-rat antibody or anti-rabbit antibody (Pierce, Rockford, IL) was used at 1:2,500 dilution. The signal was detected using an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL).

Microscopy

Cells from axenic culture were seeded onto acid-washed coverslips and fixed with 1% glutaraldehyde, 0.1% Triton X-100 in KK2 buffer (20 mM potassium phosphate, pH 6.2, 2 mM MgCl₂) for 10 min. Autofluorescence was quenched using 5 mg/ml NaBH₄ for 10 min. Actin filaments were stained with 100 nM Rhodamine- or Texas red-conjugated phalloidin (Sigma Chemical Co. and Molecular Probes, Inc., Eugene, OR, respectively) and nuclei with 500 nM Hoechst 33258 or 33342. Cells were observed using an epifluorescence microscope (Axiophot; Zeiss, Inc., Thornwood, NY) or a scanning confocal microscope (MRC1024; Bio Rad, Hercules, CA). Images were recorded with a Hamamatsu CCD camera or on Kodak Ektachrome 400 film, and digitized with an Agfa Arcus II scanner in conjunction with Adobe Photoshop 3.0 software.

Cells were synchronized to observe cytokinesis using aphidicolin (Pedrali-Noy et al., 1987). The medium of axenically growing cells was supplemented with 10 μ g/ml aphidicolin (final concentration) for 16 h. The aphidicolin was removed with three washes of fresh axenic medium and

the cells allowed to continue through the cell cycle. At the first signs of mitotic activity (usually about 5 h after release) cells were fixed as described above.

Time-lapse video microscopy of cells was used to observe cytokinesis. Cells growing in petri dishes were synchronized as above, and a time-lapse video recorder (Betacam; Sony, Inc., Tokyo, Japan) was used to record phase contrast images collected by a CCD camera (Sony, Inc.) from an inverted microscope (Zeiss, Inc.).

To observe traction-mediated cytofission, *rasG*⁻ cells were grown for 5 d in shaking flasks and transferred to tissue culture plates. Phase-contrast photomicrographs were taken using an inverted microscope (Zeiss, Inc.) and Kodak T-Max 100 film. Alternatively, electronic images were obtained using a Panasonic video camera and a frame grabber connected to a Macintosh computer running NIH Image 1.60 software.

For the effects of azide, axenically growing cells were seeded onto coverslips and perfused with KK2 buffer for 5 min using a perfusion chamber as described in Devreotes et al. (1987). The buffer was replaced with 0.01% sodium azide in KK2 and an inverted microscope (Zeiss, Inc.) with phase contrast optics used to observe adhering cells.

To measure speed and persistence, cells were followed in a perfusion chamber, and images of several cells were recorded every minute. Images were grabbed as above, and the position of the centroids of several cells calculated using NIH Image 1.60 software and expressed as an x,y coordinate. The coordinates were used to calculate the velocity and persistence of each cell as described below.

Firstly, the length of the path between successive centroids was taken as the instantaneous velocity of each cell. Secondly, the cosine of the angle of the path between centroids was used as a measure of the persistence of movement of a cell. A cell moving in a constant direction will produce a path with angle 0° and therefore a cosine of one, while a cell changing direction at 90° will produce a cosine value of zero.

At least eight cells were followed for at least 20 min, and the velocities and persistences were pooled and used to give mean values.

Chemotaxis Assay

Bacterially growing cells were washed with KK2 buffer to remove bacteria. Cells were seeded onto coverslips and submerged in KK2 buffer. A micropipette filled with 10 μM folic acid in KK2 was placed just above the coverslip and pressurized to expel the folic acid. The assay was performed with the same pipette for both *rasG*⁻ and wild-type cells.

Results

Disruption of the *Dictyostelium rasG* Gene

Cells containing a disrupted *rasG* gene were generated by homologous recombination by the strategy shown in Fig. 1 A. A construct was made containing 1.9 kb of *rasG* genomic DNA (Robbins et al., 1992), with a *bsr* marker (Sutoh, 1993) inserted between the promoter and coding sequence of the gene, in the same orientation so the strong *act8* terminator blocked any read-through from the *rasG* or *bsr* promoters. The construct was transfected into AX2 cells, and transformants were cloned after 7 d of blasticidin selection. 2 independent clones, out of 74 examined, were found to contain a disruption in *rasG* (Fig. 1 B), with the rest apparently containing nonhomologous integration of the vector. Both independent lines were found to behave similarly, so one (IR15) was used for all the work described here. When Western blots are probed with the broad-spectrum Ras antibody Y13-259 (Furth et al., 1982),

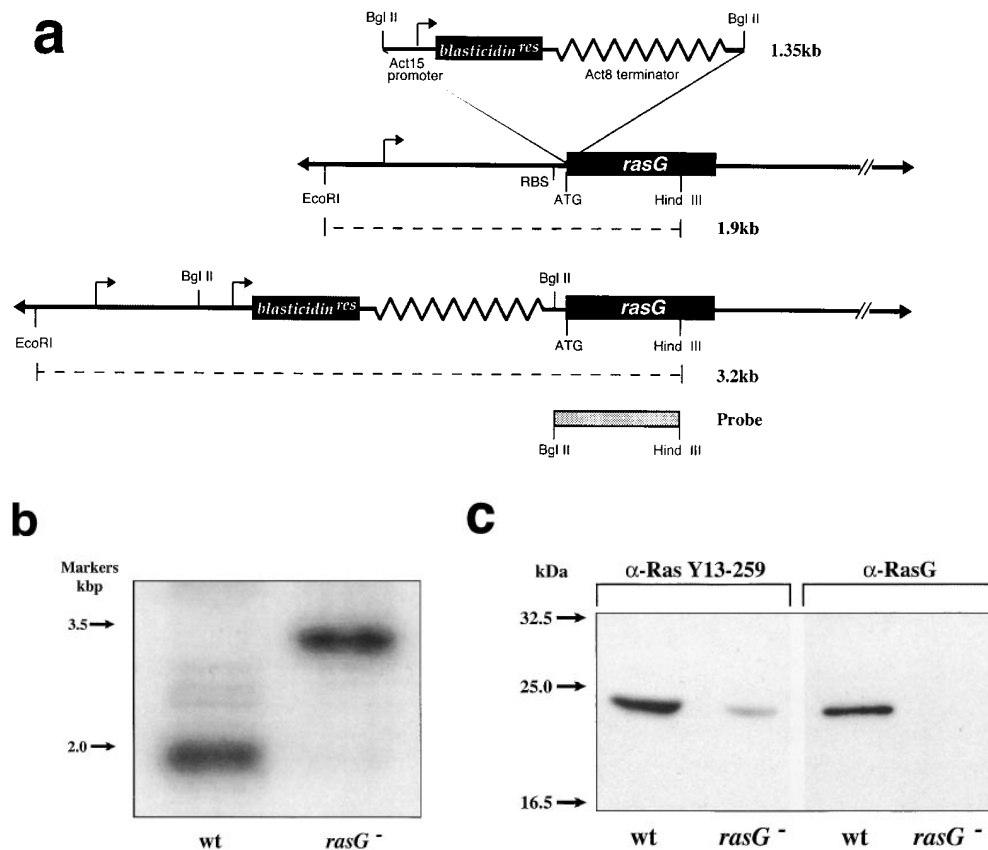


Figure 1. Disruption of the *rasG* gene. (a) Schematic representation of the cloning strategy employed to disrupt the *rasG* gene. A 1.7-kb fragment encoding the cDNA for the blasticidin resistance gene (*bsr*) driven by the constitutive *actin15* promoter was inserted by homologous recombination into the *rasG* promoter between the promoter and the ATG start codon. A probe from the *rasG* coding sequence (shaded bar) was used to detect correct disruptants by Southern blotting of genomic DNA. The expected bands in the parental strain and disruptants are indicated by dotted lines. (b) Southern blot of *rasG*⁻ and wild-type parental genomic DNA. Nuclear DNA from strains IR15 (*rasG*⁻) and AX2 (*wt*) was digested with *EcoRI* and *HindIII*, separated on an 0.8% agarose gel, blotted onto nylon, and probed with the *rasG* coding sequence (see above). The 1.9-kb parental band and 3.2-kb *rasG*⁻

disrupted band are marked. (c) Western blot of *rasG*⁻ and AX2 wild-type cells. Whole cell lysates were separated by PAGE using a 15% acrylamide gel, blotted onto PVDF, and probed with the general Ras antibody Y13-259 (left) and a RasG specific antibody (right). Y13-259 recognizes several different *Dictyostelium* Ras proteins with varying efficiency.

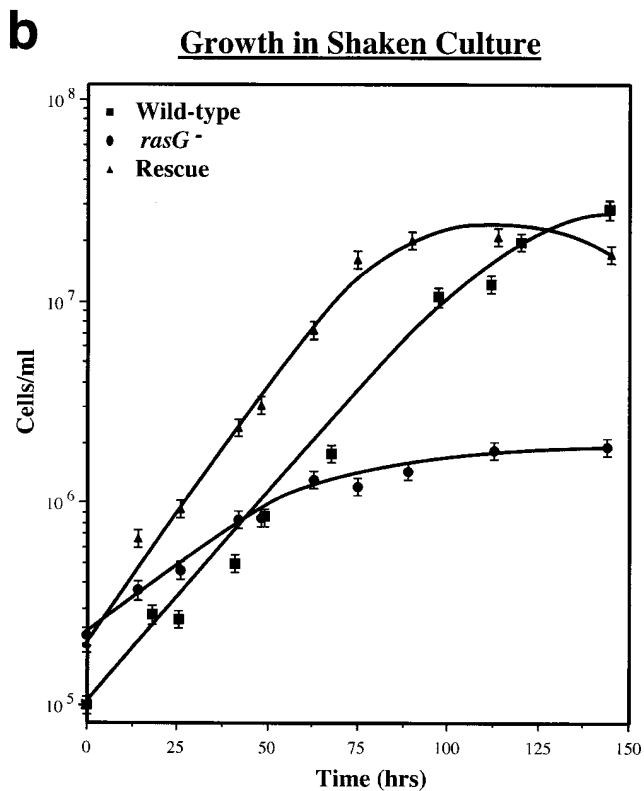
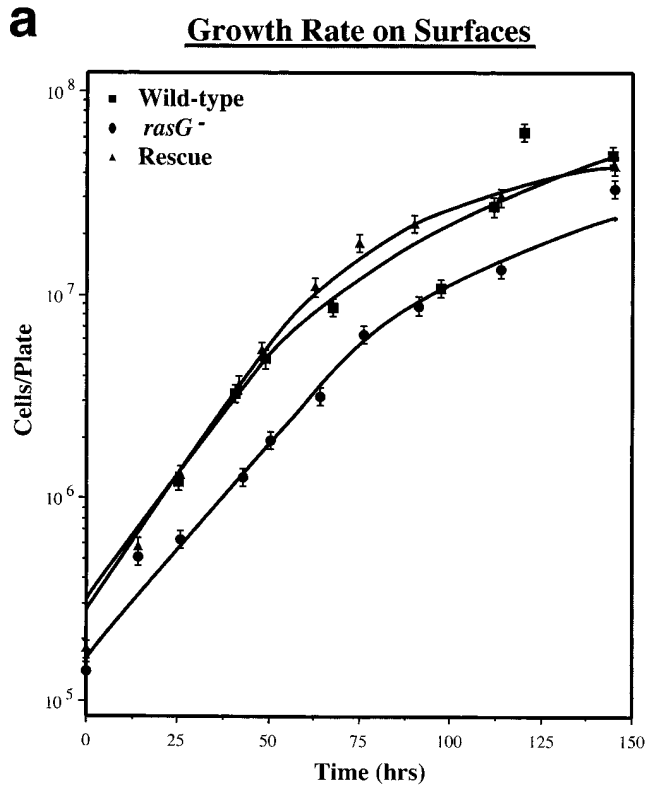


Figure 2. Growth of wild-type and *rasG*⁻ cells. (a) Growth on surfaces. Wild-type (■), *rasG*⁻ (●), and *rasG*⁻ cells rescued by expression of *rasG* (▲) were seeded in Petri plates (10⁵ cells/plate) with 10 ml axenic medium. At intervals the cells were detached from the surface, and the number of cells in a small aliquot was counted. (b) Growth in shaken culture. Wild-type (■), *rasG*⁻ (●), and *rasG*⁻ cells rescued by expression of *rasG* (▲) were transferred from Petri plates into axenic medium at time zero and counted at intervals thereafter.

disrupted cells show approximately half the wild-type level of Ras proteins (Fig. 1 C, left). Y13-259 detects the diverged RasB and RasC proteins, albeit less well than RasG or RasD. The Ras protein seen in IR15 therefore presumably derives from the *rasB* and *rasC* genes (which are expressed at lower levels than *rasG* in wild-type cells) or *rasD* (which is barely expressed in growing wild-type cells). No RasG protein is found in disruptants when a specific RasG antibody is used (Fig. 1 C, right).

Growth of *rasG*⁻ Cells

Unexpectedly, *rasG* appears to be dispensable for growth under most conditions. As shown in Fig. 2 A, *rasG*⁻ cells in liquid medium grow only slightly more slowly than wild-type (14.5 vs 13.4 h doubling time). *rasG*⁻ cells also grow somewhat more slowly than wild-type on bacteria, forming colonies that are a little smaller than AX2 (data not shown).

When cells are grown in shaken suspension, however, a different result is seen (Fig. 2 B). *rasG*⁻ cells double far more slowly than wild-type (27 vs 15 h doubling time), and cease growing at a considerably lower cell density (Fig. 2 B). This weak growth in suspension culture, despite nearly normal growth on surfaces, is reminiscent of the phenotype of *mhcA*⁻ cells (De Lozanne and Spudich, 1987), which do not produce myosin II, and therefore cannot perform cytokinesis. The role of *rasG* in cytokinesis was examined further, as described below.

Retransfection of *rasG*⁻ cells with a genomic copy of the *rasG* gene and promoter reverses both growth phenotypes, allowing a normal doubling time whether cells are grown on plates, in shaken suspension (Fig. 2, A and B), or on bacteria (data not shown). The mutant phenotypes are therefore solely due to a lack of RasG.

Aberrant Morphology and Adhesion in *rasG* Mutants

Observed by phase contrast microscopy, *rasG*⁻ cells differ from wild-type in several respects. Cells grown on bacteria (which behave more consistently than axenically grown cells) and then allowed to migrate on a glass coverslip (Fig. 3 A) are far more flattened than wild-type and adhere more tightly. They are also less polar; most wild-type cells possess a clear orientation, whereas *rasG*⁻ cells are nearly all circular (Fig. 3 A). When the motion of cells was recorded by video microscopy, *rasG*⁻ cells travelled considerably slower than wild-type (64% of the mean speed; Table I). Two aspects of the motion of *rasG*⁻ cells are surprising. The defect in polarization of *rasG*⁻ cells would suggest that they are unable to maintain a specific direction, yet they move with nearly the same persistence (a measure of the cell's ability to move in a constant direction) as wild-type (Table I). Secondly, wild-type cells alternate between polarized and rounded morphologies, with nearly all movement taking place in the polar phase. *rasG*⁻ cells seem to move nearly as fast when rounded as when polar, which explains how they are able to move at as much as 64% of wild-type speeds despite the majority being apparently unpolarized.

The distribution of F-actin is also aberrant in axenically grown *rasG*⁻ cells. Staining with fluorescent phalloidin (Fig. 3 B) reveals two major differences between *rasG*⁻

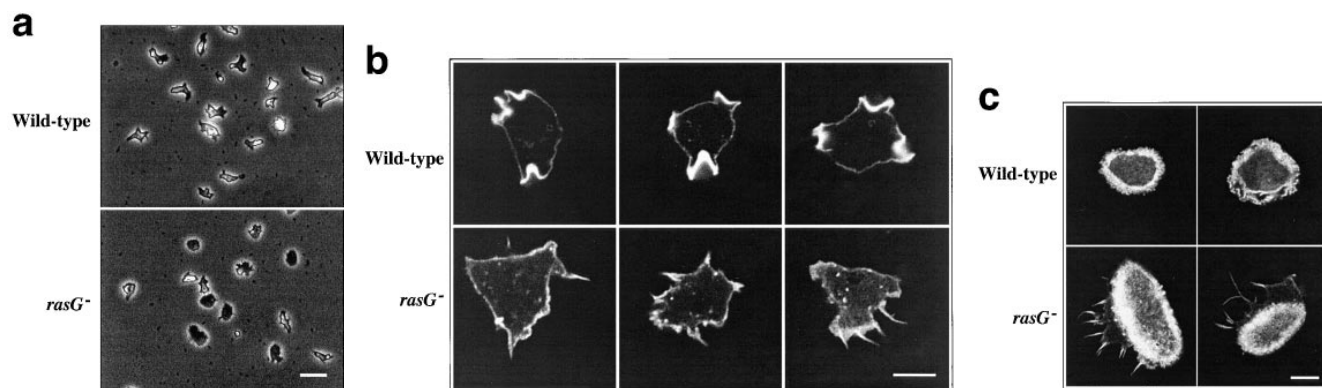


Figure 3. Morphology of wild-type and *rasG*⁻ cells. (a) Phase-contrast micrographs of cells adhering to glass. Wild-type cells (top) show a rounded and polarized morphology, while *rasG*⁻ cells (bottom) are more flattened and nonpolar. (b and c) Scanning confocal micrographs of cells stained with rhodamine-phalloidin to visualize F-actin. (b) Moving wild-type cells (top) and *rasG*⁻ cells (bottom). (c) Stationary wild-type (top) and *rasG*⁻ cells (bottom). Bar: (a) 20 μm; (b and c) 10 μm.

and wild-type. Firstly, the lamellipodia seen at the leading edges of polarized wild-type cells are replaced by large numbers of elongated filopodia. While similar filopodia are seen in a proportion of wild-type cells (~10–15%; data not shown), they are present in nearly all *rasG*⁻ cells, frequently in large numbers, often reaching considerably greater lengths than in wild-type cells (Fig. 3 B). Again, nearly all *rasG*⁻ cells show no obvious polarity; the filopodia appear to be spread randomly around the perimeter of most cells. Wild-type cells in the rounded phase frequently show a continuous, broad cortex of F-actin, with no actin-rich protrusions; equivalent *rasG*⁻ cells exhibit a similar cortex, but usually also possess long filopodia (Fig. 3 C). Various highly unusual morphologies are also common in *rasG*⁻ cells, in particular crescent and hour glass shapes (Fig. 4). In these cells the cortex, rather than being continuous, is divided into discrete F-actin-rich lobes, as if the different ends of the cell were trying to move in opposite directions. The nuclei are usually found between the lobes of F-actin, and the two halves of the cells pulling in opposite directions can squeeze the nuclei into cylindrical shapes.

A second unusual feature of *rasG*⁻ cells is the abundance of small, punctate F-actin structures within the cell body. These are conspicuously present in the majority of cells (Figs. 3, B and C, and 4; particularly clear actin structures are also visible in Fig. 6 A). Similar structures are seen in a small proportion of wild-type cells, but in lower numbers (1–2 per cell; data not shown). Three-dimen-

sional reconstruction of confocal Z-series indicates that the structures are roughly spherical and are localized to the cortex of the base and the upper surface of the cells (data not shown; a QuickTime video showing an example of a three-dimensional reconstruction is available on the World Wide Web at <http://www.ucl.ac.uk/~dmcbrob/movies.html>). The precise nature of these structures is as yet unknown. Mutant and wild-type cells contain similar quantities of F-actin per cell (data not shown); the differences appear to reflect a failure of organization, rather than an inability to polymerize actin.

Normal Chemotaxis in *rasG*⁻ Cells

Chemotaxis up chemical gradients involves both cell polarity and changes in the actin cytoskeleton. Since both the cell polarity and cytoskeleton of the *rasG*⁻ cells are aberrant (Fig. 3), and in the light of the observation that the disruption of the Aimless RasGEF demonstrates a requirement for a *ras* pathway for *Dictyostelium* chemotaxis (Insall et al., 1996), we measured chemotaxis of *rasG*⁻ cells towards folic acid, which is used by growing cells to detect bacteria. As documented in Fig. 5, *rasG*⁻ cells are almost equal to wild-type in their ability to migrate towards a micropipette filled with folic acid. Considering their impaired cytoskeletal structure and slow movement, they perform chemotaxis surprisingly successfully.

Cytokinesis and Cell Fission

The poor growth of *rasG*⁻ cells in shaken suspension suggests a defect in cytokinesis. When cells are transferred from growth on petri plates, where they maintain a wild-type number of nuclei (data not shown), to shaken culture, they rapidly become multinucleate (Fig. 6 A). Within 3 d of shaken growth, the majority of cells has several nuclei, and many have >10. Multinucleate cells the size of the one shown in Fig. 6 A are common, and considerably larger examples are sometimes observed. After 6 or 7 d of growth in shaken suspension, cells become so large that they lyse, presumably from the shear forces generated by shaking. When multinucleate cells are allowed to adhere to a surface such as a glass coverslip, they rapidly tear themselves

Table I. Speed and Persistence Measurements for Wild-type and *rasG*⁻ Cells

	Wild-type (AX2)	<i>rasG</i> ⁻ (IR15)
Mean speed (μm/min)	4.5 ± 2.6	2.9 ± 2.4
Persistence	0.46	0.44

Cells were examined in a perfusion chamber as described in Materials and Methods. Images were recorded every minute, and the distance moved by the centroids of several cells was used to calculate the mean cell speed. The persistence, which represents the constancy of direction of each cell, was calculated as the cosine of the angle between consecutive paths.

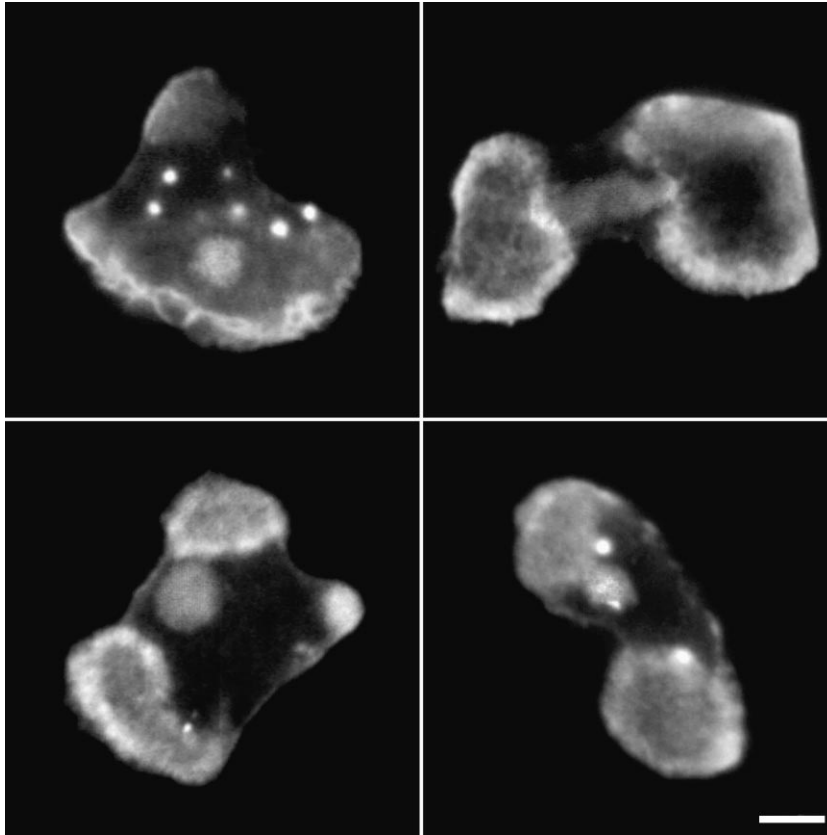


Figure 4. Epifluorescence micrographs of stationary *rasG*⁻ cells stained with Texas red-phalloidin to visualize F-actin and Hoechst 33342 to visualize the nuclei. The cells adopt abnormal morphologies and adhere to the glass through large pads of F-actin. Small, intensely stained masses of F-actin are also present in some cells. Bar, 5 μ m.

into mononucleate fragments. The single cell shown in the process of splitting in Fig. 6 *B* had divided into at least 11 daughters within 30 min of plating on a surface. This process, which has been named traction-mediated cytofission (Fukui et al., 1990), appears not to greatly harm the cells, and is the mechanism which allows *Dictyostelium* cytokinesis mutants to divide at nearly normal rates on a surface (a QuickTime video showing a *rasG*⁻ cell splitting by traction-mediated cytofission after growth in suspension culture can be viewed at the World Wide Web site <http://www.ucl.ac.uk/~dmcbrob/movies.html>).

Nearly all the cytokinesis mutants that have been previously described in *Dictyostelium* have defects in the structural proteins of the cytoskeleton. The most comprehensively studied mutants lack myosin II function (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Chen et al., 1995). These cells are unable to complete cytokinesis because of an inability to generate the contractile ring which pinches the daughter cells apart. The phenotype of *rasG* disruptants is not caused by a lack of myosin II activity. Treatment of cells with sodium azide causes a rapid depletion of cellular ATP levels, which causes myosin II to bind irreversibly to actin (Patterson and Spudich, 1995). When wild-type cells in a perfusion chamber are treated with 0.01% sodium azide, they round up and are washed off the substratum. *mhcA*⁻ cells, which lack the heavy chain of myosin II, remain flattened and stuck down. Azide treatment caused nearly normal rounding and loss of *rasG*⁻ cells (Fig. 7). The small number of mutant cells that did not wash away had rounded up in the same way as wild-type cells but were still adhering to the

glass coverslip. This seems to be caused by the aberrant adhesion of *rasG*⁻ cells rather than any lack of myosin II function.

We wished to discern whether *rasG*⁻ cells were unable to perform cytokinesis because of a mechanical defect preventing the physical separation of the daughter cells, like that in *mhcA*⁻ cells, or because of a problem with the initiation of cytokinesis due to the loss of a signal communicating the completion of nuclear division to the cytoplasm. To answer these questions, synchronized cells in mitosis were required, but we found existing methods of synchronizing *Dictyostelium* cells unsatisfactory. By releasing cells whose cell cycle progression was blocked by aphidicolin (an inhibitor of DNA polymerase; Pedrali-Noy et al., 1980), we observed good synchrony. 50% of cells passed through mitosis \sim 4 h after release. When synchronized wild-type cells are observed by time-lapse video microscopy, cytokinesis is observed as a rounding of the cell followed by a rapid pinching movement, taking \sim 5 min to complete (Fig. 8 *A*, top rows). The initial stages of cytokinesis in *rasG*⁻ cells appear indistinguishable from those in wild-type cells (Fig. 8 *A*, lower rows). However, the daughter cells appear unable to separate completely and remain attached by a thin bridge of cytoplasm, which is eventually resolved by traction between the daughter cells (Fig. 8 *A*).

Wild-type cells that are fixed during cytokinesis and stained for F-actin and chromosomes show a characteristic binucleate hour-glass shape with the chromosomes condensed throughout the process (Fig. 8 *B*, top rows). Again, with fixed cells the initial stages of cytokinesis in *rasG*⁻ cells appear identical to those in wild-type cells (Fig. 8 *B*,

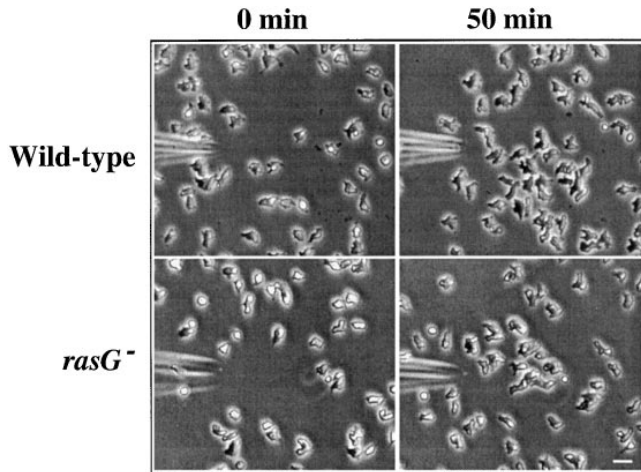


Figure 5. Chemotaxis in wild-type and *rasG*⁻ cells. Phase contrast micrographs of wild-type cells (top) and *rasG*⁻ cells (bottom) migrating towards a micropipette filled with 10 μ M folic acid. Cells are shown 0 (left) and 50 min (right) after presentation of the pipette. Bar, 20 μ m.

lower rows), but the bridge of cytoplasm that results from the failure of the *rasG*⁻ daughter cells to separate is shown clearly. The chromosomes of the two daughters decondense (Fig. 8 B, lower right image) indicating that the cells can remain attached for some time. In contrast, wild-type cells show condensed nuclei even as the daughters separate completely (Fig. 8 B, upper right image). Staining with an anti-tubulin antibody indicates that the microtubules also return to an interphase morphology before complete separation in the *rasG*⁻ cells but remain in a mitotic morphology throughout cytokinesis in wild-type cells (data not shown).

The failure of *rasG*⁻ cells late in cytokinesis suggests that they have a mechanical defect. RasG does not appear to be required for the spatial or temporal specification of the cleavage furrow. As demonstrated by Fig. 7, myosin II activity is present in *rasG*⁻ cells, but RasG may be required for the correct regulation of myosin II or other cytoskeletal proteins during cytokinesis. Alternatively, RasG may be required for the correct regulation of actin polymerization during cytokinesis with the result that an aberrant F-actin framework is established in the cleavage furrow that prevents the necessary contractile forces being generated by myosin II and other cytoskeletal proteins.

Discussion

The most obvious phenotype of *rasG*⁻ mutant cells is, unexpectedly, a defect in cytokinesis. Cells also exhibit several other cytoskeletal defects, including alterations in cell polarity, morphology, motility, and F-actin structures. The slightly reduced growth rate of mutants is most probably due to the defects in motility and cytokinesis, although we cannot rule out other minor difficulties. Clearly *rasG* is not essential for growth. These results could be interpreted in two ways. Firstly, RasG may be concerned solely with the regulation of motility, while differentiation and growth control are mediated through other Ras proteins, or not by

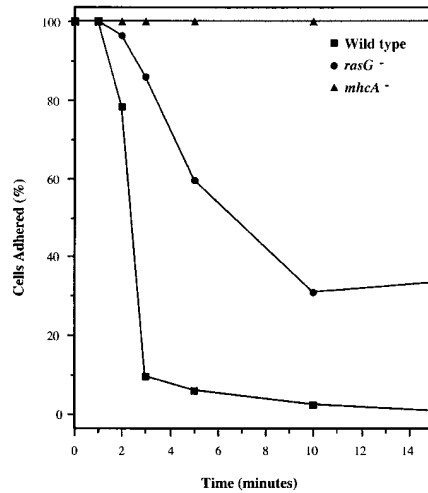


Figure 7. Normal myosin function in *rasG*⁻ cells. Cells adhering to glass coverslips were perfused with buffer containing 0.01% azide. Cells lacking myosin II (*mhca*⁻, \blacktriangle) remain flattened and adherent, while both wild-type cells (\blacksquare) and *rasG*⁻ cells (\bullet) round up and detach from the glass.

a Ras at all. Alternatively, RasG could be important for the control of a wide range of cellular processes, just like its mammalian homologues, but the phenotype of *rasG* disruption can be moderated by partial functional redundancy. If the latter is true, RasG might normally be central to regulation of growth but be substituted by RasB, RasC, or ectopically expressed RasD in *rasG*⁻ cells. RasD is highly homologous to RasG: 100% identical in the effector and effector-proximal domains and 82% identical over the entire length (Daniel et al., 1995), and therefore might be expected to substitute for some of the functions of RasG if expressed during growth. The low recovery of homologous recombinants compared to random integrants might suggest a central role for RasG, but it is notoriously hard to predict the efficiency of homologous integration. On the other hand, the phenotypes observed after overexpression of mutant forms of RasG are mainly concerned with cell morphology. Cells containing an activated RasG appear flattened, while those transformed with inhibitory mutants are polarized (Rebstein et al., 1997). Whichever explanation is true, cytokinesis and actin organization appear to be the only processes that cannot survive *rasG* disruption.

Ras and Actin

The role for Ras proteins in motility has been observed in a range of species, though until recently it has been overshadowed by the importance of growth control. Ras activation leads to enhanced motility of endothelial cells (Fox et al., 1994) and apparently mediates the chemotactic response of fibroblasts to PDGF (Kundra et al., 1995). When the genes encoding RasGAP and/or NF1 are disrupted in mice, one of the causes of embryonic lethality is malformation of blood vessels due to hypermotile endothelial cells (Henkemeyer et al., 1995). Likewise, *Saccharomyces CDC42*, which controls actin polymerization during bud formation, is regulated through Ras (Mosch et al., 1996).

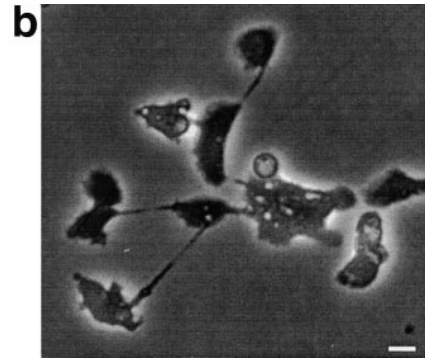
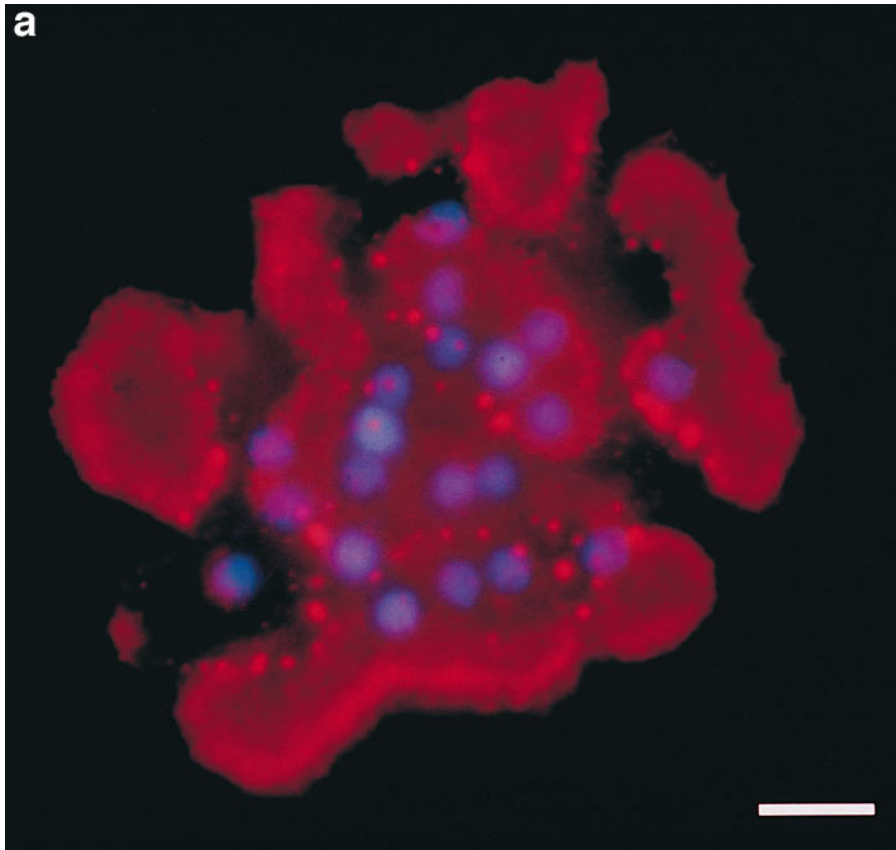


Figure 6. Defective cytokinesis in *rasG*⁻ cells. (a) Epifluorescent micrograph of a multinucleate *rasG*⁻ cell that has been growing in shaken culture for 5 d. F-actin is visualized with rhodamine-conjugated phalloidin, nuclei with Hoechst 33258. (b) Phase contrast micrograph of a multinucleate *rasG*⁻ cell undergoing traction-mediated cytofission, 10 min after plating. Bars, 20 μ m.

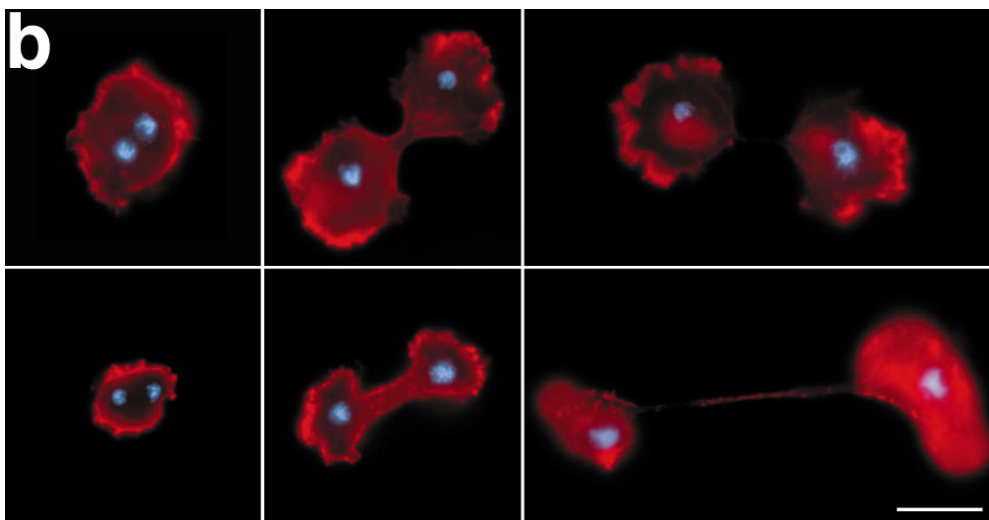
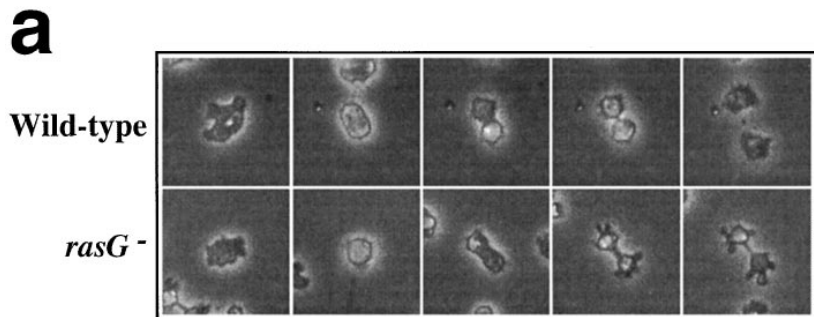


Figure 8. Cytokinesis in wild-type and *rasG*⁻ cells. (a) Video frames showing cytokinesis in wild-type and *rasG*⁻ cells. Cells were observed by time-lapse videomicroscopy after synchronization by aphidicolin. Representative cytokineses from each strain are shown. (b) Synchronized wild-type and *rasG*⁻ cells were stained with Texas red-conjugated phalloidin and Hoechst 33342 to visualize F-actin and nuclei, respectively. *RasG*⁻ cells show a characteristic failure to separate after formation of the cleavage furrow. Bar, 10 μ m.

In *Dictyostelium rasG⁻* cells, all of the phenotypes we observe are connected with the control of the actin cytoskeleton. The lamellipodia at the leading edge of wild-type cells, and the filopodia found in *rasG⁻* cells, are both transitory structures made of freshly polymerized actin (Hall et al., 1988), and the cleavage furrow is also actomyosin rich (Fukui and Inoue, 1991). The absolute quantity of F-actin in *rasG⁻* cells is normal, though the part played by the unusual punctate actin structures is unknown.

A range of experiments has shown that small GTPases such as Rac and Cdc42 proteins are controlled by Ras pathways (Nobes and Hall, 1995). *Dictyostelium* contains a large number of *rac* homologues (Bush et al., 1993), so the different effects of RasG on the cytoskeleton and motility could be transmitted through several Rac proteins with separate roles. The specific role of the *racE* gene in cytokinesis (Larochelle et al., 1996), described below, is a possible example. Another known effector of Ras proteins is p110 PI 3-kinase (Rodriguez-Viciano et al., 1994), which may constitute the direct connection between Ras and Rac proteins. Three different p110 homologues have recently been cloned in *Dictyostelium* (Zhou et al., 1995), and it is significant that mutants also show defects in the control of the cytoskeleton. We look forward to observing the effects of *rasG* disruption on PI 3-kinase and Rac activity.

Ras and Cytokinesis

Ras proteins in other organisms have been associated with both control of the cytoskeleton and cell proliferation but not usually with cytokinesis. However, since in other systems Ras is essential for cell growth, and therefore nuclear division, its role in the division of the cytoplasm thereafter has been difficult to study. Despite this, there are several reports connecting Ras activity with cytokinesis. *Schizosaccharomyces byr4*, a suppressor of *ras1* mutations (Song et al., 1996), appears to encode a regulated inhibitor of cytokinesis. Furthermore, activating Ras mutants increase the rate of cytokinesis in mammalian cells (Ng et al., 1992). There is also an increasing amount of evidence that the small GTPases Rac, Rho, and Cdc42 are centrally involved in cytokinesis (Dutartre et al., 1996), and Ras appears to act as an upstream regulator of their activities. It is therefore likely that the central role of RasG in *Dictyostelium* cytokinesis is mirrored in other organisms.

There are several likely candidates for proteins downstream of RasG. Interestingly, the disruption of one GAP gene (Lee et al., 1997) and overexpression of the other (Faix and Dittich, 1996) both result in deficiencies in cytokinesis, as does overexpression of activating RasG mutants (Rebstein et al., 1997). This may reflect a need for limited, local activation of RasG during cytokinesis. RasG activation at an inappropriate place or time could be just as harmful to efficient cytokinesis as the complete lack of RasG in disrupted cells. The protein encoded by the *racE* gene is required for cytokinesis (Larochelle et al., 1996); unlike *rasG⁻* cells, however, *racE⁻* mutants appear normal in all other respects, including the formation of lamellipodia, adhesion, and rate of motility. Again, since Rac proteins in other systems can be activated by Ras, a direct connection between RasG and RacE seems possible. Similarly, cells containing concurrent deletions in two PI 3-kinase

genes (*pik1* and *pik2*) lose the ability to grow in shaken culture (Zhou et al., 1995), despite nearly normal growth on surfaces, which suggests that they have similar defects in cytokinesis to *rasG⁻* cells. Alternatively, the defect in cytokinesis in *rasG⁻* cells may be indirect, caused by the general disruption of the actin cytoskeleton rather than the loss of a specific signaling pathway.

Like the *ras* genes themselves, the biochemical pathways between Ras and the cytoskeleton are apparently conserved between *Dictyostelium* and mammalian cells. This again suggests that cytokinesis in mammalian cells may turn out to be affected by Ras as it is in *Dictyostelium discoideum*. The fascinating prospect is that Ras proteins through their involvement in the signaling pathways that commit cells to division and by regulating the cytoskeleton during cytokinesis are major players at the start and at the end of the cell cycle.

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