Journal of Cell Science 113, 1427-1434 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS1128

Functional overlap of the *Dictyostelium* RasG, RasD and RasB proteins

Meenal Khosla[1], George B. Spiegelman[1,2], Rob Insall[3] and Gerald Weeks[1,2,*]

[1]Department of Microbiology and Immunology and [2]Department of Medical Genetics, University of British Columbia, Vancouver, BC V6T 1Z3 Canada
[3]Department of Biochemistry University of Birmingham, Birmingham, UK
*Author for correspondence

Accepted 14 February; published on WWW 21 March 2000

SUMMARY

Disruption of the *rasG* gene in *Dictyostelium discoideum* results in several distinct phenotypes: a defect in cytokinesis, reduced motility and reduced growth. Reintroduction of the *rasG* gene restores all of the properties of the *rasG*$^-$ cells to those of the wild type. To determine whether the defects are due to impaired interactions with a single or multiple downstream effectors, we tested the ability of the highly related but non identical *Dictyostelium ras* genes, *rasD* and *rasB*, to rescue the defects.

Introduction of the *rasD* gene under the control of the *rasG* promoter into *rasG* null (*rasG*$^-$) cells corrected all phenotypes except the motility defect, suggesting that motility is regulated by a RasG mediated pathway that is different to those regulating growth or cytokinesis. Western blot analysis of RasD protein levels revealed that vegetative *rasG*$^-$ cells contained considerably more protein than the parental AX-3 cells, suggesting that RasD protein levels are negatively regulated in vegetative cells by RasG. The level of RasD was enhanced when the *rasD* gene was introduced under the control of the *rasG* promoter, and this increase in protein is presumably responsible for the reversal of the growth and cytokinesis defects of the *rasG*$^-$ cells. Thus, RasD protein levels are controlled by the level of RasG, but not by the level of RasD.

Introduction of the *rasB* gene under the control of the *rasG* promoter into *rasG*$^-$ cells produced a complex phenotype. The transformants were extremely small and mononucleate and exhibited enhanced motility. However, the growth of these cells was considerably slower than the growth of the *rasG*$^-$ cells, suggesting the possibility that high levels of RasB inhibit an essential process. This was confirmed by expressing *rasB* in wild-type cells; the resulting transformants exhibited severely impaired growth. When RasB protein levels were determined by western blot analysis, it was found that levels were higher in the *rasG*$^-$ cells than they were in the wild-type parental, suggesting that RasG also negatively regulates *rasB* expression in vegetative cells. Overexpression of *rasB* in the *rasG*$^-$ cells also reduced the level of RasD protein.

In view of the fact that alternate Ras proteins correct some, but not all, of the defects exhibited by the *rasG*$^-$ cells, we propose that RasG interacts with more than one downstream effector. In addition, it is clear that the levels of the various Ras proteins are tightly regulated in vegetative cells and that overexpression can be deleterious.

Key words: *Dictyostelium*, Ras protein, Cytoskeleton, Cytokinesis, Growth, Motility

INTRODUCTION

Ras proteins belong to a large super-family of monomeric G proteins that have regulatory switch functions. This group of proteins is active when bound to GTP and inactive when bound to GDP and their regulatory switch function involves the interconversion of the GTP and GDP bound forms. They have been implicated in the regulation of growth and cytoskeletal function in a variety of mammalian cells lines and in the development of *Drosophila* and *Caenorhabditis elegans* (McCormick, 1994).

Dictyostelium has an unusually large sub-family of *ras* genes and each of the genes has a distinctly different pattern of expression during differentiation, suggesting their products may have distinct developmental functions (Reymond et al., 1984; Robbins et al., 1989, 1990; Daniel et al., 1993a,b). Two of the *ras* gene products, RasG and RasD, are closely related to each other and to the mammalian Ha-Ras protein (Reymond et al., 1984; Robbins et al., 1989). Although the remaining proteins, RasB, RasC, RasS and Rap, are less closely related, they are all clearly members of the Ras sub-family (Robbins et al., 1990; Daniel et al., 1993a,b). Of these, RasB most closely resembles RasG and RasD (Daniel et al., 1993a).

Although all the genes are expressed to a greater or lesser extent during growth, *rasG* is most clearly classified as a growth specific gene. There are high levels of *rasG* mRNA at all stages of vegetative growth and these drop dramatically by the aggregation stage of development, in response to pulses of the chemoattractant, cyclic AMP (Khosla et al., 1990). *rasG* null cells containing a disrupted *rasG* gene (*rasG*$^-$ cells) have a number of defects (Tuxworth et al., 1997). In shaken suspension in rich nutrient medium, they grow more slowly and

to a much lower final density than the parental axenic cells. Under these conditions, large multinucleate cells are formed, suggesting a defect in cytokinesis. These multinucleate cells rapidly divide by traction mediated cytokinesis when plated onto a solid surface. When the mutants are maintained on a plastic surface they are the same size and have the same nuclear content as the parental strain. In addition, *rasG*$^-$ cells exhibit reduced polarity and motility and these changes are accompanied by an unusual distribution of F-actin (Tuxworth et al., 1997). It is not clear if these defects in vegetative cells are due to single or multiple functions of RasG.

In an attempt to investigate further the complex growth phenotype of the *rasG*$^-$ cells, we have tested the ability of *rasD* and *rasB* to rescue the *rasG*$^-$ defects. Since RasD and RasG share over 85% overall identity, and have common effector and effector flanking domains (Robbins et al., 1989), it was anticipated that *rasD* would rescue most, if not all, of the defects exhibited by the *rasG*$^-$ cells. In contrast, there are a number of differences in the sequences flanking the effector domain between RasB and RasG (Daniel, 1993a) and it was therefore considered to be less likely that overexpression of *rasB* would revert the *rasG* defects. The effects of expressing *rasD* and *rasB* in *rasG*$^-$ cells are described in this report.

MATERIALS AND METHODS

Growth and transformation conditions

Cells were grown in HL5 medium (Watts and Ashworth, 1970) either on Nunc (BRL) tissue culture plates at 22°C or in rotatory agitated (150 rpm) suspensions at 22°C. Cells were also grown clonally on lawns of *Klebsiella oxytoca* at 22°C.

Transformations were performed essentially as described previously (Tuxworth et al., 1997). About 20 μg of DNA was used to transform 2×10^7 *rasG*$^-$ cells by electroporating at 1.1 mV, 3 μF with a 5 Ω resistance in series. After a 10 minute incubation on ice, cells were plated in Nunc tissue culture dishes and allowed to recover overnight in HL5 medium. After 24 hours, G418 (Gibco) was added at 10 μg/ml and the transformants that appeared as plaques after 8-10 days of selection were isolated.

Plasmid construction

The genomic copy of *rasG* (Robbins et al., 1992) was cloned into the Bluescript plasmid, which contained the G418 resistance cassette. This same plasmid was used previously to introduce the *rasG* gene into the *rasG* disrupted strain IR15 (Tuxworth et al., 1997). To produce *rasB* and *rasD* expressing vectors, the *rasG* coding sequence was removed by digestion with *Bgl*II and *Xho*I. This DNA was blunt ended with Klenow and then ligated to blunt ended cDNAs of *rasD* and *rasB*.

Chemotaxis and motility

For determining chemotaxis to cyclic AMP, cells were shaken at 150 rpm for 4 hours in PDF/MES at 5×10^6 cells per ml and then plated (10^6 cells in 1 μl) on 2% agar plates containing PDF/MES and 10 μM cAMP (Khosla et al., 1996). The average distance migrated by the halo of cells that escape from the cell mass was determined after 24 hours (Browning et al., 1995). Random motility was determined by plating 2.5×10^4 cells in 1 μl on 2% agar plates containing PDF/MES. After 24 hours, the average distance migrated by the 24 most rapidly moving cells was determined (Khosla et al., 1996).

Production of RasD and RasB antiserum

GST-RasD protein was prepared as previously described (Khosla et al., 1994), except that it was purified by binding to Sepharose 4B beads (Sigma) and then eluted with 15 mM reduced glutathione. Antibody against RasD was raised by the methodology described previously (Khosla et al., 1994), except that 100 μg of purified GST-RasD protein mixed 1:1 with Titremax (Sigma) was used for the primary intramuscular injection and identical booster injections were given after 14, 28 and 56 days. Serum was collected after 70 days and the RasD antibodies were bound to GST-RasD Affigel and then eluted with 3.5 M $MgCl_2$. Antibodies that recognized common epitopes to the other Ras subfamily proteins and the GST portion of the fusion protein were removed as described previously (Khosla et al., 1994).

Antibody against a 22 amino acid peptide corresponding to the carboxy-terminal (amino acids; 173-194) of RasB protein was generated as described previously (Robbins, 1991). RasB specific antibodies were bound to RasB peptide-Affigel 10 beads, eluted with 0.1 M glycine, pH 2.8, and immediately neutralized with 1 M Tris-Cl, pH 8.0. The eluted antibody was dialysed at 4°C against PBS and concentrated using a Centricon concentrator (Micon).

Western blot analysis

Between 1×10^7 and 4×10^7 cells were washed twice in Bonner's salts, resuspended in cold 75 mM β-glycerol phosphate-20 mM morpholinepropanesulfonic acid (MOPS; pH 7.2)-15 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)-2 mM EDTA-1 mM sodium vanadate-1 mM dithiothreitol-20 μg of antipain per ml 20 μg of leupeptin per ml-20 μg of aprotinin (Sigma Chemical Co.) per ml, and lysed by freezing and thawing. Protein concentrations were determined as previously described (Bradford, 1976). An aliquot containing 20 μg of protein from each transformant was mixed with an equal volume of 2× sample buffer (0.5% β-mercaptoethanol, 0.5% SDS, 50 mM Tris-Cl, pH 6.8, 12.5% glycerol, 0.04% Bromophenol Blue), boiled for 5 minutes, and then subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

After electrophoresis, the proteins were transferred to nitrocellulose membranes for 1 hour at 90 V (Towbin et al., 1979) and probed with purified polyclonal anti-RasG or anti-RasD antibodies. The membranes were incubated in 5% powdered milk (Carnation) in Tris-buffered saline (TBS)-Tween (25 mM Tris-Cl, pH 8.0, 1.0% NaCl, 0.5% Tween-20) overnight at 4°C and were then washed twice for 5 minutes with TBS. The primary antibody was diluted 1/5,000 in TBS-Tween containing 0.5% powdered milk and incubated with the nitrocellulose membranes at room temperature for 2 hours. The membranes were then washed four times (for 5 minutes each) in TBS-Tween, and the amount of bound primary antibody was detected using secondary antibody, diluted 1/10,000 in TBS-Tween containing 0.5% powdered milk, and the ECL detection system (Amersham). The membranes were exposed to X-ray film for 10 to 30 seconds, the time depending on the amount of antibody binding obtained.

Nuclear staining

To determine nuclear number, cells in HL5 were grown in shake suspension for five days. 3×10^3 cells/cm^2 were allowed to settle onto glass coverslips for 30 minutes and then washed 3 times with KK2. The cells were fixed in 3.7% formaldehyde for 10 minutes and washed a further 3 times in KK2. Cells were then permeabilised with −20°C acetone, dryed, rehydrated with KK2 and then stained with Hoechst 33258 dye. After further washing in KK2, coverslips were mounted on glass slides using 50% glycerol and viewed using a Zeiss Axiophot microscope with epifluorescence.

Measurement of fluid phase endocytosis and phagocytosis

IR17 (*rasG*$^-$) and *rasG*$^-$/rasB cells were grown on Nunc (BRL) tissue culture plates while AX3 and pVEII-*rasB* cells were grown in shake suspension. Growth was for two days in HL5 in the absence of folate to induce *rasB* expression. Cells were harvested and resuspended at $3\text{-}4\times10^6$ cells/ml in fresh HL5 containing 2 mg/ml FITC dextran (M_r

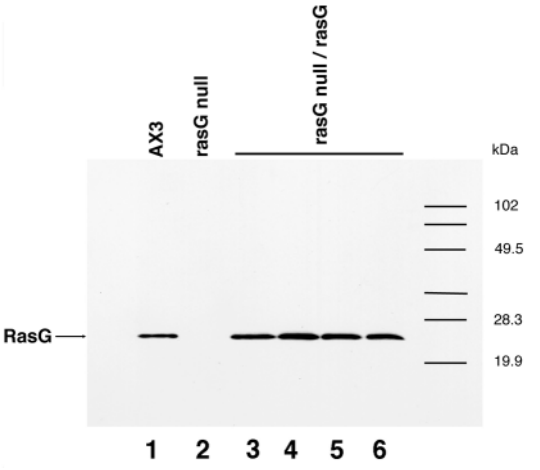

Fig. 1. Elevated level of RasG protein in *rasG*$^{-}$*/rasG* cells. Protein extracts (20 μg) from AX-3 (lane 1), IR17 *rasG*$^{-}$ cells (lane 2), and four independent *rasG*$^{-}$*/rasG* isolates (lanes 3, 4, 5 and 6) were electrophoresed on polyacrylamide gels, blotted onto nitrocellulose filters and probed with RasG antibody as described in Materials and Methods. The molecular masses of a range of standard proteins (102 kDa, 78 kDa, 49.5 kDa, 34.2 kDa, 28.3 kDa and 19.9 kDa) are indicated by the lines.

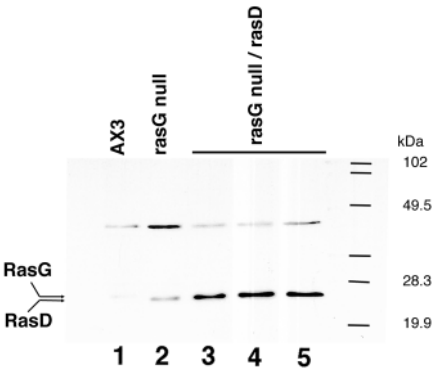

Fig. 2. Elevated level of RasD protein in *rasG*$^{-}$*/rasD* cells. Protein extracts (20 μg) from AX-3 (lane 1), IR17 *rasG*$^{-}$ (lane 2), and three independently isolated *rasG*$^{-}$*/rasD* cell lines (lanes 3, 4 and 5) were electrophoresed, blotted and probed with RasD antibody as described in Materials and Methods. The protein size markers are the same as in Fig. 1.

70,000; Sigma) to measure fluid phase endocytosis as described by Vogel et al. (1980). At the indicated time intervals, 1 ml samples were removed and further fluid phase endocytosis was stopped by diluting cells in 9 ml of ice-cold 20 mm phosphate buffer, pH 6.2. Cells were centrifuged, washed two times with cold phosphate buffer, resuspended in 2 ml of 50 mM Na_2HPO_4 solution and then lysed by addition of Triton X-100 (0.2% final concentration). The retained fluorescence was measured using a Perkin Elmer spectrofluorometer (excitation, 470 nm and emission, 520 nm). Fluorescence measurements were normalized relative to total protein levels to account for differences in cell size between various strains (Seastone et al., 1999). All values were corrected for suface adhesion of FITC dextran by subtracting the value determined at time zero. Rates of phagocytosis were measured using latex beads as described by Temevari et al. (1996).

RESULTS

Expression of *rasG*, *rasD* and *rasB* in *rasG*$^{-}$ cells

Dictyostelium rasG null (*rasG*$^{-}$) cells exhibit several distinct defects: impaired cytokinesis, altered cell polarity, reduced motility, and slow growth (Tuxworth et al., 1997). Transformation of the *rasG*$^{-}$ cells with a genomic clone of *rasG* reversed all these phenotypic changes (Tuxworth et al., 1997). To determine if *rasD* and *rasB*, the *Dictyostelium ras* genes most related to *rasG*, could also reverse the *rasG*$^{-}$ phenotypes, these genes were transformed into *rasG*$^{-}$ cells and expressed under the control of the *rasG* promoter. This was accomplished by removing the *rasG* coding sequence from the genomic *rasG* clone and replacing it with either *rasD* cDNA or *rasB* cDNA sequences. The resulting plasmids were transformed into *rasG*$^{-}$ cells and transformants were selected in HL5 containing 10 μg/ml of G418. The previously described *rasG*$^{-}$ strain (IR15) that had been generated in an AX-2 background (Tuxworth et al., 1997) was not used for this study, because it was found that some of the phenotypic defects were lost during repeated passage of the cells, suggesting phenotypic suppression. We therefore used a second, more stable *rasG*$^{-}$ mutant (IR17), generated in an AX-3 background.

RasG protein levels in the *rasG*$^{-}$ cells, the *rasG*$^{-}$*/rasG* transformants and the parental AX-3 cells were determined by western blot analysis using a RasG specific antibody (Fig. 1). These results showed, as expected, that this *rasG*$^{-}$ strain contained no *rasG* protein (Fig. 1, lane 2) and that the *rasG*$^{-}$ cells transformed with *rasG* (lanes 3-6) expressed levels of RasG protein only slightly higher than those in the AX-3 cells (lane 1).

The level of RasD protein in *rasG*$^{-}$*/rasD* transformants was determined by western blot analysis using an antibody specific to RasD. Since it had been shown previously that *rasD* is expressed at only very low levels in vegetative cells (Robbins et al. 1990), it was not surprising that no *rasD* protein was detected in vegetative AX-3 cells (Fig. 2, lane 1). In contrast, RasD protein in the *rasG*$^{-}$ cells was elevated sufficiently to be clearly detectable (Fig. 2, lane 2). Introduction of the *rasD* transgene into the *rasG*$^{-}$ mutant resulted in a further elevation in the level of RasD (Fig. 2, lanes 3-5). The barely visible band of slightly lower mobility than RasD in the AX-3 extracts (lane 1) was RasG, detectable because the polyclonal RasD antibody had low activity against RasG (data not shown). However, since RasG and RasD were sufficiently resolved by SDS-PAGE, the levels of RasD in the various strains could be unambiguously compared, even in the presence of RasG. The increased level of RasD protein in the vegetative *rasG*$^{-}$ cells may account for the survival of these cells since RasD may be partially substituting for the function of RasG. The identity of the component of approximately 45 kDa that cross reacts with the rasD Ab is not known.

To determine if the reintroduction of *rasG* reduced RasD protein to wild-type levels, the western blot of the *rasG*$^{-}$*/rasG* extracts (Fig. 1) was stripped and reprobed with the RasD antibody (Fig. 3). The blot was overexposed relative to the blot shown in Fig. 2 to emphasize the fact that there is no RasD in vegetative AX-3 cells and the RasG protein in the AX-3 extract (lane 1), detected as a result of its cross reactivity with the RasD antibody, is now more clearly seen. No RasD protein was

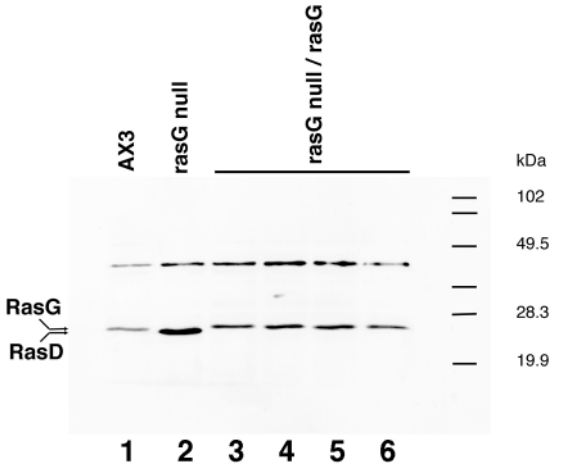

Fig. 3. Reduced RasD protein level in *rasG⁻/rasG* cells. The western blot shown in Fig. 1 was stripped and reprobed with RasD antibody.

detected in the *rasG⁻/rasG* extracts (Fig. 3, lanes 3-6), indicating that RasD had reverted to AX-3 levels (Fig. 3, lane 1). These results suggest the level of RasD in vegetative cells is regulated by the product of the *rasG* gene.

RasB protein levels were also determined by western blot analysis (Fig. 4). The RasB level in the *rasG⁻*cells (Fig. 4, lane 2) was clearly higher than that found in the parental AX-3 cells (Fig. 4, lane 1) and a further increase in RasB level was evident in the *rasG⁻* cells transformed with *rasB* (Fig. 4, lane 3). In order to determine if the overexpression of *rasB* also reduced levels of RasD the amount of RasD in the *rasG⁻/rasB* transformants was determined (Fig. 5). The levels of RasD in these transformants (Fig. 5, lanes 3-6) were significantly lower than those in the *rasG⁻* cells (lane 2), although higher than the levels in AX-3 (lane 1).

Cytokinesis and cell shape

After growth in shake suspension, several nuclei were observed

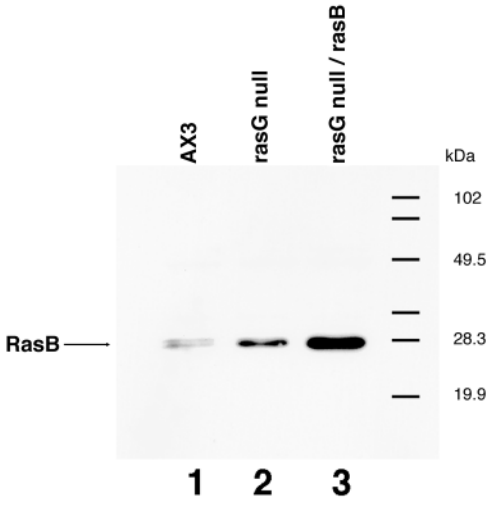

Fig. 4. Elevated RasB protein level in *rasG⁻/rasB* cells. Protein extracts (20 μg) from AX-3 (lane 1), IR17 *rasG⁻* cells (lane 2) and a *rasG⁻/rasB* isolate (lane 3) were electrophoresed on polyacrylamide gels, blotted onto a nitrocellulose filter and probed with RasB antibody as described in Materials and Methods.

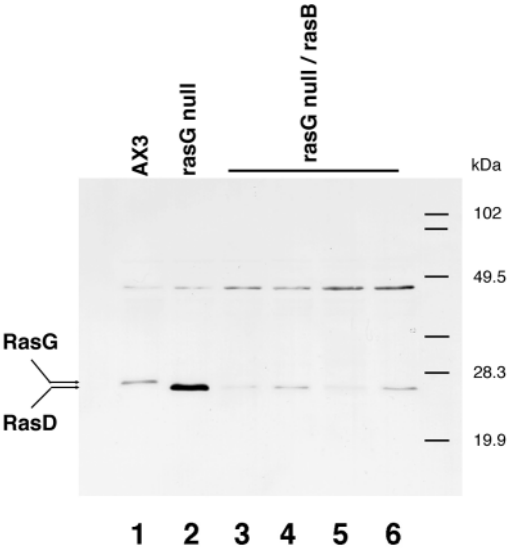

Fig. 5. Reduced RasD proteins in *rasG⁻/rasB* cells. Protein extracts (20 μg) from AX-3 (lane 1); IR17 *rasG⁻* cells (lane 2) and four independent isolates of *rasG⁻/rasB* cells (lanes 3, 4, 5 and 6) were electrophoresed, blotted and probed with RasD antibody.

in each IR15 cell, indicative of a defect in cytokinesis (Tuxworth et al., 1997). The *rasG⁻* mutant IR17 was also multinucleate when grown under the same conditions (Fig. 6, plate 2). To determine if this defect could be rescued, the *rasG⁻/rasG*, *rasG⁻/rasB* and *rasG⁻/rasD* transformants were grown in shake suspension for five days and then subjected to nuclear straining. All three *ras* genes were able to rescue the cytokinesis defect (Fig. 6, plates 3-5). Although a proportion of the *rasG⁻/rasG* and *rasG⁻/rasD* cells were binucleate (Fig. 6, plates 3 and 4), similar numbers of binucleate cells were present in the wild-type AX-3 population (plate 1, Fig. 6). In contrast, the *rasG⁻/rasB* cells were almost all mononucleate and were extremely small (Fig. 6, plate 5). The average nuclear content for a large number of cells is shown in Table 1. These numbers indicate no difference between AX-3, *rasG⁻/rasG* and *rasG⁻/rasD*, but a significant decrease in nuclear content in *rasG⁻/rasB* cells.

Motility and chemotaxis

It had been previously reported that IR15 cells exhibited slightly reduced motility (Tuxworth et al., 1997). To determine if this phenotypic defect was also a characteristic of the new *rasG⁻* mutant, IR17, and to determine if the phenotype could

Table 1. Number of nuclei of cells grown in shake suspension

Strain	Number of nuclei per cell*
AX3	1.7
rasG⁻	3.9
rasG⁻/rasG	1.7
rasG⁻/rasD	1.7
rasG⁻/rasB	1.1

*An average value calculated by counting the number of nuclei in 200-350 cells.

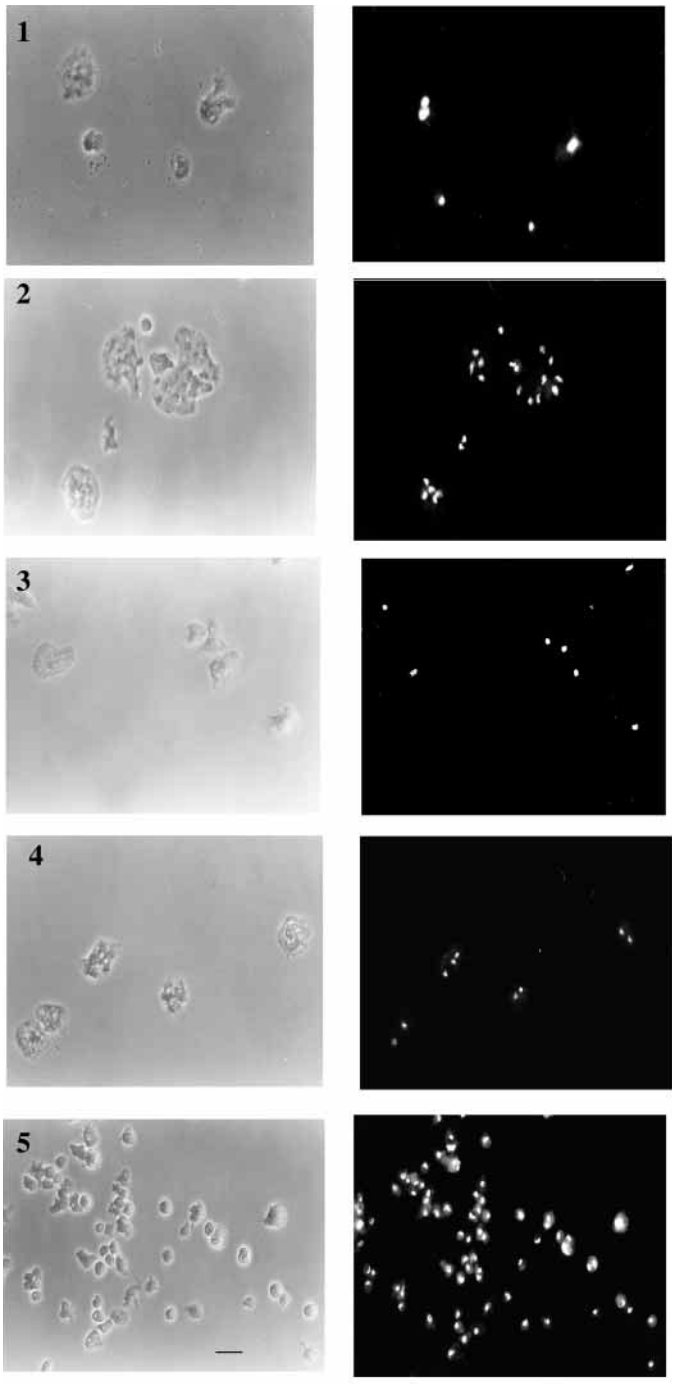

Fig. 6. Cell size and nuclear number. AX-3 cells (1), IR17 *rasG*$^-$ cells (2), *rasG*$^-$*/rasG* cells (3), *rasG*$^-$*/rasD* cells (4) and *rasG*$^-$*/rasB* cells (5). The plates on the left are phase contrast micrographs while the plates on the right are epifluorescence micrographs. All plates are at the same magnification and the bar in plate 5 equals 10 μm. Cells were grown in shake suspension for five days and then stained with Hoechst 33342 as described in Materials and Methods. In the interests of clarity, fewer cells are shown in plates 1-4 than in plate 5. The number of cells shown are plate 1, 4 cells; plate 2, 6 cells; plate 3, 7 cells; plate 4, 6 cells. The cells shown are representative of all the cells in the population.

be rescued by the introduction of other *ras* genes, random motility and chemotaxis to cAMP were quantified as described previously (Khosla et al., 1996). The data in Table 2 show that

Table 2. Random motility and chemotaxis to cyclic AMP

Strain	Distance migrated (mm)* Random motility‡	Chemotaxis to cAMP§
AX3	5.9±0.2	6.7±0
rasG$^-$	4.3±0.4	5.0±0.2
rasG$^-$*/rasG*	5.6±0.1	6.6±0.7
rasG$^-$*/rasD*	4.3±0.3	5.6±0.5
rasG$^-$*/rasB*	7.0±0.5	8.1±0.1

*Mean of 2 independent experiments ± the standard error of the mean.
‡The average distance migrated by the 24 most motile cells from the point of deposition of the population.
§Average distance migrated by the cells that escaped from the deposited population.

IR17 exhibited slightly lowered random motility than AX-3, confirming the observations made by time lapse photography for IR15 and AX-2 (Tuxworth et al., 1997) and that the motility of the *rasG*$^-$*/rasG* transformant was almost equal to that of wild-type cells (Table 2). However, the increased expression of *rasD* in the *rasG*$^-$*/rasD* cells did not enhance motility above that observed for the *rasG*$^-$ strain. Surprisingly, *rasG*$^-$*/rasB* cells moved faster than AX-3 cells (Table 2).

It was previously shown, using a qualitative micropipette assay, that *rasG*$^-$cells could chemotax towards folate. We determined the ability of cells to perform chemotaxis towards cAMP, by measuring the movement of cells away from their point of deposition on agar plates containing cAMP, as described previously (Browning et al., 1995; Khosla et al., 1996). The *rasG*$^-$ cells were chemotactic, but exhibited reduced chemotaxis to cAMP as assessed by this assay (Table 2). Similar results were obtained for chemotaxis to folate (data not shown). Chemotaxis to cAMP was restored to wild-type levels as a result of *rasG* expression in the *rasG*$^-$ cells. In contrast, expression of *rasD* did not significantly increase the rate of cAMP chemotaxis over that for the *rasG*$^-$ cells. The *rasG*$^-$*/rasB* cells exhibited higher chemotaxis towards cAMP than the parental AX-3 cells, a result consistent with their increased random motility.

Growth of the *rasG*$^-$ transformed cells

It was reported previously that the *rasG*$^-$ strain, IR15, grew slowly in HL5 medium in shaken suspension and ceased growing at a much lower final cell density than is customary for AX-2 cells (Tuxworth, 1997). We, therefore, compared the growth characteristics of the *rasG*$^-$*/rasG*, *rasG*$^-$*/rasD* and the *rasG*$^-$*/rasB* transformants with IR17 and the parental AX-3. IR17 grew more slowly than AX-3 and ceased growing at a much lower final cell density, and these properties were reversed in the *rasG*$^-$*/rasG* cells (Fig. 7). This experiment also revealed that increased expression of RasD restored the growth characteristics to those of the AX-3 parental strain (Fig. 7). However, the *rasG*$^-$*/rasB* cells grew even more slowly than the *rasG*$^-$ cells and ceased growing at a lower final cell density. The growth defect of the *rasG*$^-$ cells in HL5 medium was reported to be far less dramatic when cells were grown in HL5 medium in Petri plates (Tuxworth et al., 1997) and this property was also true for the IR17 strain. In contrast, the *rasG*$^-$*/rasB* transformants also grew extremely slowly under these conditions (data not shown).

The IR15 cells formed smaller plaques than AX-2 when grown on bacteria (Tuxworth, 1997), a property that could be

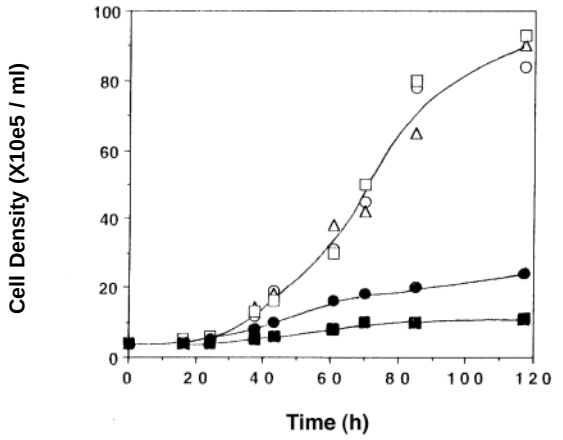

Fig. 7. Growth in suspension culture. Cell numbers of AX-3 (○), IR17 *rasG*⁻ (●), *rasG*⁻*/rasG* (△), *rasG*⁻*/rasD* (□) and *rasG*⁻*/rasB* (■) strains were determined at the indicated times and the plotted values are the means of duplicate hemocytometer counts. The data plotted are for a single experiment, but similar data were obtained for three independant experiments.

due to either reduced growth rate or reduced motility, or both. The IR17 cells also exhibited a smaller plaque size than AX-3 when grown on bacteria (data not shown). The plaque size for the *rasG*⁻*/rasG* cells was similar to that for AX-3, indicating the expected reversal of the mutant phenotype. The *rasG*⁻*/rasD* cells had a plaque size part way between those of *rasG*⁻ and AX-3, which could be due to the fact that although growth was fully restored in these cells, motility was not. *rasG*⁻*/rasB* plaques were larger than the AX-3 plaques despite the slow growth of the cells in HL5 medium, and this property may reflect the increased motility of these cells.

The growth characteristics of the *rasG*⁻*/rasB* cells suggested the possibility that these cells might exhibit impaired fluid phase endocytosis, but normal phagocytosis. We therefore compared the rates of pinocytosis and phagocytosis for IR17, *rasG*⁻*/rasB* and AX-3. The data clearly demonstrated that the rate of fluid phase endocytosis in the *rasG*⁻*/rasB* cells was significantly lower than the rate for IR17 and AX3 (Fig. 8; Table 3). In contrast, the rate of phagocytosis in the *rasG*⁻*/rasB*

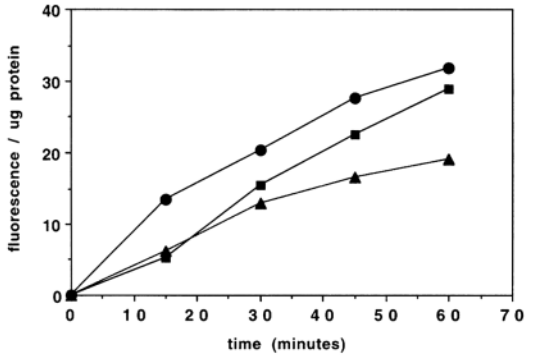

Fig. 8. Determination of fluid phase endocytosis. The uptake of FITC-dextran by AX-3 (■), IR17 *rasG*⁻ (●) and *rasG*⁻*/rasB* (▲) was determined at the indicated time points as described in the Materials and Methods. The data are from a single representative experiment.

Table 3. Comparison of rates of fluid phase endocytosis

Strain	Rate of fluid phase endocytosis* (uptake/μg protein/minute)
AX3	0.60±0.09
IR17, *rasG*⁻	0.64±0.04
rasG⁻*/rasB*	0.44±0.07
pVEII-*rasB*	0.30±0.07

*The mean ± standard deviation of four determinations.

cells was very similar to the levels determined for IR17 and AX-3 (data not shown).

Expression of *rasB* in a wild-type cell background

The possibility that the slow growth of *rasG*⁻*/rasB* cells was a deleterious effect of RasB overexpression on fluid phase endocytosis, was investigated further by isolating transformants that expressed *rasB* under the control of the discoidin promoter in an AX-3 background. These pVEII-*rasB* transformants also grew considerably more slowly in HL5 medium than the parental AX-3 cells (data not shown) and exhibited a lower rate of fluid phase endocytosis (Table 3). These results are consistent with the idea that *rasB* overexpression is deleterious to fluid phase endocytosis and that this defect results in slow growth in HL5 medium. These cells produced plaques that were of similar size to those of AX-3 when grown on bacteria (data not shown) and exhibited normal rates of phagocytosis, confirming that *rasB* overexpression is not deleterious to phagocytosis or growth on bacteria.

DISCUSSION

The problem of understanding cellular roles of Ras protein in multicellular organisms is complicated by the fact that there are multiple Ras family proteins and complex intersecting pathways (McCormick, 1994). We have begun to study this network in *Dictyostelium*, a simple eucaryotic organism in which multiple Ras proteins have been identified (Daniel et al., 1993b). In this study, we expressed the *rasG*, *rasD* and *rasB* genes under control of the *rasG* promoter in *rasG*⁻ cells in order to obtain information on the functional overlap between the three *Dictyostelium* Ras proteins that are most highly related to the mammalian Ras subfamily proteins. The phenotypes of the isolated transformants are summarized in Table 4.

A number of important conclusions can be drawn from the protein expression results. Firstly, we had concluded previously that the *rasG* promoter was tightly regulated, since the introduction of high copy numbers of *rasG* under the control of its own promoter did not raise the cellular level of RasG protein (Thiery et al., 1992). The *rasG*⁻*/rasG* transformant generated in the present study contained a level of RasG protein that was only slightly higher than the level in the parental AX-3 strain, a result consistent with the idea that the *rasG* promoter is regulated by the amount of RasG protein in the cell.

Secondly, whereas RasD protein was not detectable in extracts of the parental AX-3 cells, it was clearly detectable in extracts of *rasG*⁻ cells, under the conditions of the western blot

Table 4. Summary of the transformant phenotypes

	AX-3	*rasG*$^-$	*rasG*$^-$*/rasG*	*rasG*$^-$*/rasD*	*rasG*$^-$*/rasB*
Growth	Normal	Slow	Normal	Normal	Very slow
Motility/chemotaxis	Normal	Reduced	Normal	Reduced	Increased
Nuclear number	Normal	High	Normal	Normal	Normal

assay used in these studies. Similar results were observed for the IR15 *rasG*$^-$ transformant and its AX-2 parent (data not shown). The finding that RasD protein was not detectable in the axenic parental strains is consistent with earlier measurements of *rasD* mRNA which indicated that *rasD* is expressed at very low levels in vegetative cells (Reymond et al., 1984; Robbins et al., 1989). Since RasD protein levels were greatly enhanced in *rasG*$^-$ cells but again not detectable in *rasG*$^-$*/rasG* cells, the level of RasD protein appears to be regulated, at least indirectly, by RasG. When *rasD* was expressed from the *rasG* promoter in the *rasG*$^-$*/rasD* cells, the RasD level was considerably enhanced relative to that in the *rasG*$^-$ cells. The very low levels of RasD in growing wild-type cells, suggests that RasD is normally unimportant during growth. This is consistent with the recent finding that *rasD*$^-$cells exhibit normal growth and morphology (A. Wilkins et al., unpublished).

Thirdly, RasB protein levels were higher in the *rasG*$^-$ cells than in AX-2, also suggesting the possibility that RasG regulates the level of RasB in wild-type cells. When *rasB* was expressed in a *rasG*$^-$ background, RasD protein levels were also reduced, although to levels that were still detectable. Thus, RasB also regulated the level of RasD but not as efficiently as did RasG. The regulation of RasD and RasB levels by RasG and the regulation of RasD levels by RasG and by RasB, suggests amounts of all Ras proteins in vegetative cells are tightly controlled. This tight regulation is probably important since we have shown previously that RasG overexpression has a marked inhibitory effect on growth (Khosla et al., 1996) and in this study we have shown that RasB overexpression is also inhibitory.

The *rasG*$^-$*/rasG* transformant had properties that were very similar if not identical to those of the parental AX-3 cells. However, despite the high level of identity between RasD and RasG, *rasD* did not completely rescue the phenotypic defects in the *rasG*$^-$ cells. The *rasG*$^-$*/rasD* cells grew in shake suspension with the characteristics of the wild-type parental strain and the overexpression of RasD rescued both the defect in cytokinesis and the delay in the onset of development seen in *rasG*$^-$ cells. Clearly RasD was able to substitute for RasG for these functions, but only when the level of protein was elevated above the level found in the *rasG*$^-$ cells. However, expression of *rasD* in the *rasG*$^-$ cells did not restore motility to normal levels. These results suggest that the signalling pathway for normal motility involves a function of RasG that is separate from the function required for cytokinesis, growth and the regulation of the onset of development. This is consistent with our previous data suggesting that RasG performs more than one role in *Dictyostelium* (Zhang et al., 1999).

Recently a Ras interacting protein, R1P3 was identified in *Dictyostelium* (Lee et al., 1999). Since this protein interacts strongly with activated RasG but only weakly with activated RasD and RasB, it is possible that RasD fails to correct the *rasG*$^-$ motility defect because it is unable to interact with R1P3. However, although R1P3$^-$ cells have a defect in chemotaxis, the defect is far more pronounced than the defect reported for the *rasG*$^-$ strains. Activated RasG also interacts better than RasD with the PI3 kinase PI3K1 (Lee et al., 1999) suggesting another potential molecular interaction that might explain the *rasG*$^-$*/rasD* phenotype. However, cells with a disrupted PI3K1 have a wild-type phenotype whereas cells with deletions in both PI3K1 and the highly related PI3K2 have more serious defects than the *rasG*$^-$*/rasD* cells (Zhou et al., 1995, 1998; Buczynski et al., 1997). Therefore, although two proteins have been identified that interact more efficiently with RasG than RasD, there is no evidence to suggest that a defect in the interaction with either of these proteins is responsible for the *rasG*$^-$*/rasD* phenotype.

Overexpression of *rasB* did not restore the growth of *rasG*$^-$ cells to wild-type values. In fact, the expression of *rasB* in *rasG*$^-$ cells dramatically inhibited both the rate of growth and the extent of growth when cells were grown in HL5 medium. Suspension grown *rasG*$^-$*/rasB* cells were extremely small and predominantly mononucleate, while in contrast, many of the suspension grown AX-3 cells were binucleate. It is possible that although RasB is not capable of restoring cell growth, it is capable of restoring normal cytokinesis. RasG and RasB share considerable identity, including their effector domains (Daniel et al., 1993a), and cytokinesis could be regulated by a downstream effector that interacts with one of these common domains. However, it is also possible that the rate of growth of the *rasG*$^-$*/rasB* cells in suspension is sufficiently slow to allow the defective cytokinesis to maintain the population as mononucleate cells. In this scenario, RasB would be incapable of correcting either the growth or the cytokinesis defect of the *rasG*$^-$ cells. Because the RasB overexpression so dramatically inhibits growth, it is not possible at this time to distinguish between these possibilities.

rasB overexpression also markedly inhibited cell growth in a wild-type background. This result raises the possibility that the slow growth rate of *rasG*$^-$ cells is due to the increased levels of RasB protein in these cells. Since the impact of *rasB* expression on growth in both *rasG*$^-$ and AX-3 backgrounds was far more pronounced when cells were grown in axenic medium than when cells were grown on bacteria, we considered the possibility that RasB overexpression might interfere with fluid phase endocytosis. In fact, fluid phase endocytosis was significantly lower in *rasG*$^-$*/rasB* and pVEII *rasB* cells than in either of the parental cells, *rasG*$^-$ or AX-3, whereas phagocytosis levels were normal. However, the finding that fluid phase endocytosis is normal in *rasG*$^-$ cells is not consistent with the idea that the slow growth of *rasG*$^-$ cells is due to the increased level of RasB in these cells, although it does not eliminate this possibility. RasS is essential for fluid phase endocytosis (J. R. Chubb et al., unpublished observations), but it is unlikely that RasB has a dominant negative effect on RasS, since phagocytosis is reduced in *rasS*$^-$ cells, but unaffected in cells overexpressing *rasB*. Interestingly, however, both *rasS*$^-$ cells and *rasB* overexpressing cells exhibit increased motility. We are currently attempting to isolate *rasB*$^-$ cells and the phenotype of these cells may help to address these questions.

What is the nature of the growth defect in the *rasG*$^-$ cells? In contrast to the wealth of knowledge on growth regulation in

yeast, we know little of how growth is regulated in a unicellular amoeboid organism such as the vegetative *Dictyostelium* cell. However, it is reasonable to suppose that growth rate and extent of growth are controlled both by sensing the external nutrient environment and by sensing the internal metabolic status. If RasG is a positive effector for one or both of the signalling pathways involved in this sensing, the absence of RasG might lead to an impaired response to the presence of nutrients and impaired nutrient uptake. However, fluid phase endocytosis appears to be normal in *rasG*$^-$ cells, a result not consistent with this explanation.

In conclusion, although we cannot rule out the possibility that the multiple phenotypes of the *rasG*$^-$ cells are due to a single defect in the regulation of cytoskeletal function, the results presented here are more consistent with the idea that RasG is involved in regulating several independent signal transduction pathways. Our results also indicate complex interactions between pathways involving the various Ras proteins, suggesting complex signalling networks.

This research was supported by a grant from the Medical Research Council of Canada.

REFERENCES

Bradford, M. M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Browning, D. D., The, T. and O'Day, D. H. (1995). Comparative analysis of chemotaxis in *Dictyostelium* using a radial bioassay method. *Cell. Signalling* **7**, 481-489.

Buczynski, G., Grove, B., Nomura, A., Kleve, M., Bush, J., Firtel, R. A. and Cardelli, J. (1997). Inactivation of two *Dictyostelium discoideum* genes, *DdPIK1* and *DdPIK2*, encoding proteins related to mammalian phosphatidylinositide 3-kinases, results in defects in endocytosis, lysosome to postlysosome transport, and actin cytoskeleton organization. *J. Cell Biol.* **136**, 1286-1271.

Daniel, J., Spiegelman, G. B. and Weeks, G. (1993a). Characterization of a third *ras* gene, *ras*B, that is expressed throughout the growth and development of *Dictyostelium discoideum*. *Oncogene* **8**, 1041-1047.

Daniel, J., Bush, J., Spiegelman, G. B. and Weeks, G. (1993b). Isolation of two novel *ras* genes in *Dictyostelium discoideum*: evidence for a complex developmentally regulated *ras*-gene family. *Oncogene* **9**, 501-508.

Khosla, M., Robbins, S. M., Spiegelman, G. B. and Weeks, G. (1990). Regulation of DdrasG gene expression during *Dictyostelium* development. *Mol. Cell. Biol.* **10**, 918-922.

Khosla, M., Spiegelman, G. B., Weeks, G., Sands, T. W., Virdy, K. J. and Cotter, D. A. (1994). RasG protein accumulation occurs just prior to amoebae emergence during spore germination in *Dictyostelium discoideum*. *FEMS Microbiol. Lett.* **117**, 293-298.

Khosla, M., Spiegelman, G. B. and Weeks, G. (1996). Overexpression of an activated *rasG* gene during growth blocks the initiation of *Dictyostelium* development. *Mol. Cell. Biol.* **16**, 4156-4162.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 592-596.

Lee, S., Parent, C. A., Insall, R. and Firtel, R. A. (1999). A novel Ras-interacting protein required for chemotaxis and cyclic adenosine monophosphate relay in *Dictyostelium*. *Mol. Biol. Cell* **10**, 2829-2845.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY.

McCormick (1994). Activators and effectors of *ras* p21 proteins. *Curr. Opin. Genet. Dev.* **4**, 71-76.

Reymond, C. D., Gomer, R. H., Mehdy, M. C. and Firtel, R. A. (1984). Developmental regulation of a *Dictyostelium* gene encoding a protein homologous to mammalian *ras* protein. *Cell* **39**, 141-148.

Robbins, S. M., Williams, J. G., Jermyn, K. A., Spiegelman, G. B. and Weeks, G. (1989). Growing and developing *Dictyostelium* cells express different *ras* genes. *Proc. Nat. Acad. Sci. USA* **86**, 938-942.

Robbins, S. M., Suttorp, V. V., Weeks, G. and Spiegelman, G. B. (1990). A *ras*-related gene from the lower eukaryote *Dictyostelium* that is highly conserved relative to the human rap genes. *Nucl. Acids Res.* **18**, 5265-5269.

Robbins, S. M. (1991). Characterization of a *ras* and *ras*-related gene and their developmental expression in the cellular slime mould *Dictyostelium discoideum*. PhD thesis. University of British Columbia, Vancouver, Canada.

Robbins, S. M., Williams, J. G., Spiegelman, G. B. and Weeks, G. (1992). Cloning and characterization of the *Dictyostelium discoideum rasG* genomic sequences. *Biochim. Biophys. Acta* **1130**, 85-89.

Seastone, D. J., Zhang, L., Buczynski, G., Rebstein, P., Weeks, G., Spiegelman, G. B. and Cardelli, J. (1999). The small M_r Ras-like GTPase Rap1 and the phospholipase C pathway act to regulate phagocytosis in *Dictyostelium discoideum*. *Mol. Biol. Cell* **10**, 393-406.

Temesvari, L. A., Rodriguez-Paris, J. M., Bush, J. M., Zhang, L. and Cardelli J. A. (1996). Involvement of the vacuolar proton translocating ATPase in multiple steps of the endo-lysosomal system and in the contractile vacuole system of *Dictyostelium discoideum*. *J. Cell Sci.* **109**, 1479-1495.

Thiery, R., Robbins, S., Khosla, M., Spiegelman, G. B. and Weeks, G. (1992). The effects of expression of an activated *rasG* mutation of the differentiation of *Dictyostelium*. *Biochem. Cell Biol.* **70**, 1193-1199.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Nat. Acad. Sci. USA* **79**, 4350-5354.

Tuxworth, R. I., Cheetham, J. L., Machesky, L. M., Spiegelman, G. B. and Weeks, G. and Insall, R. H. (1997). *Dictyostelium* RasG is required for normal motility and cytokinesis, but not growth. *J. Cell Biol.* **138**, 605-614.

Vogel, G., Thilo, L., Schwarz, H. and Steinhart, R. (1980). Mechanism of phagocytosis in *Dictyostelium discoideum*: phagocytosis is mediated by different recognition sites as disclosed by mutants with altered phagocytotic properties. *J. Cell Biol.* **86**, 456-464.

Watts and Ashworth (1970). Growth of myxamoebae of the cellular slime mold *Dictyostelium discoideum* in axenic culture. *Biochem. J.* **119**, 171-174.

Zhang, T., Rebstein, P. J., Khosla, M., Cardelli, J., Buczynski, G., Bush, J., Spiegelman, G. B. and Weeks, G. (1999). A mutation that separates the RasG signals that regulate development and cytoskeletal function in *Dictyostelium*. *Exp. Cell Res.* **247**, 356-366.

Zhou, K., Takegawa, K., Emr, S. D. and Firtel, R. A. (1995). A phosphatidylinositol (PI) kinase gene family in *Dictyostelium discoideum*: biological roles of putative mammalian p110 and yeast Vps34p pI 3-kinase homologs during growth and development. *Mol. Cell. Biol.* **15**, 5645-5656.

Zhou, K., Pandol., S., Bokoch, G. and Traynor-Kaplan, A. E. (1998). Disruption of *Dictyostelium* PI3K genes reduces [^{32}P]-phosphatidylinositol 3, 4 biophosphate and [^{32}P]-phosphatidylinositol triphosphate levels, alters F-actin distribution and impairs pinocytosis. *J. Cell Sci.* **111**, 283-294.