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Mediation of Cell-Substratum Adhesion by RasG in *Dictyostelium*

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Abstract Previous studies on the functions of the RasG gene in the cellular slime mold, *Dictyostelium discoideum,* have revealed that it is required for normal motility and cytokinesis. To further understand how the RasG gene regulates various cellular processes, we transformed an activated form of RasG, that is, RasG (G12T), a mutation from glycine to threonine at amino acid position 12 into wild type KAX-3 cells. This produced moderate but constitutive RasG(G12T) protein expression, which causes cells to become significantly more adherent to the substratum than are wild type cells. The RasG(G12T) transformants also grow slowly on bacterial plates, and engulf fewer bacteria on filter surfaces, indicating a defect in phagocytosis when cells are adhered. The expression of the activated RasG also dramatically reduces the number of filopodia on the cell surface. Tyrosine phosphorylation on a 43 kDa protein (most likely actin) of the RasG (G12T) transformants is highly elevated. Taken together, our observations suggest that RasG is crucial for *Dictyostelium* cell-substratum adhesion during growth and that RasG may play a role in adhesion-mediated phagocytosis. Our results also suggest that RasG is important in filopodial formation and that RasG is involved in the signal pathway that is regulated by tyrosine phosphorylation. J. Cell. Biochem. 79:139–149, 2000.

Key words: Ras proteins; *Dictyostelium*; cell-adhesion; phagocytosis; filopodia; tyrosine phosphorylation

INTRODUCTION

Ras genes play a crucial role in growth, development, and differentiation and are involved in various signal transduction pathways mediated by growth factors [Satoh et al., 1992; Lowy and Willumsen, 1993; Burgering and Bos, 1995; Marshall, 1996]. Point mutations in Ras genes can cause acute transformation in mammalian cells and have been found in up to 30% of most human cancers [Bos, 1989]. In the simple eukaryotic organism, *Dictyostelium discoideum,* six Ras homologues have been identified, RasD [Reymond et al., 1984], RasG [Robbins et al., 1989], RasB [Daniel et al., 1993], RasS, and RasC [Daniel et al., 1994], as well as rap1 [Robbins et al., 1990]. The first cloned *Dictyostelium* Ras gene, RasD, plays an important role in development. [Reymond et al., 1986; Van Hasstert et al., 1987]. The functions of other Ras genes during the life cycle of *Dictyostelium* are largely unknown.

Three recent studies investigated RasG functions in *Dictyostelium*. The study by Weeks and colleagues documents that RasG is important for the early development of *Dictyostelium* [Khosla et al., 1996]. The second study suggests that a major role of RasG is in control of cell architecture, rather than growth or differentiation [Tuxworth et al., 1997]. A very recent study suggests that RasG regulates development and cytoskeletal functions by direct interaction with more than one downstream effector [Zhang et al., 1999].

To further understand the functions of RasG, we used a constitutive promoter to express an activated form of RasG(G12T) in wild type KAX-3 cells. This study shows that RasG plays a role in phagocytosis during the growth of *Dictyostelium*, since the RasG(G12T) transformants grow poorly on bacterial agar plates and are defective in engulfing bacteria when adhering to a filter surface. More important, we now report that RasG is crucial for *Dictyostelium*

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cell-substratum adhesion. The expression of the activated form of RasG causes a dramatic increase in adhesion on glass surfaces compared to wild type cells. Additionally, our RasG(G12T) transformants display a reduction of filopodia on cell surface and an increase in tyrosine phosphorylation on a 43 kDa protein during growth.

MATERIALS AND METHODS

Construction of Plasmids

An activated form (G12T) of the RasG cDNA was obtained from Dr. G. Weeks at University of British Columbia (Canada). The substituted nucleotides in the RasG(G12T) mutant change a glycine (GGT) to a threonine (ACT) at amino acid position 12. A DraI fragment of RasG(G12T) cDNA was then linked to a BamHI adapter and subsequently subcloned into the multiple cloning site of the pCFC5 vector [Chen and Katz, 1998], which contains a constitutive Actin15 promoter and an N-terminal T7 tag with codons favorable to *Dictyostelium*. This results in the plasmid pCFC4.

In order to demonstrate that the phenotypes seen in transformants are not due to the interactions of the T7 tag with other cellular proteins, a control plasmid was also constructed. The control plasmid (pCFC6FS) contains a frame-shift mutation in the middle of the RasG gene (at the single AccI site) resulting a nonfunctional protein. The frame shift was generated by first digesting the site with restriction enzymes, then filling the site with dNTPs using T4 DNA polymerase (New England BioLabs). The AccI site after the fill-in gained two additional bases, T and C, and thus the sequence of RasG was frame-shifted from this point. Restriction enzyme digestions and DNA sequencing confirmed the deletion of AccI site and the frame-shifting by fill-in on the plasmid DNA.

Cell Culture and Transformation

The wild type strain KAX-3, a derivative of AX-3, was used in these studies. AX-3 has the ability to grow either by phagocytosis of bacteria (in liquid culture or on nutrient plates) or by pinocytosis of HL5 medium axenically. Cells were transformed with pCFC4 plasmid DNA by electroporation [Howard et al., 1988]. The electroporation was done by using a BioRad Gene Pulsar set with 3 μF capacitor supplied voltage of 2.5kv/cm, resistance (Ω) between 200–1,000, yielding time constants (τ values) of 0.4–0.6 msec. After electroporation, cell aliquots were transferred to a Falcon 100× 20 mm OPTILUX Petri dish, and allowed to adhere and recover in HL5 medium for 5–12 h at 22°C before being treated with drugs. Selection was performed in OPTILUX Petri dishes for 8 days in HL5 medium containing 10 μg/ml G418 (Geneticin) and 50 μg/ml streptomycin. Clonal isolates were obtained after selection by plating the cells with *Klebsiella aerogenes* on SM plates.

Western Blots

Cell lysates were prepared from 5×10^7 exponentially grown cells, boiled in Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT and 60 mM Tris, pH 6.8) for 2 min. Generally, 10% of the cell suspension was reserved for estimating the total protein content, using the Bio-Rad protein kit. Proteins (100–150 μg per lane) were separated by SDS-PAGE (12.5% polyacrylamide gel) and transferred to a nitrocellulose membrane (Schleicher & Schuell BA 85, 0.45 μm) in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). Detection of primary antibody on the membrane was performed by using horseradish peroxidase-chemiluminescent method (Amersham ECL). The membranes were exposed to X-ray film for 1–10 min, depending on the strength of the signals. For the anti-phosphotyrosine immunoblot, the blocking reagent was 3% BSA, 1 mM EDTA, 1 mM $NaVO_3$, and 0.05% Tween in PBS.

The primary antibody Anti-T7 (T7-Tag) was obtained from Novagen. Anti-Ras (pan-Ras) was from Oncogene Science, Anti-actin from Sigma (A-2066) and antiphosphotyrosine from Upstate Biotechnology (clone 4G10).

Adhesion Assays

Coverslip adhesion assay: Log-phase axenically growing cells were washed and resuspended in 17 mM Sorensen buffer (KH_2PO_4/Na_2HPO_4, pH6.1) at 1×10^6 cell/ml and incubated at 22°C on a shaker for 3 h. Cells were then resuspended at 5×10^7 cell/ml in the same buffer and a total of 1×10^6 cells were plated on glass coverslips for incubation at 22°C for 30 min in a moist chamber. The coverslips for the control and experimental cells were paired and placed in a ceramic boat and washed extensively (20×) by dipping the ce-

ramic boat into a beaker containing Sorensen buffer. The remaining adherent cells were harvested by adding 100 μl of Sorensen buffer to the coverslip and scraping the cells into a microcentrifuge tube (1.5 ml) with a rubber policeman. Another 100 μl of Sorensen buffer was applied to each coverslip to remove any remaining cells. The cell suspension was briefly spun in an Eppendorf microcentrifuge. Cell pellets were resuspended in 100 μl of Sorensen buffer and the cell number was counted using a hemacytometer.

Vogel [1987] adhesion assay: Log-phase axenically growing cells were washed and resuspended in Sorensen buffer at a density of 1×10^6 cell/ml and incubated in 25 ml tissue culture glass flasks at room temperature at 120 rpm on a gyratory shaker (New Brunswick Scientific, Model G10) for 10 min. Cells were then incubated and adhered for 40 min in the glass flasks without shaking. The flasks were then gently agitated for 3 min at 60 rpm and the supernatants were transferred to test tubes. Nonadherent cells in each supernatant was determined using a hemacytometer.

Growth Rate on Bacterial Plates

The growth rate of *Dictyostelium* cells on bacterial plates was determined by the following method: Plaque size after 3-day growth: Vegetative HL5 axenically grown cells were plated clonally with bacteria on SM nutrient plates [Sussman, 1987]. The plates were then incubated at 22°C for 3 days. The diameters of emerged plaques were measured with a ruler.

Quantification of Phagocytosis on Filters

Dictyostelium amoebae phagocytosis was quantified by the procedures modified from Cohen et al. [1994]. The bacteria *Klebsiella aerogenes* were first labeled by fluorescein isothiocyanate (FITC) (Sigma Immunochemicals, St. Louis, MO). This was achieved by growing bacteria in Luria broth (LB) at 37°C overnight until OD_{600} was around 2. Bacteria were then pelleted and resuspended in 10 ml of 50 mM phosphate buffer (Na_2HPO_4, pH 9.2), containing 0.1 mg/ml FITC. The mixture was incubated at room temperature for 1 h, with 150 rpm shaking on a gyratory shaker. The labeled bacteria were washed three times in 20 mM phosphate buffer (Na_2HPO_4, pH 6.3) by repeated centrifugation, and resuspended with the same buffer at a density 5×10^{10} bacteria/ml.

1×10^7 exponentially growing *Dictyostelium* amoebae were obtained from either axenic or bacterial grown culture. The amoebae were washed three times in 20 mM phosphate buffer (pH 6.3), and resuspended with 100 μl of FITC-labeled bacteria (5×10^{10} bacteria/ml). This yields a multiplicity of 500 (500 bacteria to each 1 amoeba). Amoebae were immediately deposited onto a circle of 2 cm diameter on Whatman filter paper #50 (4.25 cm), with Millipore AP10 MF support pads underneath. The filter paper and support pads were presaturated (presoaked) in 20 mM phosphate buffer (pH6.3) before the experiment. After a 40-min incubation in a humid Petri dish at 22°C, the filter was lifted from the dish and vortexed in 10 ml ice-cold 20 mM phosphate buffer (PH6.3) in a 50 ml Corning centrifuge tube. Cell were then pelleted at 1,500 rpm in an SS34 rotor of a Sorval RC2-B centrifuge, washed two more times in 20 mM phosphate buffer (pH 6.3) and resuspended in 2 mls of 50 mM phosphate buffer (pH 9.2). Cells were first counted by a hemacytometer to determine the cell number and then lysed in 0.1% Triton X-100 for measurement of fluorescent bacteria.

The amount of fluorescence in the lysate was determined with a spectrofluorometer (SPEX Fluorolog 1680, 0.22 mm, Double Spectrometer) using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. A standard curve was generated by lysing a defined number of FITC-labeled bacteria in 50 mM phosphate buffer (pH 9.2) containing 1% SDS. The average of three numerical readings was recorded for each sample.

F-actin Staining by Rhodamine-Phalloidin

Amoebae at a density of 1×10^6 cell/ml were harvested from the growth zone on bacterial plates and washed three times in 17 mM Sorensen buffer (KH_2PO_4/ Na_2HPO_4 pH6.1). 100 μl of cell suspension was deposited onto glass coverslips at room temperature in a humid chamber for 30 min. Buffer was poured off from the coverslips and amoebae were fixed with 3.7% formaldehyde in Sorensen buffer for 15 min at room temperature in a humid chamber. The coverslips were placed in a ceramic boat and washed four times in 200 ml of PBS buffer (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl 4.3 mM NaH_2PO_4 and 24.6 mM Na_2HPO_4 pH7.4). The cells were then equilibrated three times on coverslips each in 200 μl of PBS for

5 min. After equilibration, cells were stained with 200 μl of rhodamine-phalloidin (Molecular Probes) at 2 unit/ml in PBS for 1 h in a dark, humid chamber. The coverslips again were placed in a ceramic boat and washed four times in 200 ml of PBS. Additional washes were performed by adding the PBS buffer on coverslips and then incubating at room temperature for 30 min with three changes of buffer. Adherent cells were examined with an Olympus reflected light fluorescent microscope (Model BH2-RFCA).

RESULTS

Expression of the RasG(G12T) Proteins

In order to distinguish the introduced RasG protein from the endogenous gene products, we placed an epitope tag (T7) in front of the activated RasG protein. To control for the 'T7-tag' effect, we constructed a frame-shift mutation in the RasG(G12T) gene in the plasmid, which results in a truncated and inactivated RasG protein. The truncated mutant protein was confirmed by a two-dimensional SDS-PAGE (data not shown). We compared the phenotypes of cells carrying the frame-shift mutations of RasG(G12T) with both wild type cells and RasG(G12T) transformants. The frame shift showed no abnormal growth or development. In addition, several assays, included chemotaxis and coverslip adhesion revealed that frame-shift mutants of RasG(G12T) exhibit wild type phenotypes (data not shown).

We have isolated six independent RasG(G12T) transformants (six different experiments) from four independent strains of KAX-3. We first examined whether the transformants expressed the expected T7-tagged RasG protein. By blotting with an anti-T7 monoclonal antibody, all transformants expressed a 28 kDa protein, which is not seen in the vector control EXP4(+) plasmid transformants (Fig. 1A). As shown in Figure 1B, the result from a Western blot using a RasG-specific polyclonal antibody suggested that the 28 kDa protein expressed in all transformants was indeed a RasG protein. The expression level of T7-tagged RasG(G12T) protein represented about 25% of the endogenous RasG protein, as quantified by densitometry.

To assess the level of T7 RasG(G12T) proteins that are expressed during the *Dictyostelium* life cycle, protein abstracts were obtained from different time points during development. As shown in Figure 1C, transformants expressed the T7-tagged RasG(G12T) proteins during vegetative growth. This level of expression was maintained throughout the first 9 h of development, then reduced to a very low level by 20 h after starvation (Fig. 1C, lanes 2 and 3). This expression pattern is consistent with the known expression pattern of genes driven by the Actin15 promoter [Cohen et al., 1986] that we used for the RasG(G12T) expression.

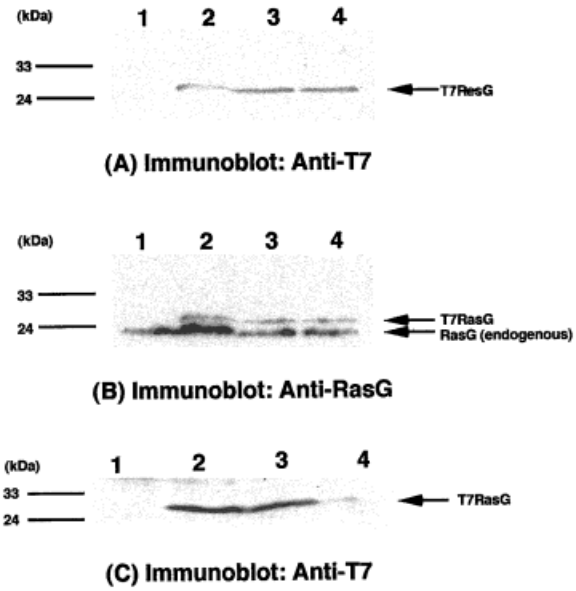

Fig. 1. /B Expression of T7-tagged RasG protein in RasG(G12T) transformants. Immunoblots of lysates from vegetative amoebae of three independent RasG(G12T) transformants, along with an EXP4(+) vector control transformant. **(A)** and **(B):** Lane 1, EXP4(+) vector control transformant; Lanes 2–4: three independently derived transformants. **A:** Immunoblot using a monoclonal anti-T7 antibody. **(B).** Same filter stripped and reblotted with a polyclonal anti-RasG antibody. **(C):** Expression of T7-tagged RasG protein during growth and development. Immunoblot was performed using a monoclonal anti-T7 antibody. Cell lysates were isolated from different developmental stage RasG(G12T) transformants, along with cell lysate from vegetative amoebae of an EXP4(+) vector control transformant. Lane 1, EXP4(+) vector control transformant; Lane 2, vegetative RasG(G12T) transformants; Lane 3, RasG(G12T) transformants after 9-h starvation; Lane 4, RasG(G12T) transformants after 20-h starvation.

Activated RasG Causes Growth Inhibition on Bacterial Plates

When RasG(G12T) transformants were grown on bacterial plates, they showed a greatly reduced growth rate and a thin growth zone (figure not shown). The growth rate was quantified by measuring the plaque diameter after 3 days of growth. As shown in Table 1,

TABLE I. Growth Rate of RasG(G12T) Transformants on the Bacterial Plates*

Cell type	Plaque size (3 days)[a]
KAX-3 (wild-type)	1.06 ± 0.15 cm[b]
Vector control	1.06 ± 0.09 cm[b]
RasG(G12T)	0.70 ± 0.12 cm[b]

*A RasG(G12T) transformant was compared with a wild type strain (KAX-3) and a vector control transformant (EXP4(+)).

[a]The diameter of a single plaque on a bacterial plate after plating single HL5 grown vegetative amoeba; average of 20 samples (cm) ± S.E..

[b]A t-test indicated that there is a significant difference between vector control and RasG(G12T) transformants (Ts = −10.7, $P < 0.001$). There was no difference between the KAX-3 wild type and vector control transformant cells as suggested by a *t*-test.

after 3 days, the plaque diameter of the RasG(G12T) transformants was only about 70% of wild type cells. The rate of plaque expansion in the transformants was also less than half of the rate of the wild type (data not shown). These results suggest that the activated RasG protein inhibits the growth of *Dictyostelium* amoebae on bacterial plates. In contrast to the growth defect, the development of RasG(G12T) transformants appeared to be normal on these plates (figure not shown).

To extend our observations on the growth of RasG(G12T) transformants, we then measured their growth rate in axenic liquid culture. As shown in Figure 2A, surprisingly, RasG(G12T) transformants grew normally in axenic medium: the doubling time of RasG(G12T) transformants was the same as the wild type, 8 h on average. This observation thus suggests that the growth inhibition on bacterial plates is not caused by a defect in the general growth machinery. Instead it could be due to the presence of bacteria, or to the attachment of cells to a substratum. To distinguish these we compared the growth rate of the RasG(G12T) transformants and wild type cells growing in liquid in the presence of bacteria. As shown in Figure 2B, the transformants do not show any difference in generation time compared to wild type cells. Taken together, the above observations suggest that attachment to substratum is the key factor causing slow growth of RasG(G12T) transformants on bacterial plates.

Increased Cell-Substratum Adhesion by RasG(G12T) Transformants

Cell-substratum adhesion plays a key role in *Dictyostelium* phagocytosis (e.g., see Cohen et al., 1994), we thus conducted cell-substratum adhesion assays using two independent methods. First assay (shown in Fig. 3) quantifies the ability of *Dictyostelium* amoebae to attach to a glass surface. Cells were allowed to attach to glass coverslips for a period of 30 min and then the coverslips were rigorously washed several times in buffer in a ceramic boat. The remaining adherent cells were then counted. Wild type controls were always paired with experimentals in the ceramic boat to insure equal treatment in the assay. As summarized in Figure 3, in each paired experiment RasG(G12T) transformants are dramatically (about 10×) more adherent on the glass surface than the wild type cells. The result is statistically significant, as analyzed by the Wilcoxon's *sign-ranked test* [Sokal and Rohlf, 1981], n = 30, $P < 0.001$, df = 29, and two-tailed. In a second assay method adopted from Vogel [1987] (data not shown), cells, allowed to adhere to the bottom of glass tissue culture flasks were agitated gently for short period of time. Nonadherent cells were then counted. Vogel's adhesion method, yielded a similar result: RasG(G12T) transformants exhibited approximately a tenfold increase in adhesion on the glass surface. The result was also statistically significant ($P < 0.01$).

In sum, our observations suggest that the expression of an activated RasG causes a dramatic increase in cell-substratum adhesion in *Dictyostelium*.

Adherent RasG(G12T) Transformants Are Defective in Phagocytosis

The slow growth phenotype of RasG(G12T) transformants occurred only when they were growing on bacterial plates. A previous study by Cohen et al. [1994] suggested that phagocytosis in *Dictyostelium* is dependent on proper cell-substratum adhesion. To address whether the dramatic increase in adhesiveness of RasG(G12T) transformants might lead to their inability to phagocytose bacteria effectively on plates, we measured phagocytosis of bacteria by adhered *Dictyostelium* amoebae [Cohen et. al, 1994]. As demonstrated in Table 2, adherent Ras(G12T) transformants were defective in

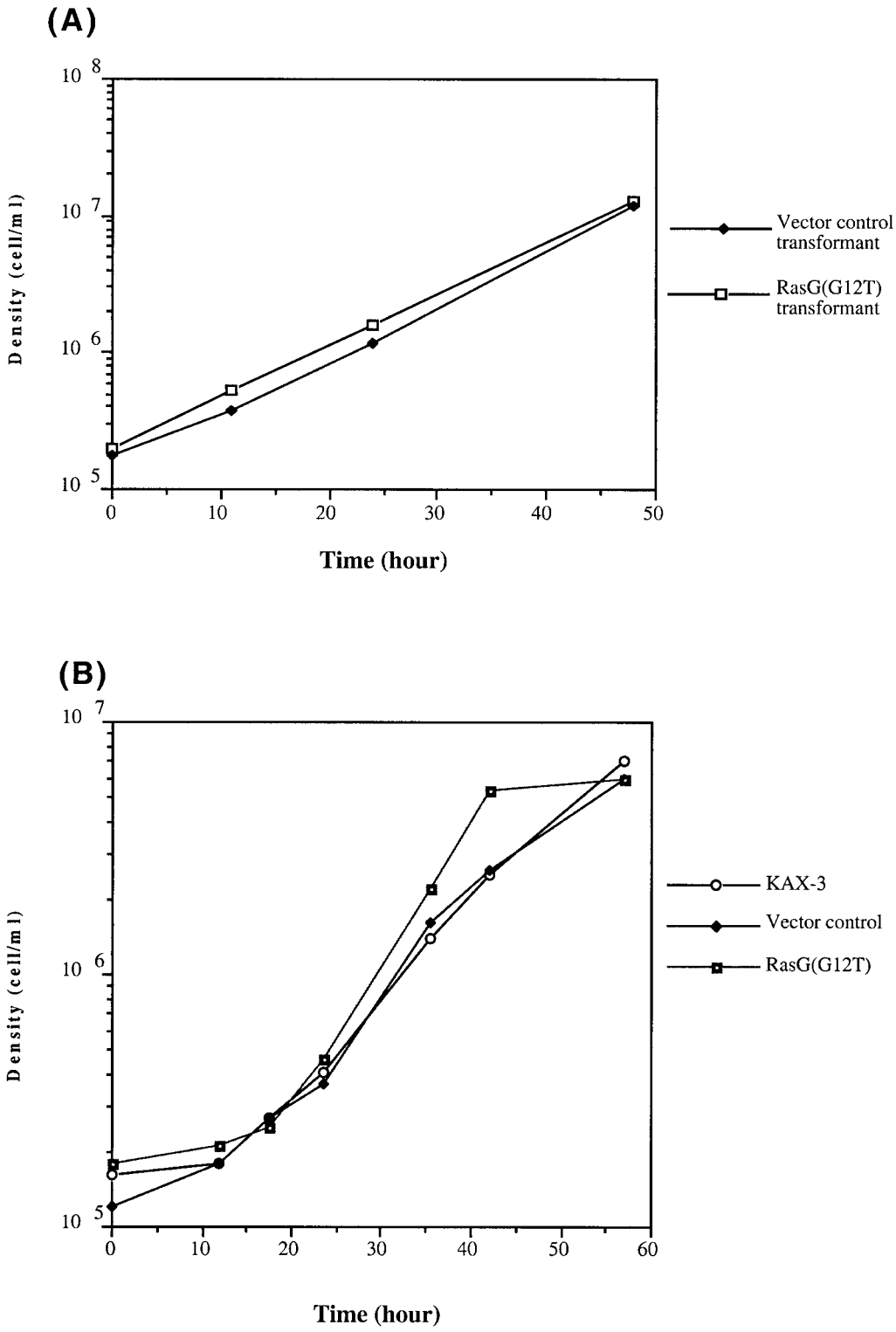

Fig. 2. Comparison of growth rates for RasG(G12T) transformants and wild type cells. **(A):** in axenic liquid culture. EXP4(+) vector control transformants were compared with RasG(G12T) transformants. **(B):** in bacterial liquid culture. Wild type cells, KAX-3 and EXP4(+) vector control transformants are compared with RasG(G12T) transformants. Growth was measured by determining cell density with a hemacytometer at the times indicated.

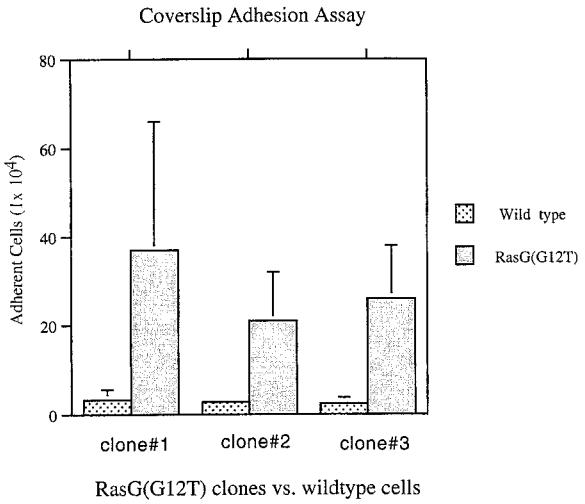

Fig. 3. Coverslip cell adhesion assay for wild type cells and RasG(G12T) transformants. Representation of typical paired experiments. Cell numbers were determined by harvesting the adherent cells from coverslips (see Materials and Methods).

TABLE II Phagocytosis of Bacteria by RasG(G12T) Transformant Cells*

Phagocytic index[a]		
Experiment	Vector control	RasG(G12T) transformant
I.	85	45
II.	82	30
III.	119	74
IV.	48[b]	46

*A RasG(G12T) transformant was compared with a vector control transformant (EXP4(+)).

[a]Determined by measuring the number of ingested FITC-labeled bacteria per amoeba during the 40 min incubation period after amoebae were adhered to the surface of filter at room temperature. All the samples (n = 4) were pooled and a statistical analysis was carried out using the Mann-Whitney *U*-test [Sokal and Rohlf, 1981; pp. 433–437]. The result indicates that there is significant difference (at 0.05 level) between the two means ($U_S = 16$, $P < 0.05$; one-tailed).

[b]Note that we consider this particular reading of 48 as a statistical 'outlier' and it should not obscure the overall trend of the observations. When not counting the outlier, the average number of engulfed bacteria (for three samples) by wild type cells, was 95 bacterium/per amoeba and RasG(G12T) was 50 bacterium / per amoeba.

phagocytosis (measured by the number of FITC-labeled bacteria engulfed). RasG(G12T) transformants exhibit approximately a 48% reduction in phagocytosis of bacteria, compared to wild type cells.

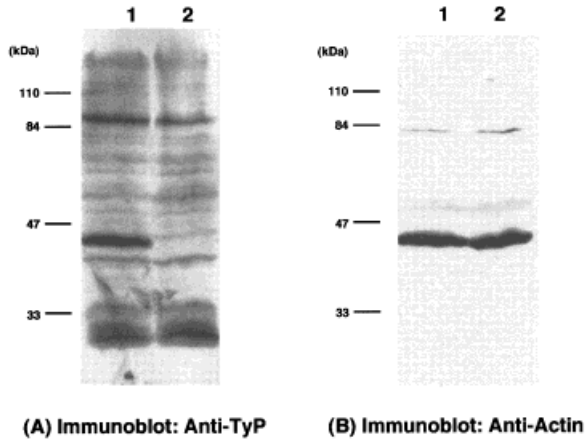

Fig. 4. Tyrosine phosphorylation on actin in RasG(G12T) transformant and EXP4(+) vector control transformant. **(A):** Immunoblot using a monoclonal antiphosphotyrosine antibody. **(B):** Same filter stripped and reblotted with a polyclonal anti-actin antibody. Lane 1, RasG(G12T) transformant; Lane 2, EXP4(+) vector control transformant. Lysates were prepared from vegetative cells growing in liquid axenic cultures.

RasG(G12T) Transformants Exhibit a High Level of Tyrosine Phosphorylation on a 43 kDa Protein During Vegetative Growth

Tyrosine phosphorylation on proteins plays a crucial role in signal cascades that lead to cell growth and differentiation in many organisms. It is well established that Ras proteins are among the major mediators of these biological pathways, stimulated by both receptor and nonreceptor tyrosine kinases [for a review, see Schlessinger, 1993]. These observations prompted us to examine the pattern of tyrosine phosphorylation in RasG(G12T) transformants.

Cell lysates for protein analysis were prepared from cells in the vegetative growth phase and immunoblotted with a phosphotyrosine specific monoclonal antibody. As revealed in Figure 4A, a protein of apparent molecular weight 43 kDa was highly phosphorylated on tyrosine in the RasG(G12T) transformants but not wild type cells.

Two recent reports [Howard et al., 1993; Schweiger et al., 1992] have documented that actin, with an apparent molecular weight between 43–45 kDa undergoes significant changes in tyrosine phosphorylation during the *Dictyostelium* life cycle. We therefore reblotted the same membrane with an anti-actin antibody. The result shows that the anti-actin antibody recognized the same 43 kDa band (Fig. 4B). Since the MAP kinases (ERKs) also run at

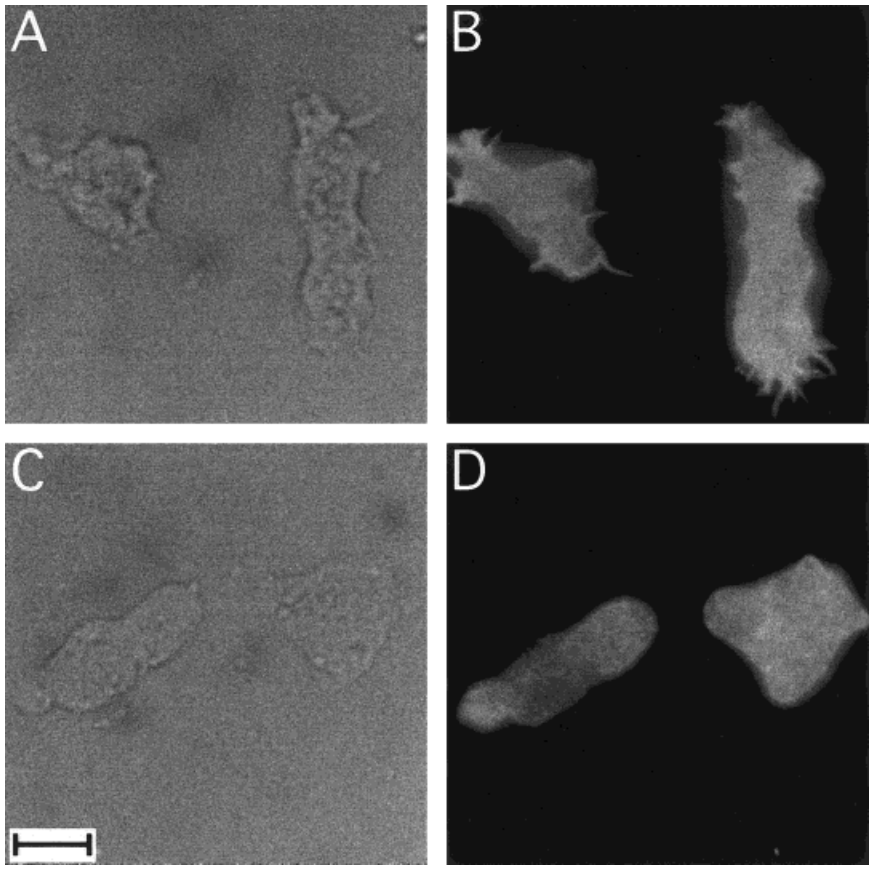

Fig. 5. Visualization of filopodia on wild type cells and RasG(G12T) transformants. F-actin was stained with rhodamine-phalloidin after fixation of vegetative *Dictyostelium* amoebae on a glass surface. Panels **A, B:** wild type KAX-3 cells; Panels **C, D:** RasG(G12T) transformants.

a similar position on SDS gel, we studied whether ERK2 phosphorylation is affected in our RasG(G12T) transformants. By using a phosphorylation-specific antibody to ERK2, we found that RasG(G12T) transformants display a normal ERK2 phosphorylation pattern during growth and development (data not shown). We therefore favor the notion that the highly phosphorylated 43 kDa protein is, in fact, actin.

RasG(G12T) Transformants Display Less Filopodia (Microspikes)

Actin is a major component of eukaryotic cell skeleton. In normal *Dictyostelium* cells, actin filaments are enriched in the cortical region and in cell surface projections. We examined whether in RasG(G12T) transformants there were any changes in actin organization by staining the F-actin on adherent cells using rhodamine-conjugated phalloidin. As shown in Figures 5B and 5D, some of the actin structures, such as pseudopodia and the cortical actin ring, are normal in RasG(G12T) transformants when compared to wild type cells. A closer examination nevertheless reveals a major difference between wild type cells and RasG(G12T) transformants. In the wild type cells, most of the adherent cells (more than 95%) exhibit numerous filopodia on their cell bodies (Fig. 5B). Filopodia (also known as 'microspikes') are actin filaments structured as thin, finger-like (0.1–0.2 μm wide) projections on the cell surface [Garrod and Born, 1971]. In contrast, it appears that RasG(G12T) transformants display very few filopodia (Fig. 5D). Some RasG(G12T) transformant cells are almost completely lacking filopodia on the cell surface.

DISCUSSION

This study has demonstrated that expression of an activated form of RasG protein in *Dictyostelium* can produce a dramatic increase in cell-substratum adhesion. Our results also show that RasG is important for phagocytosis during growth. Furthermore, RasG(G12T) transformants exhibit a reduction of filopodial projections on the cell body and an increase of ty-

rosine phosphorylation on a 43 kDa protein (most likely actin) during vegetative growth.

Some of our observations on the effects of the activated RasG(G12T) are different from the previous study by Weeks and colleagues [Khosla et al., 1996] who used a repressible promoter for the expression of the RasG protein. They reported that slow growth occurred in axenic culture. In contrast, we find normal growth in axenic medium and slow growth only on bacterial plates. A recent study from the same group reported that overexpression of RasG(G12T) in an AX-2 background caused an increase in the number of filopodia on the cell surface, while maintaining a wild type tyrosine phosphorylation pattern. Our RasG(G12T) transformants in an AX-3 background produce very few filopodia and exhibit a high level of tyrosine phosphorylation on a 43 kDa protein. There are several ways to reconcile these differences. The level of RasG(G12T) expression in transformants constructed by the Weeks group was much greater than in the current study and could account for the phenotypes seen in their study. The Weeks group used a discoidin promoter-driven construct that maintained at least twice the normal level of endogenous RasG protein. Our Actin15 promoter construct produced only 1/4 the level of expression compared with wild type RasG. The reason for these differences in expression may stem from the selection procedure. The discoidin promoter construct was turned off during transformation selection, whereas our Actin15 construct was expressed during that time. If overexpression of RasG(G12T) protein is deleterious to cells during the selection, but less so later, our selection scheme would thus only tolerate low levels of expression compared with the selection scheme using the repressible discoidin promoter.

Alternatively, the differences seen could result from the different genetic backgrounds of the strains used in the studies. The Weeks group used the AX2 strain whereas we used KAX-3, a derivative of AX-3. The two strains were derived independently and differences in the phenotypes produced by the same mutant in the two different backgrounds have been detected previously (Brink et al., 1990; Cox et al., 1996).

In present study the RasG(G12T) transformants were about 10× more adhesive to glass surfaces than were wild type cells. Although previous work on *Dictyostelium* has described the isolation of nonadhesive mutants [Bozzaro and Ponte, 1995; Vogel, 1987] and mutants which are reduced in adhesion [Weber et al., 1995], our RasG(G12T) transformants may be the first case in *Dictyostelium* where altered gene expression leads to a dramatic increase in the ability of cells to adhere to a glass surface.

In *Dictyostelium* there is a close connection between cell-substratum adhesion and phagocytosis. The phagocytosis impaired mutants isolated by Vogel [Vogel et al., 1980] were also defective in cell adhesion to glass or plastic. Similarly, Cohen et al. [1994] found that their phagocytosis mutants were defective in adherence and that the phagocytosis-less phenotype could be rescued by cells attaching on agar or polycarbonate filters. Our result suggests that too much cell-substratum adhesion, as well as too little adhesion, can diminish phagocytosis.

Early work on protein tyrosine phosphorylation in *Dictyostelium* indicated that actin is one of the major proteins undergoing transient regulation during the switch between growth and development [Schweiger et al., 1992; Howard et al., 1993]. When starved (or developing) cells are reexposed to growth medium, actin exhibits a transient, dramatic increase in tyrosine phosphorylation, presumably preparing the cytoskeletal organization for reentering growth phase. The significance of this transient tyrosine phosphorylation on actin is currently unknown. We did not study this developmental regulation of tyrosine phosphorylation since our transformants display normal development. Our RasG(G12T) transformants did, however, reproducibly exhibit a higher level of tyrosine phosphorylation on a 43 kDa protein (presumably actin) during vegetative growth (Fig. 4B) compared to wild type cells. In contrast, RasG(G12T) transformants in an AX-2 background (by the Weeks group) exhibited a wild type pattern for this transient tyrosine phosphorylation even while they exhibit a phenotype of being unable to aggregate [Zhang et al., 1999]. Apparently this developmental regulation of tyrosine phosphorylation of actin is not straightforward and requires further study.

The cell-shape change in *Dictyostelium* correlates with the temporal change of tyrosine phosphorylation of actin in response to growth conditions [Howard et al., 1993]. But cell-shape change is not a direct result of tyrosine phos-

phorylation on actin per se. For example, cells overexpressing the rap1 protein show a dramatic cell-shape change, that is, a flattened morphology but exhibited a wild type temporal tyrosine phosphorylation change on actin [Rebstein et al., 1993]. Similarly, RasG(G12T) transformants in an AX-2 background [Zhang et al., 1999] show a pronounced flattened morphology while also exhibiting a wild type tyrosine phosphorylation pattern. Finally, our RasG(G12T) transformants, with an altered tyrosine phosphorylation on the 43 kDa protein (presumably the actin), maintain normal cell shape changes from growth to development.

Tyrosine phosphorylated proteins have been detected in filopodia of neuronal growth cones in mammalian cells [Wu and Goldberg, 1993]. The level of tyrosine phosphorylation throughout the growth cone is negatively correlated with the elongation of filopodia. That is, the elongation of filopodia occurs at a faster rate when there were low levels of tyrosine phosphorylation of proteins in the growth cone. In *Dictyostelium*, the level of tyrosine phosphorylation and the number of filopodia also seems to be negatively correlated. For example, Firtel and coworker have observed that PTP1-overexpressing cells, with less tyrosine phosphorylation of actin, contain more 'microspikes' projections (i.e., filopodia) on the cell surface [Howard et al., 1993]. More importantly, PTP1 null cells, with a high level of tyrosine phosphorylation on actin, exhibit fewer filopodia than wild type cells. This is consistent with our observation that RasG(G12T) transformants, with a high level of tyrosine phosphorylation on the 43 kDa protein (presumably actin), also display very few filopodia. It should be noted that our observation on the lack of filopodia on the cell surface of RasG(G12T) transformants is complementary to the recent RasG knock-out study [Tuxworth et al., 1997]. Our activated RasG(G12T) transformants exhibit very few filopodia on the cell surface, whereas the RasG knock-out strain (*RasG* $^-$) displays more filopodia compared to wild type cells. These observations taken together suggest that RasG is a negative regulator of filopodial formation.

In contrast, it is more difficult to explain why RasG(G12T) transformants in an AX-2 background [Zhang et al., 1999] display more filopodia than their parental strain. It is interesting to point out that the wild type AX-2 has very few filopodia on the cell surface [Zhang et al., 1999], in contrast to the abundant filopodia on wild type AX-3 cells that we observed in this study. This different morphology between AX-2 and AX-3 reinforce the notion that genetic background may play a role in determining phenotypes in *Dictyostelium*.

In conclusion, besides a role in early development and in controlling cell architecture as previous studies suggested, the present study further places RasG in a central position during *Dictyostelium* growth. Our result suggests that RasG may be crucial for regulation of cell-substratum adhesion and phagocytosis during growth. RasG could execute these functions through tyrosine phosphorylation.

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