Dictyostelium RasD is required for normal phototaxis, but not differentiation

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RasD, a *Dictyostelium* homolog of mammalian Ras, is maximally expressed during the multicellular stage of development. Normal *Dictyostelium* aggregates are phototactic and thermotactic, moving towards sources of light and heat with great sensitivity. We show that disruption of the gene for *rasD* causes a near-total loss of phototaxis and thermotaxis in mutant aggregates, without obvious effects on undirected movement. Previous experiments had suggested important roles for RasD in development and cell-type determination. Surprisingly, *rasD*⁻ cells show no obvious changes in these processes. These cells represent a novel class of phototaxis mutant, and indicate a role for a Ras pathway in the connections between stimuli and coordinated cell movement.

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Members of the Ras family of small GTPases are central to a wide range of biological processes, including growth control, signaling, differentiation, and cell motility (Sorcher et al. 1993; Valencia and Sander 1995). Mammalian Ras proteins were discovered originally in an activated form as the products of oncogenes from tumor-promoting viruses (Shih et al. 1979). Expression of a *ras* gene with an activating mutation is sufficient to transform fibroblast cell lines, resulting in invasive, malignant cells. Microinjection of an activated Ras protein has been shown to cause many of the features of the transformed phenotype, including mitogenesis, altered cell morphology, and membrane ruffling (Bourne et al. 1990). Ras has since been shown to mediate the response to receptor tyrosine kinase activation in several different species, including mammals, *Drosophila* and *C. elegans* (Mulcahy et al. 1985; Han and Sternberg 1990; Fortini et al. 1992). In *Saccharomyces*, Ras proteins are required for control of adenyl cyclase and for progression through the G1-phase of the cell cycle (Toda et al. 1985).

Dictyostelium contains an unexpectedly large number of Ras subfamily proteins (Reymond et al. 1984; Robbins et al. 1989; Daniel et al. 1993, 1994). Two of these proteins, RasG and RasD, are closely related to mammalian Ha-Ras. The *rasG* gene is expressed at high levels in growing cells, whereas *rasD* is expressed at only low lev-

els during growth, but is induced after the cells initiate multicellular development (Reymond et al. 1984; Khosla et al. 1990; Esch and Firtel 1991). Null mutants containing a disrupted *rasG* gene exhibit several defects connected with motility, in particular a marked reduction in cell polarity, impaired cytokinesis in suspension, and slow growth (Tuxworth et al. 1997). Cells expressing an activated *rasG* fail to initiate development unless provided with exogenous cAMP pulses (Khosla et al. 1996). RasG therefore resembles Ras from higher eukaryotes in that it appears to control differentiation, cytoskeletal function, and cell division.

Several experiments have suggested a role for RasD in the correct proportioning of the presumptive stalk and spore (prestalk and prespore) cells during differentiation (Reymond et al. 1986; Louis et al. 1997). The *rasD* gene is expressed at higher levels in prestalk than prespore cells, suggesting a possible role in prestalk cell differentiation (Jermyn et al. 1987). Furthermore, cells that have been transfected with a *rasD* gene containing an activating mutation, *rasD*^{G12T}, arrest development after forming multitipped mounds (Reymond et al. 1986). These mutants express enhanced levels of the prestalk cell-specific genes *ecmA* and *tagB*, and very low levels of the prespore cell specific gene *cotC*, suggesting that RasD is involved in the choice of the prestalk cell fate (Louis et al. 1997).

Another Ras pathway member, the presumptive Ras guanine nucleotide exchange factor (RasGEF) encoded by the *aimless* gene, is required for both cAMP production

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and chemotaxis (Insall et al. 1996). We initially made mutants containing disruptions of different *ras* genes in an attempt to reproduce the *aimless* phenotype as part of a search for targets of the Aimless protein. As we describe here, disruption of the *rasD* gene does not result in any obvious defect in differentiation or a similar phenotype to *aimless*. Instead, *rasD*[−] cells develop successfully, producing culminants morphologically indistinguishable from the parental strain. However, *rasD*[−] slugs show impaired phototactic and thermotactic accuracy, while both processes are normal in *rasG*[−] slugs. That *rasD*[−] cells should exhibit such a subtle but definitive phenotype is highly unexpected and suggests a specific role for a RasD signaling pathway in photosensory and thermosensory responses in *Dictyostelium*.

Results

Disruption of the *Dictyostelium rasD* gene

Cells containing a disrupted *rasD* gene were generated by homologous recombination. A construct was made containing 2.0 kb of *rasD* genomic DNA (Reymond et al. 1986), with a blasticidin resistance cassette (Sutoh 1993) inserted into the 5' end of the *rasD*-coding sequence in the same orientation, so that its strong *act8* termination sequence blocked any transcriptional readthrough. The construct was transfected into AX3 cells, and transformants were cloned following seven days of selection in blasticidin S. Out of seven independent clones examined, six were found to contain a simple disruption in *rasD*. All clones were indistinguishable in growth and colony morphology and one, designated *rasD*[−], was used for all subsequent work. The wild-type *rasD* gene was transfected into this clone, using a G418 resistance cassette, as a control for nonspecific effects of transformation. This cell line will be referred to as *rasD*[−]*rasD*.

To confirm complete loss of RasD protein, an antibody against RasD was prepared using the approach used previously to generate a specific antibody to RasG (Khosla et al. 1994). Western blot analysis of bacterially expressed *Dictyostelium* Ras-GST fusion proteins (data not shown) revealed that the antibody exhibited the highest activity against the RasD protein, but still had residual activity against RasG, the closest cellular homolog of RasD, despite extensive cross absorption. Nonetheless, the antibody was sufficiently specific to allow determination of RasD levels.

Western blot analysis of extracts from AX3 cells at various stages of development using the RasD antibody detected two proteins of slightly different mobility, each with a different expression pattern (Fig. 1A). Because the higher mobility band showed the same expression profile as *rasD* mRNA, it was concluded that this represents the RasD protein. This band was completely absent throughout the development of *rasD*[−] cells (Fig. 1B). In light of the cross reactivity of the antibody with RasG, the blot was stripped and reprobed with the highly specific RasG antibody. This clearly identified the source of the lower mobility band as RasG (Fig. 1C). Reintroduction of a ge-

Figure 1. Expression of RasD and RasG proteins during *Dictyostelium* development. Cells were developed for the indicated times (in hours) then lysed in sample buffer. Twenty μ g of total protein was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted as described in Materials and Methods. (A) Parental cells probed with anti-RasD; (B) *rasD*[−] cells probed with anti-RasD; (C) Parental cells probed with anti-RasG; (D) Parental, *rasD*[−] and *rasD*[−] rescued with genomic *rasD*, all after 14 hrs of development, probed with anti-RasD.

nomeric *rasD* fragment into *rasD*[−] cells restored the lower band (Fig. 1D).

In experiments using purified proteins, we have found that the RasD antibody shows much more activity towards RasD than RasG (~fivefold; data not shown). The strength of the lower-mobility bands in blots from wild-type cells therefore indicates that cells always contain at least as much RasG as RasD, even late in development when RasD levels are at their peak.

Development of *rasD*[−] cells

Deletion of the *rasD* gene caused no obvious changes in cell growth or proliferation. Unexpectedly, it also caused no discernible alterations in development—all developmental stages from aggregation through to culmination were seen at the usual times. It has been shown recently that the overexpression of an activated *rasD* gene leads to a marked increase in prestalk cell-specific gene expression and a decrease in the expression of prespore cell-specific genes during development relative to wild type (Louis et al. 1997). We therefore investigated the expression of several cell-type specific genes during the development of *rasD*[−] cells to determine if loss of *rasD* had an effect on cell-type patterning. Accordingly, the same Northern blot was cut and simultaneously probed for the prestalk-specific genes *ecmA* and *ecmB* and the prespore-specific gene *pspA* (Fig. 2). The blot was stripped and reprobed with the IG7 probe as a control for loading. Unexpectedly, there was no obvious difference in the timing or level of expression of any of the three genes during development of *rasD*[−] cells. This result is dramatically different from the gross changes seen in cells expressing an activated *rasD* gene, and indicates that RasD is not required for normal proportioning of prestalk and prespore cells in the developing aggregate. Similarly, the expression of the *spiA* gene, which is a marker for terminal differentiation of spore cells, is indistinguishable in wild-type and *rasD*[−] cells (data not shown).

Figure 2. Expression of cell type-specific mRNA during development. Parental and *rasD*⁻ cells were harvested at the indicated times of development (in hours) and total RNA was prepared. Twenty μ g of RNA was separated on an agarose gel and transferred to nitrocellulose. The filter was cut into strips and hybridized simultaneously with *ecmA*, *ecmB*, *pspA*, and IG7 (loading control) probes.

Normal pattern formation in *rasD*⁻ aggregates

Previous work had implicated RasD in pattern formation during development (Esch and Firtel 1991), in particular in the spatial regulation of gene expression. To test this hypothesis, both *rasD*⁻ cells and the parental strain were transfected with *lacZ* reporter constructs under the control of different prestalk- and prespore-specific promoters. Cells were allowed to develop to either the slug or preculminant stage, then histochemically stained for β -galactosidase activity. The prestalk-specific *ecmA*O promoter (Early et al. 1993) drives expression of *lacZ* in the anterior fifth of the slug and the basal disc, stalk, tip, and upper and lower cups of the culminant. The *ecmO* region of the *ecmA*O promoter causes expression in the rear portion of the tip of the slug. The *ecmB* promoter is expressed in a cone of cells in the tip of the slug, and in the culminant shows a similar expression pattern to *ecmA* except that there is no staining of the tip/papilla outside the stalk tube. The prespore specific promoter *pspA* is active in the posterior four-fifths of the slug and in the spore mass of the culminant. For each of these reporter constructs, patterning in *rasD*⁻ aggregates was not appreciably different from that of parental strain (Fig. 3).

One powerful way of identifying otherwise hidden developmental defects is to mix cells of the mutant and parental strains to produce chimaeric aggregates, and observe whether the two cell types behave differently. Accordingly, *rasD*⁻ cells constitutively expressing *lacZ* from the *act15* promoter were mixed with either *rasD*⁻ cells or the parental strain in a 1:4 ratio. Again, cells were developed to either the slug or preculminant stage, then stained for β -galactosidase activity. In both mixtures, blue staining could be seen distributed randomly throughout the slugs and preculminants, suggesting that the development of *rasD*⁻ cells is as robust as the parental strain (Fig. 3O–R). Identical staining patterns were also obtained when these experiments were repeated with *act15/lacZ* expressing AX2 cells instead of *rasD*⁻ cells (data not shown).

Impaired phototaxis and thermotaxis in *rasD*⁻ aggregates

We did, however, see an obvious defect in phototaxis and

thermotaxis in slugs from *rasD*⁻ null cells. *Dictyostelium discoideum* cells form motile slugs after aggregation. These seek out optimal conditions for culmination, using phototaxis and thermotaxis, in which the slugs move with great sensitivity towards sources of light (Fisher and Williams 1981) and heat (Smith et al. 1982). When slugs from wild-type cells are kept in the presence of lateral light, they move almost directly towards the light source (Fig. 4). The *rasD*⁻ slugs were obviously less able to orient correctly (Fig. 4). To verify that this phenotype was caused by loss of RasD, and not an incidental consequence of transformation or selection, the *rasD*⁻ null cells were retransformed with a genomic copy of *rasD*. Several different transformants were examined, all of which exhibited normal phototaxis (Fig. 4B). We also examined strains in which *rasD* had been disrupted with different selectable markers; again, loss of RasD caused defective phototaxis in every case (data not shown).

Figure 5 shows quantitative measurements of phototaxis and thermotaxis (Fisher et al. 1981). Data were analyzed using the von Mises distribution, which yields a quantitative parameter κ describing the accuracy of ori-

Figure 3. Spatial expression of cell type-specific *lacZ* reporter constructs during development. *rasD*⁻ and parental cells were transformed with various *lacZ* reporter constructs, developed on KK2 agar to either the slug (15 hr) or early culminant (18 hr) stage, and fixed and stained as described in Materials and Methods. *ecmA*O-*lacZ* marker in parental (A,C) and *rasD*⁻ (B,D) aggregates; *ecmB*-*lacZ* marker in parental (E,F) and *rasD*⁻ (G,H) culminants; *ecmO*-*lacZ* marker in parental (I) and *rasD*⁻ (J) first fingers; *pspA*-*lacZ* marker in parental (K,L) and *rasD*⁻ (M,N) aggregates; 50:50 mixes of *act15*-*lacZ* marker in *rasD*⁻ cells with unlabeled parental (O,P) or *rasD*⁻ (Q,R) cells.

Figure 4. Phototaxis of wild-type and *rasD*⁻ cells. (A) Wild-type and *rasD*⁻ cells were inoculated onto charcoal agar plates, incubated for 48 hr in the presence of a lateral light source, and the resultant slug trails photographed. (B) Wild-type, *rasD*⁻ and *rasD*⁻*rasD* cells were inoculated onto charcoal agar plates, incubated for 48 hr in the presence of a lateral light source, and the resultant slug trails were transferred to PVC disks, stained with Coomassie Blue, and digitized. The light source was towards the right of the figure in each case.

entation. A κ of zero reflects no phototaxis and infinity indicates perfect orientation along the gradient. For the parental strain, κ varied from 100–500 (Fig. 5A). The *rasD*⁻ slugs were substantially less phototactic, although a slight positive response was measured, with a κ of 5–10 (Fig. 5A, inset). As reported in Fisher et al. (1981), the initial cell density has a marked effect on the efficiency of phototaxis, so measurements were made at a range of densities. The *rasD*⁻ slugs were clearly defective at all densities tested. Other reported mutants, such as those lacking the actin-binding protein ABP-120 (Fisher et al. 1997), can be even more severely impaired with accuracies of phototaxis (κ) as low as 0.5–1.0. Again, *rasD*⁻ slugs which had been rescued with genomic *rasD* exhibited similar phototaxis to the wild type. As a control for nonspecific effects caused by alteration of Ras levels,

Figure 5. Accuracy of phototaxis and thermotaxis. (A) Variation of phototactic efficiency with initial cell density. Parent ($\square$), *rasD* null ($\triangle$), *rasG* null ($\circ$), and *rasD* null rescued with a genomic *rasD* construct ($\blacksquare$) were plated at different densities, and slug trails were observed as in Figure 2. Data were digitized and analyzed according to Fisher et al. (1981). The inset panel shows the *rasD* null data plotted on a narrower scale to show slight phototaxis. (B) Variation of thermotactic efficiency with mean temperature. Cells were inoculated at 2.4×10^6 cells/cm² onto water agarose plates and incubated in the presence of a lateral light source (A) or in darkness on a heat bar producing 0.2°C/cm temperature gradient at the agarose surface (B). Arbitrary temperature units correspond to a temperature range of 14°C (T1) to 28°C (T8), as measured at the center of the plates in separate calibration experiments.

slugs containing a deletion in the related *rasG* gene were examined. These again showed wild-type phototaxis (data not shown).

Cells lacking the *rasD* gene also exhibit impaired thermotaxis (Fig. 5B), in common with several other phototaxis mutants. Thermotaxis by the *rasD*⁻ mutant was almost undetectable, while the *rasD*⁻*rasD* rescue strains and the *rasG*⁻ strain again behaved in a similar way to the parent.

Discussion

We have successfully generated a *Dictyostelium* cell line with a disrupted *rasD* gene. Unexpectedly, in the light of reports that RasD was important for differentiation (Reymond et al. 1986) and cell type determination (Esch et al. 1992; Louis et al. 1997), the disruptants grow, aggregate, and develop indistinguishably from the wild type. This contrasts sharply with the strong phenotypes caused by loss of RasG (Tuxworth et al. 1997) and RasS (Chubb et al. 2000).

Previous reports have shown that expression of a RasD protein containing an activating mutation (G12T) has a profound effect on development. Although cells aggregated normally, the mounds that formed were multitipped and unable to develop further (Reymond et al. 1986). In addition, pattern formation was markedly disrupted, with prestalk gene expression greatly enhanced and prespore gene expression greatly reduced (Louis et al. 1997). There are several plausible explanations for this phenomenon.

First, RasD might play one or more major roles in wild-type development. Its loss could, for the most part, be compensated for by the modulated activity of other Ras proteins in the cell. Indeed, there are at least three other Ras proteins present in the cell during multicellular development: RasB, RasC, and RasG (Reymond et al. 1984; Robbins et al. 1989; Daniel et al. 1993, 1994). RasG would be a particularly suitable candidate, sharing 100% identity in the effector and effector-proximal domains, and 82% identity over its entire length, and al-

though *rasG* mRNA levels decline early in development (Khosla et al. 1990), a substantial quantity of protein is present throughout development (see Fig. 1).

Alternatively, the role of RasD might be limited to the signal transduction pathways common to phototaxis and thermotaxis in the slug stage. In this case, the profound effects of activated RasD expression could be due to interference with a pathway that is normally controlled by a different Ras protein. Previous genetic studies on slug phototaxis (Darcy et al. 1994) have suggested that there are about 20 genes involved in slug phototaxis (that is, mutations in about 20 genes cause loss of phototaxis but allow the formation of otherwise normal slugs). This suggests the existence of a fairly complex signaling system. In the current model of phototaxis, light and temperature gradients modulate the slug tip activation/inhibition system to cause slug turning by stimulating lateral shifts in tip position (Darcy et al. 1994; Fisher 1997). Accordingly, many phototaxis mutants exhibit one of two developmental abnormalities: They either form multiply-tipped culminants with few or no spores, or they form stumpy fruiting bodies with little or no stalk (Darcy et al. 1994). These phenotypes are consistent with impairment of tip inhibition and activation pathways, respectively.

In the case of RasD, continuous high activity of the protein causes the multiple-tip phenotype (Reymond et al. 1986; Esch et al. 1992; Louis et al. 1997), whereas its complete absence causes impaired phototaxis and thermotaxis without any noticeable developmental defects (Wilkins et al., this issue). Together, these findings suggest that the RasD protein, when activated, stimulates the tip activation signaling pathway, but that this activity is normally only induced in response to light and temperature gradients. Thus, the role of RasD in slugs may not be in morphogenetic signaling per se, but in the modulation of morphogenetic signaling by the photo- and thermo-receptors. This highlights the danger that high-level expression of a constitutively active Ras protein can affect processes not normally controlled by it. The point is particularly relevant in the light of the small amounts of RasD protein found at any stage in development when compared with RasG (Fig. 1; below).

Previous work has implicated Ras in other types of directed cell migration during the development of *Dictyostelium*, *C. elegans*, and *Drosophila*. However, to our knowledge this is the first report of a role for a Ras protein in either phototaxis or thermotaxis. It will be extremely interesting to see whether a similar pathway exists in any other species.

Materials and methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Company and all restriction enzymes from New England Biolabs.

Cell strains, growth, and transformation

Dictyostelium discoideum AX3 cells were either grown axenically in HL5 medium or on a bacterial food source at 22°C

(Sussman and Sussman 1967). For bacterially grown cells, SM agar plates were inoculated with 10^5 – 10^6 *Dictyostelium* cells plus a suspension of *Klebsiella aerogenes* in Luria broth. To follow differentiation, cells growing exponentially from bacterial plates or axenic growth media were washed three times in KK2 (16.5 mM KH_2PO_4 , 3.8 mM K_2HPO_4 at pH 6.0) and plated on KK2 agar or nitrocellulose filters (Millipore).

Transformation was performed by a modification of Howard et al. (1988); briefly, cells growing exponentially were mixed with 25 μg of linearized DNA and electroporated in a BioRad gene pulser at 1.0 or 1.1 kV, 3 μF with a 5-ohm resistance in series. After 10 min incubation on ice, cells were placed at 22°C for 15' in the presence of 2 μl healing solution (100 mM MgCl_2 , 100 mM CaCl_2) and then HL-5 added. Ten $\mu\text{g}/\text{ml}$ Blasticidin-S (ICN) or G418 (Calbiochem) was added 24 hr after electroporation. After 7–8 days antibiotic selection, transformants were cloned on lawns of *Klebsiella* growing on SM agar. For uracil auxotrophic selection, DH1 cells were used, and FM medium (Franke and Kessin 1977) was added in place of HL-5.

*ecmA*O/*lacZ*, *ecmB*/*lacZ*, *ecmO*/*lacZ*, *pspA*/*lacZ*, and *act15*/*lacZ* strains were generated by CaPO_4 transfection of both *rasD*[−] cells and the parental strain (Harwood et al. 1992). Initial transformants were selected at 50 $\mu\text{g}/\text{ml}$ G418 whereas stable transformants were maintained at 20 $\mu\text{g}/\text{ml}$ G418.

Vector construction and Southern analysis

The *rasD* knockout vector was constructed by inserting the Blasticidin resistance gene (*bsr*) from pBsr δ Bam (Sutoh 1993) into the single *Pst*I site of a 2.0-kb, *Eco*RI/*Bcl*I fragment of *rasD* genomic DNA (Reymond et al. 1984) cloned into the *Eco*RI and *Bam*HI sites of pBluescript KS⁺. The *bsr* gene was oriented in the same direction as the *rasD* gene to ensure the presence of a transcriptional terminator in the middle of the *rasD*-coding sequence. From the resulting vector (pATW5) the 3.3-kb *rasD*/*bsr* fragment was excised using *Eco*RI and *Spe*I and used to transform *Dictyostelium* strain AX3 as described above. To analyze clones, AX3 or *rasD*[−] genomic DNA, prepared by the method of Sun et al. (1990), was digested with *Eco*RI and *Bcl*I, blotted onto nylon membrane (Amersham), and probed with a [α -³²P]dATP labeled *Clal*/*Bcl*I fragment of *rasD* genomic DNA (Reymond et al. 1984). The *rasD* rescue vector was constructed by ligating an *Xba*I fragment containing the G418-resistance cassette from pDNeo2 and an *Xba*I/*Eco*RI fragment containing the complete *rasD* gene (including the endogenous promoter) into pBluescript KS⁺.

Protein isolation and Western analysis

To determine Ras protein levels in cell extracts, $\sim 5 \times 10^7$ washed cells were lysed by resuspension in 1% sodium dodecyl sulphate (SDS) and mixed with an equal volume of 2 $\times$ sample buffer (0.5% β -mercaptoethanol, 0.5% SDS, 50 mM Tris at pH 6.8, 12.5% glycerol, 0.04% bromophenol blue). The specificity of the RasD antibody was determined against different levels of purified RasD–GST and RasG–GST. Samples were boiled for 3 min and then subjected to SDS-PAGE. The separated proteins were transferred onto nitrocellulose membranes and the anti-RasG and anti-RasD specific antibodies were used to detect RasG and RasD, respectively. The blots were blocked with TBS (50 mM Tris-HCl, 150 mM NaCl at pH 7.5) containing 0.5% Tween 20 and 5% nonfat dry milk for at least 1 hr at room temperature. After filters were washed, they were incubated with the primary antibody (1:1000) in TBS containing 1% nonfat dry milk for 1 hr at room temperature. The bound antibody was detected by incubating blots with a secondary conjugated donkey anti-rabbit IgG antibody (1:10,000) in TBS containing

0.5% nonfat dry milk for 1 hr at room temperature. The bound secondary antibody was detected using enhanced chemiluminescence (ECL, Amersham). Western blots were stripped in 100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 at 50°C for 30 min. They were then washed in TBS for 20 min and incubated with 5% nonfat dry milk and reprobed as described previously.

Production of anti-RasD antiserum

GST-RasG protein was produced as described previously (Khosla et al. 1994). GST-RasD protein was generated by the same general procedure except it was bound to glutathione-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 μ g of purified GST-RasD protein was mixed 1:1 with Titremax (Sigma) prior to intramuscular injection and booster injections of the same amount of protein were given after 14, 28, and 56 days. Serum was collected after 70 days, the IgG fraction was prepared and antibodies directed to the GST portion of the protein were removed as described previously (Khosla et al. 1994). The remaining antibody was bound to GST-RasD-Affigel 10 beads and then eluted with 3.5 M MgCl_2 . The eluted antibody was dialyzed overnight at 4°C against TBS and concentrated in a dialysis bag immersed in PEG 20,000 (BDH). Antibodies that recognized common epitopes to the other Ras subfamily proteins were removed by absorption to GST-RasG-Affigel 10 by the method described previously (Khosla et al. 1994).

Phototaxis and thermotaxis assays

Qualitative phototaxis tests were performed as described previously (Darcy et al. 1994) by using sterile spatula-style toothpicks to transfer cells to charcoal agar plates from the edges of colonies growing on *Klebsiella aerogenes* lawns. Phototaxis was scored after 48 hr incubation at 21°C with a lateral light source.

For quantitative phototaxis experiments, washed amoebae were inoculated onto the centers of charcoal agarose plates (pH 6.5) at various densities and incubated with a lateral light source for 48 hr at 21°C.

For quantitative thermotaxis experiments, washed amoebae were inoculated onto the centers of water agarose plates ($\sim 2.4 \times 10^6$ cells/cm²) and incubated for 72 hr in darkness on a heat bar producing a 0.2°C/cm temperature gradient at the agarose surface. Arbitrary temperature units correspond to a temperature range of 14°C (T1) to 28°C (T8), as measured at the center of plates in separate calibration experiments.

Slug trails were transferred to PVC disks, stained with Coomassie Blue, and digitized. Slug orientation was analyzed using directional statistics (Fisher et al. 1981).

RNA isolation and Northern analysis

Total RNA was extracted using phenol/SDS (Berks and Kay 1990). RNA (20 μ g), resuspended in 50% formamide, 40 mM 3-(N-morpholino) propanesulfonic acid (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 6% formaldehyde, was size-fractionated on 1.25% formaldehyde-agarose gels and transferred onto nitrocellulose membranes. Specific cDNAs encoding various genes were radiolabeled by the random primer method using [α -³²P]dATP (Amersham). The prehybridization and hybridization conditions of the nitrocellulose filters have been described previously. After hybridization, the filters were washed first with 2 $\times$ SSC, 0.1% SDS at room temperature, then with 0.5 $\times$ SSC, 0.1% SDS at 60°C and exposed to X-ray film. After being

hybridized by the first cDNA probe, blots were stripped with 0.1 $\times$ SSC, 0.1% SDS, 25% formamide at 65°C and then rehybridized with a second cDNA probe.

Histochemical staining for β -galactosidase activity

Aggregates and slugs were fixed for 15 min in 1% glutaraldehyde in Z-buffer (60 mM NaH_2PO_4 , 40 mM Na_2HPO_4 , 10 mM KCl, 1 mM MgSO_4 , 2 mM MgCl_2). Samples were washed twice in Z-buffer then incubated in staining solution (0.1% 5-bromo-4-chloro-3-indolyl- β -D-galactoside, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in Z-buffer) at 37°C (Dingermann et al. 1989).

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