

Expression of activated Ras during *Dictyostelium* development alters cell localization and changes cell fate

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SUMMARY

There is now a body of evidence to indicate that Ras proteins play important roles in development. *Dictyostelium* expresses several *ras* genes and each appears to perform a distinct function. Previous data had indicated that the overexpression of an activated form of the major developmentally regulated gene, *rasD*, caused a major aberration in morphogenesis and cell type determination. We now show that the developmental expression of an activated *rasG* gene under the control of the *rasD* promoter

causes a similar defect. Our results indicate that the expression of activated *rasG* in prespore cells results in their transdifferentiation into prestalk cells, whereas activated *rasG* expression in prestalk causes gross mislocalization of the prestalk cell populations.

Key words: *Dictyostelium*, Ras, Multi-tipped aggregates, Cell localization, Gene expression

INTRODUCTION

ras genes have been isolated from a wide variety of eucaryotic organisms and their encoded products are highly conserved small monomeric G proteins that participate in signal transduction pathways by cycling between an inactive GDP-bound form and an active GTP-bound form (Yamamoto et al., 1999). In growing mammalian cells, Ras proteins are components of pathways that transduce growth factor signals from the cell surface to the nucleus. In many mammalian cell lines, the expression of an activated *ras* gene produces a protein that constitutively binds GTP and causes malignant transformation. Ras proteins have also been shown to be essential components in cell type determination during the development of various tissues of *Caenorhabditis elegans* and *Drosophila melanogaster*. For example, in *C. elegans* loss of *ras* function leads to a vulva-less phenotype, whereas the expression of an activated *ras* allele leads to a multi-vulva phenotype (Sternberg and Han, 1998). In *Drosophila*, loss of *ras* function leads to the absence of the differentiation of the R7 neuronal cell in the eye and ectopic expression of activated *ras* in non-neuronal cells leads to R7 cell formation, which disrupts the normal ommatidial array and produces an abnormal rough eye phenotype (Simon, 1994). Thus, in both examples, gain-of-function and loss-of-function alleles of *ras* have opposite effects on development.

In *Dictyostelium discoideum*, several *ras* subfamily genes are expressed at various times during growth and differentiation (Reymond et al., 1984; Robbins et al., 1989; Daniel et al., 1993a; Daniel et al., 1993b). Two of the encoded

proteins, RasD and RasG are highly related to each other and to the mammalian Ha-Ras, Ki-Ras and N-Ras proteins (Robbins et al., 1989). *rasG* is expressed during growth, and early development, whereas *rasD* is expressed at later developmental stages. Despite their quite different expression patterns, there is evidence that RasD can substitute for RasG in growing cells. Thus, vegetative *rasG*-null cells contain elevated levels of RasD and when these levels are further enhanced by introduction of the *rasD* gene under the control of the *rasG* promoter, most of the phenotypic defects of *rasG* null cells are reversed (Khosla et al., 2000). Furthermore, RasG protein persists during the development of *rasD* null cells and it is possible that RasG can replace the developmental function of RasD.

During *Dictyostelium* development, cells aggregate to form a mound with a single tip; and prestalk- and prespore cell-specific gene expression commences during the latter steps of this process. The tip elongates to form a migrating slug as a prelude to final fruiting body construction. Overexpression of an activated form of the developmentally regulated *rasD* gene, under the control of its own promoter, resulted in a transformant, *rasD::rasD(G12T)* (designated originally *Dd-ras-Thr12*), that formed a multi-tipped mound during development and proceeded no further in morphogenesis (Reymond et al., 1986). Prestalk cell-specific gene expression was greatly enhanced and prespore cell-specific gene expression was greatly reduced in this transformant (Louis et al., 1997a). *rasD* expression was found to be enriched in prestalk cells during the differentiation of wild type cells, which led to the hypothesis that it was the expression of

activated *rasD* in the prestalk cell population that caused the multi-tipped aggregate (Esch and Firtel, 1991). However, as *rasD* is expressed in significant amounts in prespore cells (Jermyn and Williams, 1987; Jermyn and Williams, 1995), it is possible that this expression contributes to the phenotype of the *rasD::rasD(G12T)* transformant.

Given the fact that the developmental defects resulting from the expression of activated RasD during *Dictyostelium* development are so dramatic (Louis et al., 1997a; Raymond et al., 1986; van Haastert et al., 1987), it was a surprise to find that *rasD* null mutants exhibited no observable defect in morphology, patterning, cell type proportions or terminal differentiation (Wilkins et al., 2000). Their only defect was in the phototactic and thermotactic responses of the slugs. This finding clearly questions the significance of RasD as a regulator of normal development. However, developing Ax2 cells still contain considerable amounts of the highly homologous vegetatively expressed RasG protein, which may fulfill most of the normal functions of RasD. Certainly in *rasG* null cells, there was a compensatory increase in the level of the RasD protein during vegetative growth, and the ectopic overexpression of RasD protein rescued most of the phenotypic defects of the *rasG* null cells (Khosla et al., 2000), suggesting that the RasD protein can substitute for most of the RasG functions. We have attempted to isolate transformants with disruptions in both the *rasG* and *rasD* genes. However, perhaps not surprisingly, the double knockout appears to be lethal.

In this study, we show that transformants that express the activated form of *rasG*, under the control of the *rasD* promoter, also form multi-tipped aggregates that are blocked from further morphogenesis and then test the hypothesis that it is the expression of activated *ras* in the prestalk population that leads to the formation of the multi-tipped aggregate and the marked changes in cell type-specific gene expression. Contrary to expectations, our results conclusively demonstrate that it is the expression of *rasG(G12T)* from the prespore specific *psA* promoter that reproduces these defects. Expression of *rasG(G12T)* from the prestalk specific *ecmA*O promoter produces single tipped mounds, and only very slight changes in gene expression. However, additional defects become apparent during the subsequent development of this *ecmA::rasG(G12T)* transformant.

MATERIALS AND METHODS

Growth, transformation and development of *Dictyostelium discoideum* cells

Dictyostelium Ax2 cells were grown in HL-5 media, formulated exactly as described (Watts and Ashworth, 1970) and cell numbers were counted in a hemocytometer. For growth in association with bacteria, *Dictyostelium* amoebae were seeded along with an innoculum of *Klebsiella oxytoca* on nutrient-rich agar plates (Sussman, 1987) and incubated at 22°C. Calcium phosphate-mediated DNA transformation was performed as described previously (Early and Williams, 1987). The transformants were selected in HL-5 medium supplemented with 10 µg/ml G418 (Life Technologies, Grand Island, NY) and maintained in the presence of 10 µg/ml G418. To initiate development, exponentially growing cells were harvested, washed twice with Bonner's Salts formulated exactly as described (Bonner, 1947) and then plated on Millipore Nitrocellulose filters (Sussman, 1987).

To quantify spore formation, developed cells were washed off filters into Bonner's Salts containing 1% Triton X-100 (Fisher Scientific, Nepean, Ontario), incubated for 30 minutes and detergent-resistant spores were counted in a hemocytometer. To determine if spores arose from wild-type or transformant cells, they were either germinated on *Klebsiella* lawns and the developmental phenotype of the resulting plaques assessed microscopically, or germinated in HL5 media in microtiter plate wells and resistance to G418 determined. Cellulose in stalk cell walls was stained with Calcofluor (Sigma, St Louis, MO) as described previously (Springer et al., 1994) except that calcofluor was used at 5 µg/ml. Stained structures were viewed with a Zeiss Axioplan2 fluorescence microscope and images captured with a digital camera (Spot Diagnostics Instruments).

Vector construction

To create the *psA::rasG(G12T)* vector, the 5' portion of the *rasG(G12T)* cDNA in the *ptZ19R-rasG(G12T)* vector (Khosla et al., 1996) was modified by PCR, using 5'-GTCTAGATCTTTTAAA-AAAATGACAG-3', and 5'-CTTAGAGTTAAGGATAAGGA-3' as primers. As an intermediate step, the PCR product was digested with *Bgl*III and *Acc*I and ligated with a similarly digested *ptZ19R-rasG* vector fragment in which a second *Acc*I site in the polylinker had been destroyed. (The *Acc*I site was destroyed by digesting with *Sal*I, which recognised an overlapping site, blunting the ends and religating.) From this construct, *ptZ19R-rasG(G12T)ΔSalIΔstop*, the *rasG(G12T)* cDNA was released by digesting with *Bgl*III and *Kpn*I and ligated to the vector fragment of *Bgl*III/*Kpn*I-digested *psA-DdPK2* (Hopper et al., 1993). The resulting construct contained the *rasG(G12T)* cDNA linked to the *psA* promoter but also contained a portion of the *DdPK2* gene that was removed by digesting with *Kpn*I and *Xho*I, blunting the termini and religating. The promoter/gene fusion in the generated construct, *psA::rasG(G12T)*, was confirmed by sequencing.

To create the *ecmA::rasG(G12T)* vector, the *psA::rasG(G12T)* cDNA construct was digested with *Bgl*III and ligated to a *Bgl*III fragment of the *ecmA*O promoter from the *ecmA::lacZ* vector (Early et al., 1993). After verifying the orientation of the promoter in the vector, the construct was digested with *Kpn*I and *Xba*I to remove the *psA* promoter, blunt ended and religated to generate the *ecmA::rasG(G12T)* construct.

To create the *rasD::rasG(G12T)* construct, the *Ddras/lacZ* vector DNA (Esch and Firtel, 1991) was digested with *Bgl*III and *Nde*I to excise the *lacZ*-coding region. The digested vector was blunt ended and religated. The vector was then cut with *Eco*RI and ligated to the *Eco*RI fragment of the *pt219R-rasG(G12T)ΔSalIΔstop* vector. This construction resulted in the incorporation of an additional ATG codon. In order to delete the extra ATG in the *rasD* promoter, the construct was digested with *Xba*I and *Bgl*III, releasing the promoter. The vector termini were then blunt ended and the vector religated to a *Rsa*I-digested, shortened *rasD* promoter.

RNA isolation and northern analysis

The guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987) was used to extract total RNA from *Dictyostelium* cells. For each sample, 20 µg of RNA was resuspended in loading buffer (50% formamide, 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, 6% formaldehyde, ethidium bromide, loading dye) and electrophoresed on a 1.25% formaldehyde-agarose gel buffered with MOPS, containing formaldehyde. Ethidium bromide fluorescence of the RNA samples was used to assess equal loading. The RNA was then transferred by the capillary method to a nitrocellulose membrane (Sambrook et al., 1989). RNA blots were probed with specific cDNA fragments (for the *ecmA*, and *cotC* genes) randomly labelled with [α -³²P]dCTP (NEN Scientific, Boston, MA) (Feinberg and Vogelstein, 1983). Hybridisation was performed at 42°C overnight, after which membranes were washed first in 2×SSC, 0.1% SDS (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 42°C and then in 0.5×SSC, 0.1%

SDS at 60°C. Membranes were then exposed to X-ray film (Eastman Kodak Company, Rochester, NY).

Colony lift for β -galactosidase expression

Dictyostelium Ax2 cells were transformed with one of several reporter constructs that drive cell type-specific expression of β -galactosidase: *psA-his-gal* (Detterbeck et al., 1994), *ecmA-lacZ* (Early et al., 1993), *ecmB-lacZ* (Jermyn and Williams, 1991) and *ST-lacZ* (Ceccarelli et al., 1991), as well as with the constitutively expressed *actin15-lacZ* vector. Once colonies were visible, transformant cells were harvested as a pool and an aliquot was diluted and spread with *Klebsiella oxytoca* onto nutrient rich agar plates at a density of 75 cells per plate. After 4-5 days incubation, *Dictyostelium* plaques appeared on the lawn of *Klebsiella* and cells in the centre of the plaques had initiated differentiation. Clones were screened for β -galactosidase expression using a colony lift assay (Buhl et al., 1993). Positive clones with wild-type developmental morphology were selected. Identical protocols were followed to create *psA::rasG(G12T)* and *ecmA::rasG(G12T)* cells expressing the *lacZ* constructs, except that Ax2 cells were cotransformed with either the *psA::rasG(G12T)* or *ecmA::rasG(G12T)* constructs and with one of the *lacZ* constructs. Clones that stained positive for β -galactosidase and exhibited either the typical *psA::rasG(G12T)* or *ecmA::rasG(G12T)* morphology were selected for further analysis.

In situ detection of β -galactosidase expression

Dictyostelium transformants expressing β -galactosidase promoter fusion constructs were washed free of nutrients and plated for development (using KK2 buffer) on white Millipore filters. At various developmental time points, portions of the filters, containing several developing organisms, were cut and immersed for 10 minutes in Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 2 mM MgCl_2) containing 1% glutaraldehyde to fix the cell masses (Dingermann et al., 1989). After washing twice with Z buffer, cells were incubated in 0.1% 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-gal), 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in Z buffer at 37°C overnight (Dingermann et al., 1989). Stained organisms were rinsed with Z buffer and then photographed with a Nikon 35 mm camera and Kodak Ektachrome 100+ film.

Phototaxis assay

Cells were harvested and washed twice with Bonner's Salt and resuspended at a density of 1×10^8 cells/ml. The cells were spotted on one side of 1% water agar (Difco Laboratories, Detroit MI) plates to maintain the developing structures at the slug stage (Dormann et al., 1996). The plates were placed in a box lined with moist paper towels in an orientation such that the cells were placed

furthest away from a single slit cut into one side of the box to allow light entry. The box was incubated at 22°C in the light for 2 days and the deposited cells were then examined for evidence of slug migration.

RESULTS

Developmental phenotypes of transformants expressing activated *rasG* under the control of developmental promoters

In order to determine whether the multi-tipped phenotype of the *RasD::rasD(G12T)* transformant (Reymond et al., 1986) was due to the expression of activated *ras* in prestalk cells or prespore cells, we expressed activated *ras* under the control of cell type-specific promoters. For these studies, we used the *rasG* gene, as it has proved to be far easier to clone and we had a highly specific antibody against the gene product (Khosla et al., 1994). First we checked to make sure that the activated *rasG* gene, *rasG(G12T)*, would produce the same effect as *rasD(G12T)* when expressed under the control of the *rasD* promoter. Several *rasD::rasG(G12T)* transformants were isolated and all formed multi-tipped aggregates such as the one shown in Fig. 1A. None of these transformants proceeded beyond the multi-tipped aggregate stage of development. Thus, expression of activated *rasG* from the *rasD* promoter produces an identical developmental phenotype to that produced when activated *rasD* is expressed.

rasG(G12T) was expressed exclusively in the prespore cell population by the use of the *psA* promoter (Early et al., 1988). The *psA::rasG(G12T)* transformants formed mounds with

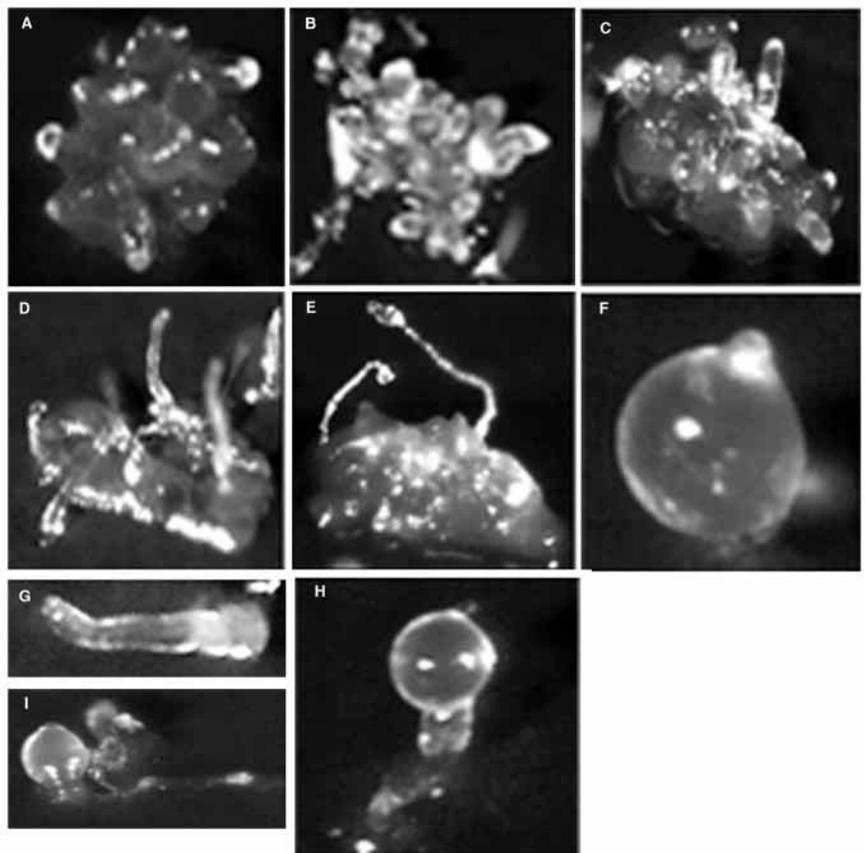


Fig. 1. Developmental structures formed by activated *rasG* transformed cells. (A,B) Mounds with multiple tips formed by a *rasD::rasG(G12T)* transformant (A), and by a *ecmA::rasG(G12T)/psA::rasG(G12T)* cotransformant (B). (C-E) A mound with multiple tips (C), a mound with multiple finger-like structures (D) and a terminal structure consisting of a basal mound with protruding stalks (E), formed by a *psA::rasG(G12T)* transformant. (F-I) A mound with a single tip (F), a slug (G) and terminal structures of irregular morphology (H,I) formed by an *ecmA::rasG(G12T)* transformant.

multiple tips (Fig. 1C), a defective phenotype that is very similar to those of the *rasD::rasG(G12T)* (Fig. 1A) and *RasD::rasD(G12T)* transformants. However, the multiple tips formed by *psA::rasG(G12T)* transformant extended to form finger-like projections (Fig. 1D) that sometimes fell to the substratum but remained anchored to the basal mound. These structures then went on to form aberrant culminants, consisting of a large basal mound to which stalk-like structures were attached (Fig. 1E). Time-lapse photography indicated that the stalk-like structures were formed from the finger-like projections that were produced initially just after the mound stage (data not shown). Although most of the stalk-like structures lacked sori, a few did produce apical pseudo-sori, which were very small and translucent (Fig. 1E) compared with a typical wild-type structure. Morphological changes ceased after about 24 hours, a time similar to that required by the wild type to complete development.

rasG(G12T) was expressed exclusively in the prestalk cell population using the *ecmA* promoter (Early et al., 1993). *ecmA::rasG(G12T)* transformants aggregated and formed mounds in approximately the same time as wild-type cells, but their subsequent development was slow and asynchronous. Most of the aggregates produced a single tip (Fig. 1F) that elongated vertically to form a finger and then subsequently fell to the substratum to form a slug (Fig. 1G). Although these transformant slugs were morphologically similar to those of the wild type, the terminal structures that subsequently formed were heterogeneous, and none was identical to wild-type fruiting bodies. For example, some consisted of a spherical sorus-like structure supported on a short irregular cell mass but other aberrant morphologies were also observed (Fig. 1H,I). The *ecmA::rasG(G12T)* transformants required 36 to 48 hours to form these terminal structures, substantially longer than the time required for the wild type to complete development.

Although the *psA::rasG(G12T)* transformant produced mounds with multiple tips (Fig. 1C), developmental morphogenesis did not terminate at this stage. Thus, it would appear that expression of activated Ras in both the prestalk and prespore cell population is necessary to block morphogenesis at the multi-tipped aggregate stage. To test this hypothesis, we cotransformed Ax2 cells with the *ecmA::rasG(G12T)* and the *psA::rasG(G12T)* constructs. When starved to induce development, the cotransformants formed mounds with multiple protruding tips (Fig. 1B) that did not progress further in morphogenesis and thus had a phenotype that was very similar to that of both the *RasD::rasD(G12T)* (Reymond et al., 1986) and the *rasD::rasG(G12T)* transformant (Fig. 1A).

Cell type-specific gene expression

As cell type-specific gene expression had been found to change drastically in the *RasD::rasD(G12T)* transformant (Louis et al., 1997a), cell type specific gene expression was measured in the *ecmA::rasG(G12T)* and *psA::rasG(G12T)* transformants in order to determine if they exhibited a similar defect. Northern blots of RNA isolated from the transformants and from control cells at various developmental time points were probed with cDNA fragments of the prespore-specific *cotC* gene (Fosnaugh and Loomis, 1989) and the prestalk-specific *ecmA* gene (Jermyn et al., 1987). It was found that *cotC* and *ecmA* mRNA levels were only slightly altered in the *ecmA::rasG(G12T)* transformant (Fig. 2B,D) relative to

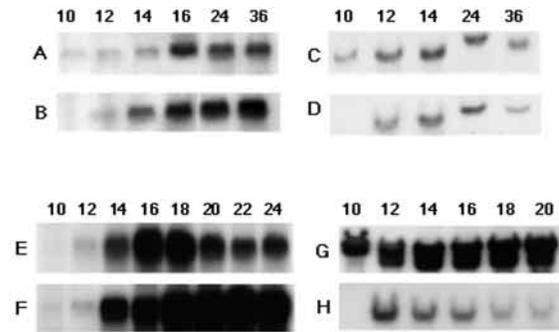


Fig. 2. Northern blots of RNA isolated from wild-type cells (A,C,E,G), an *ecmA::rasG(G12T)* transformant (B,D) and a *psA::rasG(G12T)* transformant (F,H) at various developmental times. Blots were probed with either the prestalk-specific *ecmA* cDNA (A,B,E,F) or the prespore-specific *cotC* cDNA (C,D,G,H). The numbers above the lanes refer to the hour of development at which RNA was isolated.

those in control cells (Fig. 2A,C). Thus, there was a far less pronounced effect on cell type specific gene expression in the *ecmA::rasG(G12T)* transformant than had been observed previously for *RasD::rasD(G12T)* (Louis et al., 1997a).

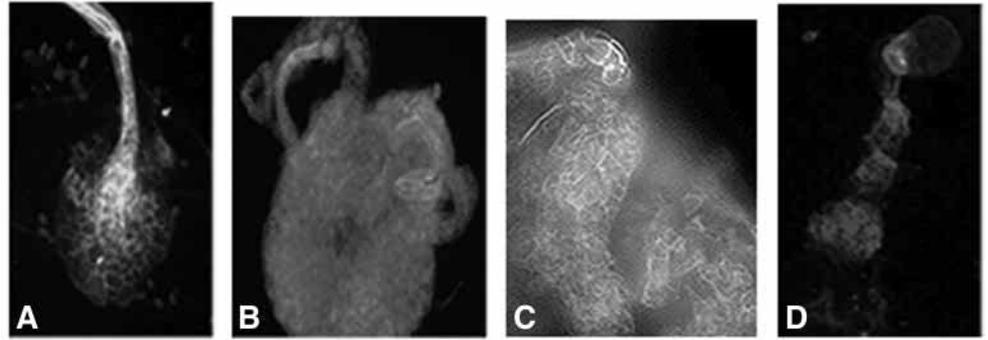
However, *ecmA* expression was markedly elevated during the development of the *psA::rasG(G12T)* cells (Fig. 2F), relative to the level in the wild-type strain (Fig. 2E). Unlike the expression pattern in the wild type cells (Fig. 2E), there was no decrease in *ecmA* mRNA late in the development of the transformant (Fig. 2F). In contrast, the level of *cotC* in the transformant was dramatically reduced (Fig. 2H), relative to that in the wild type (Fig. 2G), and the level in the transformant rapidly decreased as development progressed, such that transcript was barely detectable by 20 hours post-starvation (Fig. 2H). These results clearly demonstrated that prestalk cell gene expression was greatly enhanced, while prespore cell gene expression was significantly reduced in developing *psA::rasG(G12T)* cells, a result very similar to that obtained earlier with the *Dd-ras-Thr12* transformant (Louis et al., 1997a).

Analysis of stalk and spore cell formation by the *rasG(G12T)* transformants

As the terminal structures formed by the *psA::rasG(G12T)* transformants possessed very few sori (Fig. 1E), it was not surprising that less than 1% of the initial amoebae differentiated to form spore cells (data not shown). Calcofluor staining followed by fluorescence microscopy, revealed that the stalk-like structures contained stained cells (Fig. 3B) that were similar in appearance to wild-type stalk cells (Fig. 3A). The entire basal mound of the *psA::rasG(G12T)* culminant also exhibited marked calcofluor fluorescence and, although individual fluorescent cells were difficult to discern (Fig. 3B), stalk-like cells were visible when the structures were gently squeezed under a coverslip (Fig. 3C). These results indicate that the majority of the *psA::rasG(G12T)* transformant cells had differentiated into stalk cells.

Some of the culminants formed by the *ecmA::rasG(G12T)* transformants possessed sorus-like structures, but less than 1% of the initial amoebae differentiated to form spore cells (data not shown). In contrast, calcofluor-stained stalk-like cells were

Fig. 3. Calcofluor staining of stalk cells in terminally developed structures. (A–D) Culminants of wild-type Ax2 cells (A), a *psA::rasG(G12T)* transformant (B,C) and an *ecmA::rasG(G12T)* transformant (D) were stained with calcofluor and viewed by fluorescent microscopy to identify cellulose-lined stalk cell walls. The structure shown in C was gently squeezed under a coverslip, before photography.



observed everywhere in the terminal culminants, with the exception of the sorus-like structures, but stalk tubes were not observed (Fig. 3D).

Given their marked differences in gene expression and developmental morphology, it is perhaps surprising that both transformants form stalk cells and that neither form spore cells. In order to better understand the effects of activated Ras expression on stalk and spore cell formation, the spatial localization of the prestalk and prespore cell populations at various stages during differentiation was examined.

Prestalk and prespore cell localization in developing *psA::rasG(G12T)* transformants

To determine the localization of prestalk cells in the *psA::rasG(G12T)* transformant, we generated a *psA::rasG(G12T)/ecmA::lacZ* cotransformant and induced it to differentiate. At the multi-tipped mound stage, X-gal staining was found predominantly in the tips of the finger-like projections (Fig. 4A), a pattern reminiscent of that seen for the wild type, where intense *ecmA-lacZ* expression is observed in the single tip of each mound or finger (Early et al., 1993; Early et al., 1995). As development progressed, however, X-gal staining was observed in a gradually increasing number of cells and by 24 hours almost the entire structure had stained blue (Fig. 4B). These observations clearly indicated that the majority of *psA::rasG(G12T)* cells had adopted the prestalk cell fate during development.

In order to detect prespore cell localization and to detect any possible decrease in the proportion of prespore cells, a *psA-his-gal* construct encoding a labile β -galactosidase with a half-life of approximately 3 hours was used (Detterbeck et al., 1994) and *psA::rasG(G12T)/psA-his-gal* cotransformants were generated. These transformants expressed β -galactosidase in the mounds, but not in the multiple fingers (Fig. 4C), but as development progressed, there was a gradual decrease in staining (data not shown), a result consistent with the decrease in prespore specific mRNA (Fig. 2). These results indicate that most of the mound cells of the *psA::rasG(G12T)* transformants initially assume a prespore fate, but subsequently lose this specification and transdifferentiate to prestalk cells.

β -galactosidase expression from the *ST-lacZ* construct was also examined, to confirm that the *psA::rasG(G12T)* transformant had formed stalk-like structures. In the wild type, the *ST* region of the *ecmB* promoter, drives expression in cells as they enter the stalk tube (Ceccarelli et al., 1991). In a *psA::rasG(G12T)/ST::lacZ* co-transformant, staining was initially detected in scattered cells within the basal mound, but

not in the finger-like projections (Fig. 4D). However, as development progressed, the latter cells expressed the *ST-lacZ* marker (Fig. 4E) and the pattern resembled that of a developing stalk tube. These results are consistent with the interpretation that the protruding stalk-like structures of the *psA::rasG(G12T)* transformants are mainly composed of terminally differentiated stalk cells. β -galactosidase staining resulting from the presence of the *ST-lacZ* construct in Ax-2 is shown for comparison (Fig. 4F,G).

Spore and stalk cell formation by *psA::rasG(G12T)* in chimaeric mixtures with Ax2

Wild-type cells were mixed with the *psA::rasG(G12T)* transformant and the mixture allowed to develop, to determine if the wild-type cells in the chimaeric mixture would induce the transformant to form spores. Spore formation was totally dependant on the number of Ax2 cells in the mixture and the germinated spores all exhibited a wild-type developmental phenotype (data not shown). These results demonstrate that wild type cells are unable to induce the *psA::rasG-G12T* transformant to form spores, indicating that the defect in spore formation in the transformant is cell autonomous.

In order to determine whether the *psA::rasG(G12T)* transformant contributed to the prestalk and stalk cell populations of the chimeric mixtures, *psA::rasG-G12T* cells containing the *act15-lacZ* construct were mixed with unlabelled Ax2 cells in a 1:9 ratio and induced to develop. The *psA::rasG-G12T* cells clearly participated in development, sorting to the prestalk regions of the chimaeric slug (Fig. 5A). During culmination, the *psA::rasG-G12T* cells were present in the basal disc, the stalk, and the upper and lower cups (Fig. 5B). When wild-type cells expressing the *act15::lacZ* construct were mixed with *psA::rasG(G12T)* cells in a 1:9 ratio and induced to develop, the wild-type cells progressed along the normal developmental pathway, leaving behind a mound of *psA::rasG(G12T)* cells (data not shown).

Prestalk and prespore localization in developing *ecmA::rasG* transformants

In order to determine the PstA prestalk cell localization in the *ecmA::rasG* cells, an *ecmA::rasG(G12T)/ecmA::lacZ* cotransformant was generated and induced to differentiate. When mounds of this cotransformant were stained and viewed from above, β -galactosidase-expressing cells were restricted to the periphery and the centre of the mound (Fig. 6A), a pattern similar to that observed previously for the wild-type strain (Early et al., 1995). However, the centrally localised stained cells did not

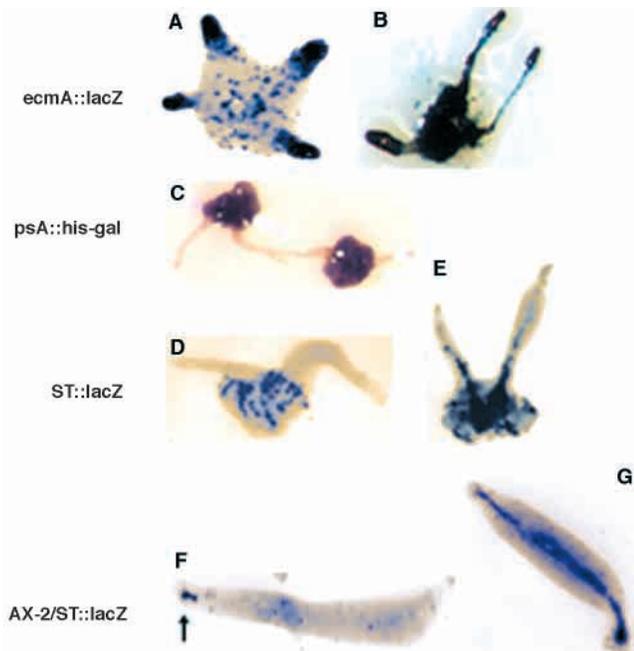


Fig. 4. Prestalk and prespore localization in *psA::rasG(G12T)* transformants. (A-G) Multiple finger mounds (A,C,D), a slug (F) and culminating stages (B,E,G) of development of a *psA::rasG(G12T)/ecmA::lacZ* cotransformant (A,B), a *psA::rasG(G12T)/psA::his-gal* cotransformant (C), a *psA::rasG(G12T)/ST::lacZ* cotransformant (D,E) and a wild-type/*ST::lacZ* transformant (F,G) were stained to detect β -galactosidase activity. The arrow in F indicates the inner cone of *ST::lacZ*-positive cells in the anterior.

migrate to the tip and the anterior cells of the slug did not stain (Fig. 6B). The vast majority of stained cells were in the posterior of the slug (Fig. 6B) and some stained cells were shed during migration (data not shown). This localization is quite different from wild-type slugs where PstA cells are restricted to the tip (Early et al., 1993). In the terminal culminants, staining was observed in all cells except those in the sorus-like structures (Fig. 6C).

In slugs formed from cells that had been cotransformed with *ecmA::rasG(G12T)* and *ecmO::lacZ* to determine the localization of PstO cells, β -galactosidase activity was very low and limited to a few scattered cells (data not shown). There was no sign of the characteristic collar of PstO cells, which is observed in the anterior region of wild-type slugs (Early et al., 1993; Early et al.,

Fig. 6. Prestalk and prespore cell localization in developing *ecmA::rasG(G12T)* transformants. (A-J) Aggregate (A,D), slug (B,E,G,I) and culminating (C,F,H,J) stages of development of an *ecmA::rasG(G12T)/ecmA::lacZ* cotransformant (A,B,C), an *ecmA::rasG(G12T)/ecmB::lacZ* cotransformant (D,E,F), an *ecmA::rasG(G12T)/ST::lacZ* cotransformant (G,H) and an *ecmA::rasG(G12T)/psA::lacZ* cotransformant (I,J) were stained to detect β -galactosidase activity. The arrows in B,E,G,I point to the tips of the slugs. The arrows in F point to the tips of two culminating structures.

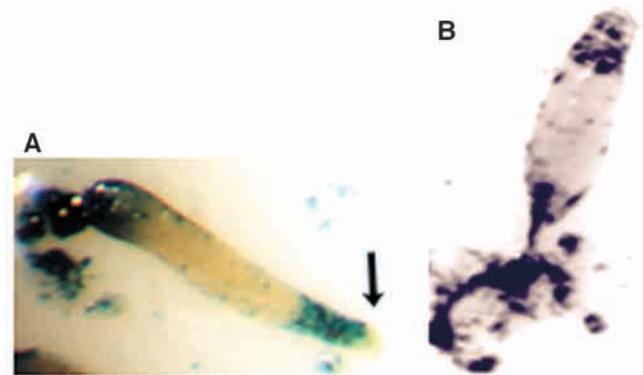
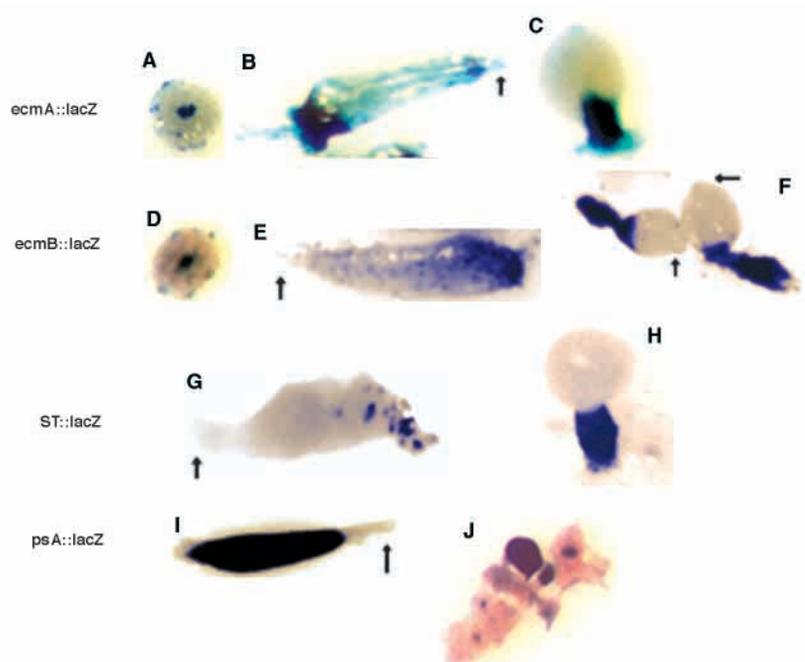


Fig. 5. The localization of *psA::rasG(G12T)* cells in developing structures formed from chimaeric mixtures with wild-type cells. (A,B) A mixture of 10% *psA::rasG(G12T)/actin15::lacZ* cotransformant cells and 90% Ax2 cells was developed until the slug (A) and culminating (B) stages and then stained to detect β -galactosidase activity. The arrow in A points to the tip of the chimaeric slug.

1995). Staining increased only slightly during culmination and was confined to the posterior of the structure (data not shown).

ecmA::rasG(G12T)/ecmB::lacZ cotransformants were generated to determine the localization of PstB cells. Staining was observed in cells at the periphery and at the centre of the mound (Fig. 6D), a pattern unlike that observed for PstB cells in the wild type (Jermyn et al., 1996). At the slug stage, PstB cells were predominantly in the rear (Fig. 6E). This is substantially different from the wild type, where PstB cells are not only found in the posterior rearward region but are also present as a group in the anterior prestalk region. PstB cells were also observed in the slime trail behind some of the transformant slugs (data not shown). As development progressed, the level of *ecmB* expression increased and staining was detected throughout the terminal culminants, with the exception of the sorus-like structures (Fig. 6F). This is again



different from the wild type, where PstB cells are confined to the stalk, the basal disc, and to the upper and lower cups (Ceccarelli et al., 1991; Jermyn and Williams, 1991; Jermyn et al., 1996).

In cells cotransformed with *ecmA::rasG(G12T)* and *ST-lacZ*, β -galactosidase staining was restricted to the posterior region of the slugs (Fig. 6G) and the onset of its expression was delayed relative to the wild type. Expression increased during culmination but remained restricted to the posterior regions of the organism (data not shown). The culminants expressed β -galactosidase in the irregular cell mass that supported the sorus-like structure (Fig. 6H). There was no indication of a developing stalk tube within the sorus-like structures of the transformant.

Prespore cell localization was determined by observing X-gal staining in a *ecmA::rasG(G12T)/psA::his-gal* cotransformant. Staining was observed only in the posterior region of the slug (Fig. 6I). In culminants, staining was reduced and only the sorus-like structures stained for β -galactosidase (Fig. 6J). The amorphous cell mass that supported the terminal structure was devoid of stain (Fig. 6J).

Spore and stalk formation in chimaeras of *ecmA::rasG(G12T)* and Ax2 cells

As prespore specific gene expression and prespore cell localization at the slug stage of development were relatively normal in the *ecmA::rasG(G12T)* transformants, the inhibition in spore formation might be a result of a defect during a late stage of development. When *ecmA::rasG(G12T)* cells and wild-type cells were mixed in a 3:1 ratio and induced to develop, spore formation was low (19% of the initial amoebae formed spores), but the majority of these spores exhibited the *ecmA::rasG(G12T)* phenotype (data not shown). Thus, the presence of wild-type cells was able to induce the transformant to form spores, indicating that the defect in spore formation is not cell autonomous. One possible explanation for the production of low numbers of wild-type spores in the chimaeric mixtures is that the wild-type cells are preferentially recruited to the prestalk region of the developing slug, and then go on to make stalk cells. To test this possibility, *ecmA::rasG(G12T)* cells were mixed in a 9:1 ratio with wild-type cells marked with the *act15-lacZ* construct. As shown in Fig. 7A, wild-type cells were preferentially localised to the slug tip. These results suggest that wild-type cells provide the stalk cells during chimeric development with the *ecmA::rasG(G12T)* transformant.

When unmarked wild-type cells were mixed in a 9:1 ratio with *ecmA::rasG(G12T)/actin15::lacZ* cotransformant cells, staining was localised in the rearguard region and throughout the prespore region of the slug (Fig. 7B). Very few stained cells were present in the anterior region, indicating that the tip of the chimaeric slug was formed by wild-type cells (Fig. 7B).

Phototaxis and motility of *ecmA::rasG(G12T)* slugs

It has been shown that the tip region is responsible for directing slug motility and phototaxis (reviewed by Fisher, 1997). Given that PstA cells did not occupy the tip of the *ecmA::rasG(G12T)* transformant slugs, we

examined the ability of the slugs to migrate towards light. After 2 days of exposure to unidirectional light, Ax2 slugs had migrated towards the point of light entry whereas *ecmA::rasG(G12T)* slugs had migrated in random directions for only very short distances (data not shown). These results indicate that the *ecmA::rasG(G12T)* slugs are not phototactic and exhibit reduced motility compared with wild-type slugs. When the *ecmA::rasG(G12T)* transformant was mixed in a 9:1 ratio with Ax2 cells, the chimeric slugs migrated towards the light (data not shown). Thus, the provision of normal tip cells by the wild type, as indicated in Fig. 7A, resulted in slugs that were now phototactic.

DISCUSSION

The fact that activated *rasG* and *rasD* generated very similar phenotypic defects when expressed during development was not surprising as the two proteins share 82% identity and *rasD* can rescue most of the phenotypic defects of *rasG*-null cells (Khosla et al., 2000). These results are consistent with the idea that RasG and RasD perform similar functions in vivo. We have further defined the defects caused by activated *ras* expression during development by restricting activated *rasG* expression to either prestalk cells or prespore cells. When *rasG(G12T)* was expressed specifically in prestalk cells, the aggregates generated single tips and exhibited only minor alterations in cell type-specific gene expression. When RasG(G12T) was specifically expressed in the prespore cell population, the aggregates were multi-tipped and marked alterations of cell type-specific gene expression were observed. These results indicate that the multi-tipped aggregates and the alterations in gene expression observed for the *RasD::rasD(G12T)* transformant (Reymond et al., 1986; Louis et al., 1997a) were largely a consequence of activated Ras expression in prespore cells, not prestalk cells.

The mechanism by which activated Ras dramatically inhibits prespore cell specific gene expression in prespore cells is not known, but the elevation of prestalk cell specific gene can most simply be explained by the transdifferentiation of the prespore cells into prestalk cells. Spore formation in monolayers in response to 8-Br-cAMP by the *psA::rasG-G12T* transformant is low (data not shown), indicating that the defect in spore formation cannot be rescued by activating the cAMP-dependant protein kinase. The formation of multi-tipped aggregates and the marked repression of prespore cell-specific

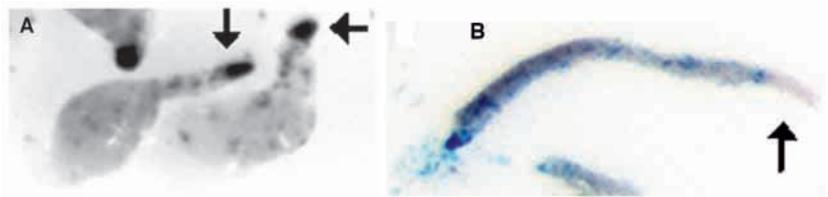


Fig. 7. Localization of wild-type and *ecmA::rasG(G12T)* transformants in chimaeric mixtures of the two organisms. (A,B) A mixture of 10% wild-type cells expressing *actin15::lacZ* and 90% *ecmA::rasG(G12T)* transformant cells (A), and a mixture of 10% *ecmA::rasG(G12T)/actin15::lacZ* cotransformant cells and 90% wild-type cells (B) were allowed to develop to the slug stage of development and then stained for β -galactosidase. The tips of the slugs are indicated by arrows.

gene expression that occurs when activated Ras is expressed in the prespore cell population could be consequences of interference with a single signal pathway. However, it is more likely that these phenotypes are due to the interference of Ras-G12T with two distinct signalling pathways. Rap1 is a Ras subfamily protein and the overexpression of the mammalian *rap1* gene can suppress the malignant phenotype of Ras transformed cells (Kitayama, et al., 1989). In *Dictyostelium*, the concurrent overexpression of *rasD(G12T)* and *rap1* did not affect multiple tip formation but did correct the defect in cell type-specific gene expression (Louis et al., 1997b). Thus, the effect of RasD(G12T) on cell type-specific gene expression patterns was antagonised by Rap, whereas the misregulation of tip formation was independent of Rap.

At the multi-tipped mound stage of development of the *psA::rasG(G12T)* transformant, the tip cells expressed prestalk-specific markers and the cells within the mound expressed prespore-specific markers. With time, however, prespore-specific gene expression in the mound decreased and prestalk-specific gene expression increased until eventually all the cells in the mound expressed prestalk cell-specific genes and these then went on to form stalk-like cells. Clearly prespore cells were produced initially and then, after expressing RasG(G12T), they transdifferentiated into prestalk cells. It is not known as yet whether RasG(G12T) simultaneously activates prestalk specific gene expression and inhibits prespore-specific gene expression, or whether one of these occurs first and then triggers the other. The inhibition of prespore cell gene expression and the inhibition of spore cell formation is similar to that observed in *gskA* null cells (Harwood et al., 1995), suggesting the possibility that the expression of RasG-G12T in prespore cells inhibits GSK-3 activity. However, we find that GSK-3 activity is not reduced in developing *psA::rasG(G12T)* transformants (M. K., unpublished observation).

As prestalk cells that do not express RasG(G12T) go on to make stalk cells, the defect in spore formation would appear to be cell autonomous. This has been confirmed by the finding that the *psA::rasG(G12T)* transformant fails to form spores when allowed to develop in chimeric mixtures with Ax2 cells. These experiments also revealed that the transformant is capable of contributing prestalk cells to the chimeric mixture, consistent with the finding that the transformant can form stalk cells.

Although the effects on spore and stalk cell formation seen in the *psA::rasG(G12T)* and *ecmA::rasG(G12T)* transformants were very similar, the mechanisms responsible are quite different. Overexpression of activated RasG from the *ecmA* promoter had no major effect on cell type specific gene expression (Fig. 2), but a number of dramatic changes in prestalk cell localization were induced. In developing *ecmA::rasG(G12T)* transformants, PstA cells were not found in their characteristic position in the tip of the slug but were localised at the rear. In addition, PstO cells were reduced in number and the few that were observed were also localised in the posterior of the slug rather than in the anterior collar. Finally, the number of PstB cells was increased and these cells were found in both the anterior and posterior of the slug. Given this level of mislocalization, it is perhaps surprising that the transformant forms a migrating slug that, in outward appearance, is identical to the wild type. Upon culmination,

stalk-like cells were formed throughout the terminal structures, except in the pseudo-sori. There was, however, no sign of a stalk tube in any of the structures. The pseudo-sori appeared to contain prespore cells: clearly the mislocalization of the prestalk cell populations prevents prespore to spore cell conversion.

We propose that the major effect of RasG(G12T) in these transformants is the mislocalization of PstA cells and that the remaining developmental aberrations are consequences of this mislocalization. Since the tip is responsible for directing slug migration (Fisher, 1997), orchestrating the movements necessary for culmination (Smith and Williams, 1980) and initiating formation of the stalk tube (Jermyn and Williams, 1991; Sternfeld, 1992), it is not surprising that the slugs of the *ecmA::rasG(G12T)* transformant exhibit defects in these properties. The prespore cells of the transformants do not form spore cells, implying a defect in a culminating signal that normally derives from the prestalk cell population and induces this conversion. Consistent with these proposals, chimaeric slugs, which contain a small percentage of wild-type cells and a large percentage of *ecmA::rasG(G12T)* cells, were capable of phototaxis and stalk tube construction, and some of the transformant cells were able to form spores. In the chimaeric slugs, the wild type provided the tip cells.

During wild-type development, the PstA cells arise at the periphery of the mound and then migrate to the tip (Early et al., 1995). It has been proposed that the sorting of PstA cells to the tip is the result of differential chemotaxis to cAMP (Early et al., 1995) and overexpression of RasG(G12T) might interfere with this progress. However, in the *ecmA::rasG(G12T)* transformants, PstA cells were observed initially in the centre of the mound, and therefore there does not appear to be a problem in PstA cell sorting. We propose that the presence of RasG(G12T) induces a more rapid conversion of PstA to PstAB cells and, as a consequence, the PstA cells do not migrate to the tip. Consistent with this idea, cells that express *ecmA* and *ecmB* co-localise in the aggregates and slugs of the *ecmA::rasG(G12T)* transformants. This could be due to a mixture of PstA and PstB cells, but as *ecmA*- and *ecmB*-expressing cells eventually migrate to the posterior of the slug, it is more likely that they are all PstAB cells. The unregulated formation of PstAB cells would also account for the absence of normal culmination and the absence of a stalk tube in the developing *ecmA::rasG(G12T)* transformants. During normal development, the conversion of PstA cells to PstB cells in the tip is an important regulatory step in culmination and is regulated by the STATA protein (Mohanty et al., 1999). It is possible that the presence of activated RasG in prestalk cells interferes with the inhibition of *ecmB* expression by STATA.

The results of *rasG(G12T)* expression in the *ecmA::rasG(G12T)* transformant help explain some of the properties of the *psA::rasG(G12T)* transformant. In *psA::rasG(G12T)* transformants, two types of prestalk cells are produced. The first is the population of the prestalk cells that initially form the multiple tips and hence do not express RasG(G12T). As these cells are in essence wild type, they are able to organise the later developmental processes. As a result, the tips extend to form finger-like and slug-like structures that even attempt migration. These tip cells are also able to initiate the synthesis of a stalk tube. The second population of prestalk

cells arise by transdifferentiation from prespore cells and, as these cells have already expressed and still contain RasG(G12T), they are defective as organisers of the morphogenetic movements necessary for culmination. They differentiate into PstAB cells and ultimately form the stalk cells that remain in the mound of the terminal structures. The results of *rasG(G12T)* expression in the *ecmA::rasG(G12T)* transformant also explains the localisation of *psA::rasG(G12T)* cells in the rear and in the region directly behind the tip of chimeric slugs (Fig. 5). Although these cells are initially prespore, we hypothesise that they transdifferentiate into PstA but are unable to occupy the tip of the slug because of their rapid conversion to PstAB cells and many of them then migrate to the rear.

The results described here go a long way towards explaining the phenotype of the previously described *RasD::rasD(G12T)* transformant. However, a notable difference between the developmental phenotypes of the *RasD::rasD(G12T)* and *psA::rasG(G12T)* transformants is that the former did not proceed beyond the multi-tipped aggregate stage, whereas the tips of the latter extended to form rudimentary stalks. The absence of stalk tube formation in the *RasD::rasD(G12T)* transformant is probably due to the expression of activated Ras in the prestalk cell population, as the initially determined prestalk cells of the *psA::rasG(G12T)* transformant did not express activated *ras*. The finding that *ecmA::rasG(G12T)/psA::rasG(G12T)* cotransformants arrest at the multi-tipped aggregate stage of development is consistent with the idea that this phenotype is the result of simultaneous expression of activated Ras in both prestalk cells and prespore cells.

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