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A Ras GAP is essential for cytokinesis and spatial patterning in *Dictyostelium*

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SUMMARY

Using the yeast two-hybrid system, we have identified developmentally regulated *Dictyostelium* genes whose encoded proteins interact with Ras·GTP but not Ras·GDP. By sequence homology and biochemical function, one of these genes encodes a Ras GAP (*DdRasGAP1*). Cells carrying a *DdRasGAP1* gene disruption (*ddrasgap1* null cells) have multiple, very distinct growth and developmental defects as elucidated by examining the phenotypes of *ddrasgap1* null strains. First, vegetative *ddrasgap1* null cells are very large and highly multinucleate cells when grown in suspension, indicating a severe defect in cytokinesis. When suspension-grown cells are plated in growth medium on plastic where they attach and can move, the cells rapidly become mono- and dinucleate by traction-mediated cell fission and continue to grow vegetatively with a number of nuclei (1-2) per cell, similar to wild-type cells. The multinucleate phenotype, combined with results indicating that constitutive expression of activated Ras does not yield highly multinucleate cells and data on Ras null mutants, suggest that Ras may need to cycle between GTP- and GDP-bound states for proper cytokinesis. After starvation, the large null cells undergo rapid fission when they start to move at the onset of aggregation, producing mononucleate cells that form a normal aggregate. Second, *ddrasgap1* null cells also have multiple developmental phenotypes that indicate an essential role of DdRasGAP1 in controlling cell patterning. Multicellular development is normal through the mid-slug stage, after which morphological differentiation is very abnormal and no culminant is formed: no stalk cells and very few spores are detected. *lac*Z reporter studies show that by the mid-finger stage, much of the normal cell-type patterning is lost, indicating that proper DdRasGAP1 function and possibly normal Ras activity are necessary to maintain spatial organization and for induction of prestalk to stalk and prespore to spore cell differentiation. The inability of *ddrasgap1* null cells to initiate terminal differentiation and form stalk cells is consistent with a model in which Ras functions as a mediator of inhibitory signals in cell-type differentiation at this stage. Third, DdRasGAP1 and cAMP dependent protein kinase (PKA) interact to control spatial organization within the organism. Overexpression of the PKA catalytic subunit in *ddrasgap1* cells yields terminal structures that are multiply branched but lack spores. This suggests that RasGAP and PKA may mediate common pathways that regulate apical tip differentiation and organizer function, which in turn control spatial organization during multicellular development. It also suggests that DdRasGAP1 either lies downstream from PKA in the prespore to spore pathway or in a parallel pathway that is also essential for spore differentiation. Our results indicate that DdRasGAP1 plays an essential role in controlling multiple, potentially novel pathways regulating growth and differentiation in *Dictyostelium* and suggest a role for Ras in these processes.

Key words: *Dictyostelium discoideum*, signal transduction, Ras, GAP, cytokinesis

INTRODUCTION

Ras proteins are members of the small GTP-binding/GTPase family that function as molecular switches controlling a variety of signaling pathways in eukaryotes (Bourne, 1995). In metazoans, Ras is involved in regulating cell growth and differentiation and is known to directly interact with the Raf serine/threonine kinase, leading to the activation of MAP kinases and downstream transcription factors, and with phosphatidylinositol-3-OH kinase (Marshall, 1995; McCormick, 1994; Rodriguez-Viciana et al., 1994; Vojtek et al., 1993; White et al., 1995). In the fission yeast *Schizosaccharomyces pombe*, Ras is essential for pheromone-mediated sexual responses that include the activation of a MAP kinase cascade (Chang et al., 1994; Wang et al., 1991). In *S. pombe* Ras1 interacts with and is required for the activation of the MEK kinase homolog byr2, which may be directly analogous to the mechanism by which Ras activates MAP kinases in metazoans (Chang et al., 1994). In the budding yeast *Saccharomyces cerevisiae*, Ras functions to regulate the adenylyl cyclase CYC1, which is required for cell growth (Wigler et al., 1988). Interaction of Ras with and activation of downstream effectors take place through an evolutionarily-conserved effector loop and require Ras to be in the GTP-bound form.

At least two other proteins are required for the Ras GDP/GTP cycle: Ras GEF and Ras GAP/NF-1 GTPase activating proteins (Boguski and McCormick, 1993; Marshall, 1995; McCormick, 1994). Ras GEFs (guanine nucleotide exchange factors) mediate the exchange of the bound GDP for

GTP and are regulated by upstream signaling pathways. Ras proteins, unlike most tested Gα subunits of heterotrimeric G proteins, have an exceptionally low, intrinsic GTPase activity. In the presence of Ras GAP, however, the rate of Ras·GTP hydrolysis is significantly enhanced. Ras GAPs interact with Ras through the same conserved effector domain by which Ras interacts with other effectors. As with other Ras effectors, interaction of Ras with GAP requires Ras to be in the GTP-bound form.

Five developmentally regulated Ras genes, encoding five distinct Ras proteins, have been identified in *Dictyostelium* (Daniel et al., 1993, 1994; Pawson et al., 1985; Reymond et al., 1984). The genes have overlapping but different patterns of expression. The five *Dictyostelium* Ras proteins show the same high level of amino acid sequence identity in the N-terminal half of the protein and are unique in their C-terminal half, except for the conserved CAAX terminus. *RasD* has been most intensely studied. It is expressed at low levels during growth and then is induced to high levels at the time of mound formation in response to extracellular cAMP (Reymond et al., 1984; Reymond and Thompson, 1991). This induction is mediated through cAMP serpentine receptors and requires the transcription factor GBF (Schnitzler et al., 1994, 1995). During the multicellular stages, *RasD* is preferentially, but not exclusively, expressed in prestalk and anterior-like cells (Esch and Firtel, 1991; Jermyn and Williams, 1995). When an activated form of RasD [RasD(G12T), a mutation equivalent to Ras(G12V)] is expressed from the cloned *RasD* promoter, the cells aggregate, form mounds with multiple tips, and then arrest in development (Reymond et al., 1986). These cells have a reduced number of cAMP receptors, show an altered adaptation of cAMP-stimulated guanylyl cyclase (which is activated in response to cAMP), and have an altered metabolism of phosphoinositols (Europe-Finner et al., 1988; Van Haastert et al., 1987; Wood et al., 1991). In addition, expression of an activated form of the RasG protein [RasG(G12T)] significantly impairs aggregation (Thiery et al., 1992; Khosla et al., 1996). Recently, a putative Ras GEF has been identified by insertional mutagenesis (Insall et al., 1996). Null mutants of this gene cannot aggregate, show reduced levels of cAMP-mediated activation of adenylyl cyclase, and are unable to properly undergo chemotaxis toward an exogenous source of cAMP.

A significant amount is understood about the receptor-mediated signaling pathways that control aggregation and cell-type differentiation in *Dictyostelium* (Devreotes, 1994; Drayer and van Haastert, 1994; Firtel, 1995; Williams and Morrison, 1994). Although the spatial patterning of the individual cell types found in the multicellular stages and the differential cell sorting required to produce these patterns have been described, little is known about the gene products required to maintain this pattern and induce culmination. We used the yeast two-hybrid system to identify potential Ras-interacting proteins (Chien et al., 1991; Fields and Song, 1989; Gyuris et al., 1993) which, together with Ras, would play an important role in mediating Ras function. Three genes were identified. One encodes a putative Ras GAP, DdRasGAP1, which we have shown has Ras GAP activity in vitro. Through the analysis of *ddrasgap1* null strains, we have shown that DdRasGAP1, and by inference Ras, is required for multiple distinct pathways during growth and multicellular development. We show that DdRasGAP1 is essential for proper cytokinesis as *ddrasgap1* null cells are extremely large and highly multinucleate during growth. Combined with other data, we suggest that Ras is essential for cytokinesis and that Ras may need to cycle between GTP- and GDP-bound states for proper regulation of cell division. DdRasGAP1 is also required for proper morphogenesis in the multicellular stages. *ddrasgap1* null cells show no observable defect in early development but display abnormal morphogenesis that includes the mislocalization of prestalk and prespore cells during the later multicellular stages, the absence of stalk cell differentiation, and the formation of very few mature spores. Expression of PKA in *ddrasgap1* null prespore cells does not bypass the inability to produce spores but results in large, multi-branched structures, indicating a role for Ras and PKA in controlling morphogenesis, apical tip differentiation, and organizer function. Our results indicate that DdRasGAP1, and presumably Ras, play distinct and important roles in controlling growth, morphogenesis, and spatial patterning.

MATERIALS AND METHODS

Molecular and cell biology methods

Cell culture, molecular biology, transformation of *Dictyostelium* cells, Southern and RNA blot analysis, and *lac*Z reporter analysis have all been described previously (Howard et al., 1988; Mann and Firtel, 1987; Mann et al., 1994a). Molecular cloning approaches are standard as described by Sambrook et al. (1989).

Screening of the yeast two-hybrid system

The yeast two-hybrid system developed in the laboratory of Roger Brent was used and screened as described by Gyuris et al. (1993). A directional cDNA library was made from poly(A)$^+$ RNA isolated from developing cells at 8-16 hours of development cloned into the 'fish' expression plasmid JG4-5. Approximately 1.5×10^6 independent library clones were obtained. The size of the inserts averaged 0.5-1.0 kb with some inserts exceeding 3-4 kb. Approximately 25% of the inserts were >1 kb.

GAP activation of Ras GTPase activity

GST-RasD (a *Dictyostelium ras* gene) (Reymond et al., 1984) and GST-DdrasGAP1 proteins were expressed in *E. coli* on GST-expression vectors (Hakes and Dixon, 1992) and purified by glutathione agarose (Sigma) affinity chromatography. *E. coli* cells were lysed in lysis buffer A (20 mM Hepes, pH 7.3, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT) containing 1 mM PMSF, 40 μg/ml aprotinin, and 1 mg/ml lysozyme. After leaving on ice for 10 minutes, Triton X-100 was added to 1%, the cells were briefly sonicated and centrifuged at 12000 rpm for 10 minutes, and the supernatant was saved. GST beads were washed twice in PBS and added to the supernatant and shaken gently for 1 hour at 4°C. The beads were gently pelleted and washed 4 times with lysis buffer containing 150 mM NaCl final concentration, 1 mM PMSF, and 0.1% Triton X-100 (buffer B). The bound proteins were eluted in buffer B containing 10 mM glutathione and then dialyzed against lysis buffer.

GAP activity was measured according to the procedure of Bollag and McCormick (1995). GST-Ras was diluted 100-fold into a buffer containing 0.1 μM [γ-^{32}P]GTP (6000 Ci/mmole), 1 mM EDTA, 20 mM Hepes, pH 7.3, and 2 mM DTT. After incubation for 5 minutes at 25°C, this was added to 20 mM Hepes, pH 7.3, 2 mM $MgCl_2$, and either 2 mM DTT and GAP was added or the Ras·GTP was allowed to incubate alone. Released $^{32}PO_4$ was quantitated by the method of Sung et al. (1995).

Isolation of the *DdRasGAP1* promoter

The *DdRasGAP1* gene was also identified by REMI insertional mutagenesis (Kuspa and Loomis, 1992) as a mutant that showed abnormal morphogenesis (Escalante and Loomis, unpublished observation). The region flanking the insertion vector was cloned as described previously (Kuspa and Loomis, 1992), mapped, and partially sequenced. An ORF was identified upstream of the *DdRasGAP1* gene in the cloned DNA (Escalante and Loomis, unpublished observation).

RESULTS

Isolation of genes encoding putative Ras-interacting proteins

We used the set of vectors for the yeast two-hybrid screen developed in the laboratory of Roger Brent (Gyuris et al., 1993) to identify *Dictyostelium* gene products that interact with Ras. For the Ras bait, we used the G12V constitutively activated mammalian Ras ORF successfully used by Vojtek et al. (1993) to identify the Raf kinase as a Ras-interacting protein since the effector domain of mammalian Ras proteins and three of the *Dictyostelium* Ras proteins, including RasD, is conserved. Moreover, use of a mammalian Ras gene for the screen might identify proteins that could interact with multiple *Dictyostelium* Ras proteins and identify a function of Ras that is held in common between *Dictyostelium* and other organisms. This gene was inserted into the pEG202 bait plasmid and a directional, oligo dT-primed cDNA library was constructed in the 'fish' expression plasmid JG4-5 using RNA isolated from *Dictyostelium* cells, which had been developed for 8-16 hours. The library was then screened according to published procedures (Gyuris et al., 1993). Sixteen positive clones were obtained after primary and secondary screening of 1.5×10^6 initial yeast transformants. All 16 clones gave a strong response in the *lac*Z reporter assay. Partial sequencing and restriction mapping of the inserts showed that they were derived from three different genes designated RIP (Ras-Interacting Protein) 1-3. One of these genes, *RIP2*, is examined here.

Known Ras effectors preferentially interact with the GTP-bound form of Ras. To examine if RIP2 specifically interacts with the GTP-bound form of Ras, we used the Ras(G15A) mutation (Vojtek et al., 1993), which should be in the GDP-bound form when assayed in the yeast two-hybrid system. When the RIP2 yeast two-hybrid plasmid was assayed with Ras(G15A) using the *lac*Z reporter, the yeast colonies did not turn blue when plated on X-gal-containing agar. Under the same screening conditions, cells co-expressing RIP2 with Ras(G12T) turned dark blue.

The inserts from the RIP2 yeast clones were then used to screen a λZap cDNA library made from RNA isolated from cells of late aggregate through the slug stage (8-16 hours) (Schnitzler et al., 1994). cDNAs containing the complete ORF were obtained (see below). Fig. 1 shows a map of the isolated clones.

RIP2 encodes a Ras GAP

Sequence analysis of *RIP2* cDNAs identified a single ORF of 841 amino acids (Fig. 2). A search of available protein data bases using the Blast program identified two Ras GAPs, human IQ GAP1 (Weissbach et al., 1994), and the *S. pombe* GAP *sar1* (Imai et al., 1991; Wang et al., 1991) as having exceptionally high scores (smallest sum probabilities of $3.0e^{-85}$ and $3.2e^{-71}$, respectively), suggesting *RIP2* encodes a Ras GAP. We have designated this gene *DdRasGAP1*. Other proteins, including Ras GAPs from *S. cerevisiae* and other vertebrate Ras GAPs, had lower scores. No homology to known Rho and Rac GAPs was identified in these searches. Homology of the *DdRasGAP1* ORF with human IQ GAP1 and *S. pombe sar1* ORFs extends beyond the previously defined GAP catalytic domain, which lies in the center of the ORF (Fig. 2). The domain that was sufficient to strongly interact with Ras in the two-hybrid screen (as determined by the smallest clone, clone D34, from the two-hybrid screen, see Fig. 2 for region of the ORF expressed in that two-hybrid clone) is at the C terminus of the *DdRasGAP1* ORF, outside the putative GAP catalytic domain.

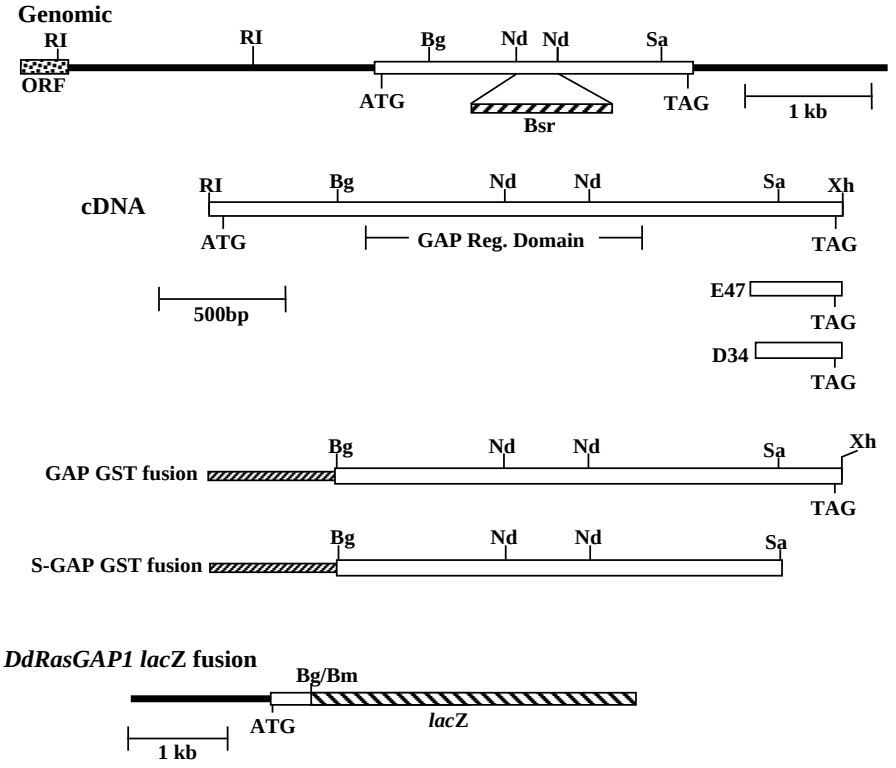

Fig. 1. Maps of *DdRasGAP1* clones. Restriction maps and diagrams of the *DdRasGAP1* genomic DNA, cDNA and other clones are shown. E47 and D34 are the inserts from the two yeast two-hybrid library. For the disruption construct, the *Bsr* gene was inserted between the *Nde*I site and the DNA digested with *Bgl*II and *Sal*I before transformation into KAx-3 wild-type cells. The *Eco*RI and *Xho*I sites on the cDNA clone are from the polylinker insertion points in the λZap library (Schnitzler et al., 1994). RI, *Eco*RI; Bg, *Bgl*II; Nd, *Nde*I; Sa, *Sal*I; Xh, *Xho*I.

To biochemically determine if *DdRasGAP1* encodes a Ras GAP, we expressed the *Dictyostelium* DdRasGAP1 and the *Dictyostelium* RasD as GST fusion proteins in *E. coli*. Two DdRasGAP1 GST fusion proteins were expressed. GST-GAP contains the entire putative GAP catalytic domain and the C terminus of the protein, whereas the GST-S-GAP contains the entire putative GAP catalytic domain, but lacks most of the C-terminal region that was part of the ORF that interacted with Ras in the two-hybrid screen. The recombinant proteins were purified by glutathione affinity chromatography and the ability of DdRasGAP1 to stimulate RasD GTPase activity was assayed (see Materials and Methods). SDS-PAGE analysis of the purified fusion proteins shows that the GST-Ras was homogeneous and of the expected size (Fig. 3A). The predominant bands of the GST-S-GAP1 and GST-GAP1 were also of the expected size. However, the purified fractions also contained some smaller molecular mass proteins, especially in the GST-S-GAP preparation. Both recombinant GAPs significantly stimulated the *Dictyostelium* RasD GTPase activity, consistent with results from the analysis of the sequence homology (Fig. 3B). This suggests that *DdRasGAP1* probably functions as a Ras GAP in vivo, although we cannot exclude that it may also function in another capacity. The assay with approx. 2 μM GST-GAP had approx. 5-fold higher GTPase stimulating activity than 0.2 μM protein. When basal activity is subtracted, the difference in stimulation is approx. 10-fold. The GST-S-GAP had an apparent lower activity than the longer GST-GAP, which could be due in part to the presence of a visible amount of smaller proteins that would affect the calculation of protein concentration and/or due to an intrinsically lower activity of the deleted gene product. The finding that the shorter form of DdRasGAP1 has Ras GTPase activity indicates that the C-terminal region, found to interact with Ras in the two-hybrid assay, is not essential for the GAP activity. We assume that the catalytic domain must also interact with Ras.

Temporal and spatial expression of *DdRasGAP1*

The *DdRasGAP1* cDNA was used as a probe for developmental RNA blots. Two transcripts of 3.2 and 3.6 kb were observed (Fig. 4A). The larger transcript was most abundant during growth and early development, while the smaller transcript was expressed at the highest level during the multicellular stages starting at 8 hours. Both transcripts are absent in the *DdrasGAP1* knockout strain (see below), which is consistent with both RNAs being *DdRasGAP1* transcripts.

A 1.2 kb 5′ flanking region of the *DdRasGAP1* gene was cloned and fused in-frame in a *lac*Z reporter vector (see Materials and Methods, Fig. 1). Stable transformants carrying the *DdrasGAP1/lac*Z reporter were allowed to develop and histochemically stained for β-gal expression. Staining of clonal isolates during the multicellular stages is seen in cells scattered throughout the migrating slug (Fig. 4B) and present in the basal disc, upper and lower cups, and to a lesser degree in the stalk

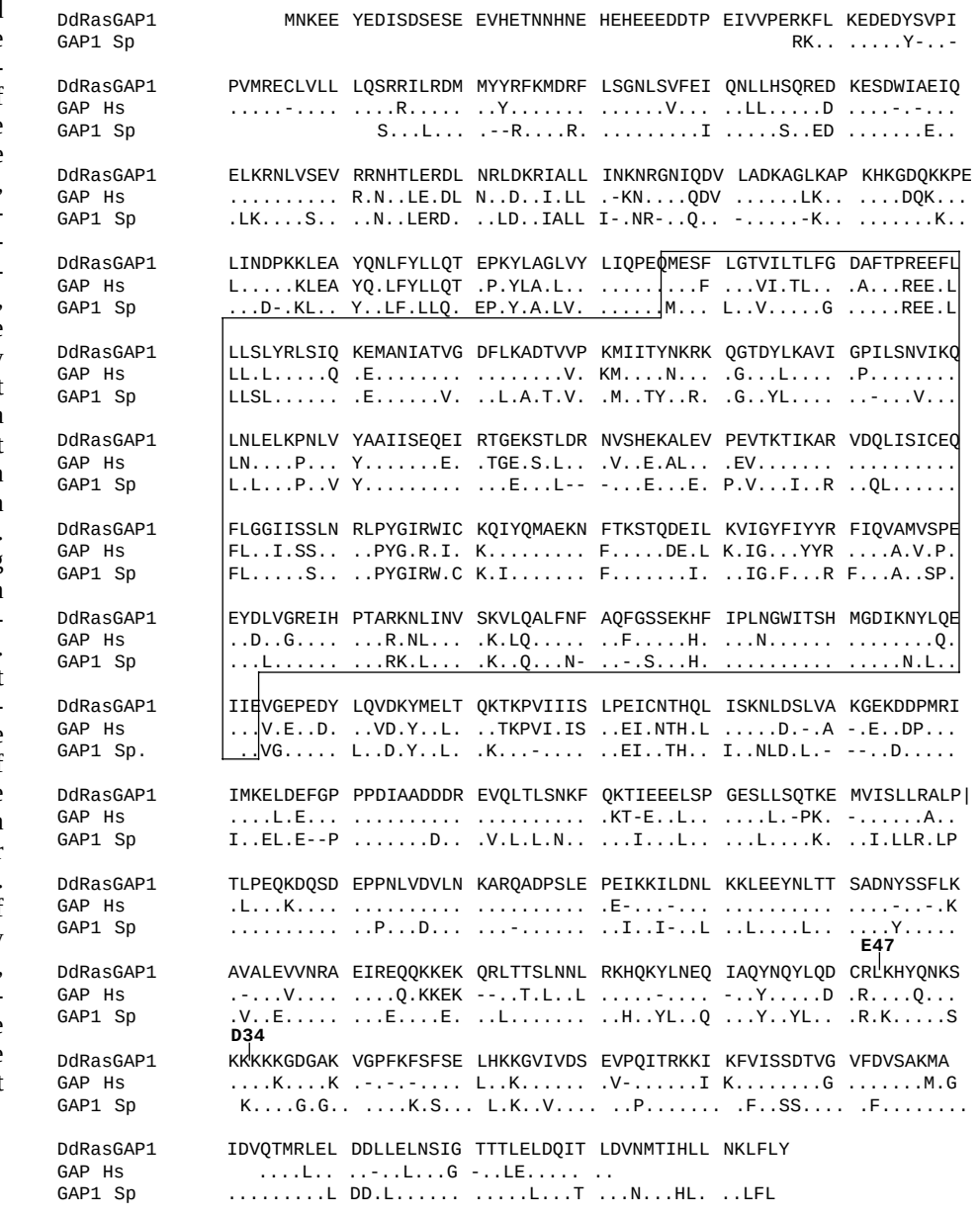

Fig. 2. Sequence comparison of DdRasGAP1 with other Ras GAP proteins. Homology is shown between DdRasGAP1, *S. pombe sar1*, and the human IQ GAP1 (see text). The boxed region is the GAP homology domain as defined by Weissbach et al. (1994). Sequence identities are shown. The N-terminal position of the two clones identified in the two hybrid screen (E47 and D34) are shown. The portion of the DdRasGAP1 ORF encoded in the yeast two-hybrid clone extends C-terminal from the position indicated. GenBank accession no. is U81156.

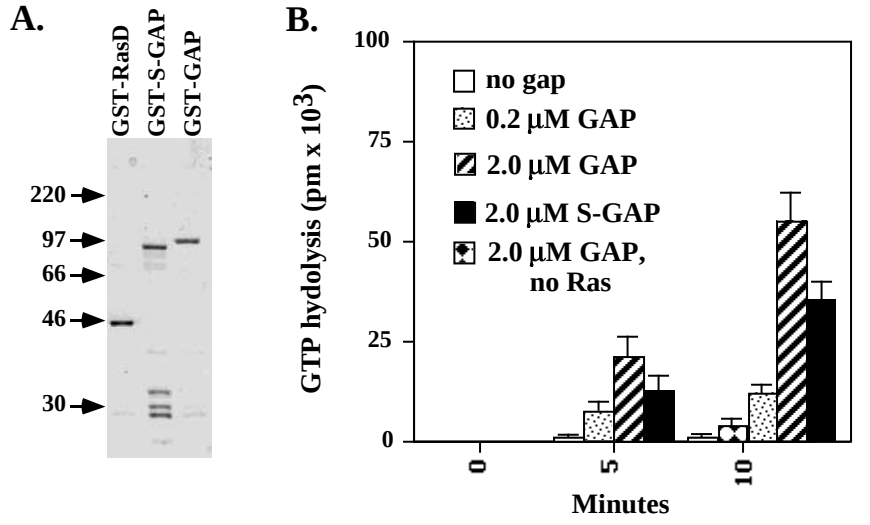

Fig. 3. Ras GAP assay. (A) SDS-PAGE gel of affinity purified GST-RasD, GST-GAP, and GST-S-GAP. See Materials and Methods for details on purification. (B) Ras GAP assay. Hydrolysis of [γ-^{32}P]GTP is measured by quantitating released $^{32}PO_4$. Duplicate samples were assayed and the assays repeated. The data presented is a combination of the results from these experiments. See Materials and Methods for specifics of the assay.

(data not shown), a pattern suggestive of anterior-like cells (Hadwiger et al., 1994; Howard et al., 1992; Jermyn and Williams, 1991). During vegetative growth in cells of clonal isolates grown on plastic, staining is observed in approx. 10-15% of the cells. Although the cells are from isolated clones, this staining pattern may indicate intrinsic differences in the expression pattern of cells within the clone. It is also suggestive of, but does not prove, a cell-cycle-regulated pattern. Interestingly, the level of expression of *RasD* has been shown to change during the cell cycle in cells synchronized by arrest at S phase (Esch, 1991).

ddrasgap1 null cells have defects in cytokinesis and in multicellular development

A *DdRasGAP1* gene disruption construct carrying resistance to blasticidin (Bs^r) (Sutoh, 1993; Fig. 1) was transformed into wild-type cells. Stable Bs^r clones were selected randomly, examined for disruption of *DdRasGAP1* by Southern blot analysis, and plated on non-nutrient agar to observe development. Two out of ten randomly picked Bs^r transformants showed a disruption of the endogenous *DdRasGAP1* gene and carried a single copy of the disruption construct as determined by Southern blot analysis (data not shown). During vegetative growth, the *ddrasgap1* null strain produced cells that appeared to clump in suspension culture. A microscopic analysis showed that these 'clumps' were extremely large cells surrounded by a halo of smaller cells (Fig. 5). Staining with the nuclear fluorescent dye Hoechst 33258 showed that the very large cells were highly multinucleate, whereas the smaller cells surrounding the large cell contained a significantly smaller number of nuclei (Fig. 5B). This phenotype suggests that the *ddrasgap1* null cells have a cytokinesis defect. Wild-type cells are predominantly mono- or dinucleate (Fig. 5C,D). The pattern of the smaller cells surrounding the very large *ddrasgap1* null cell suggests that the small cells derive from the large cell, possibly by 'budding' (see below and Fig. 5A,B). When the cells are grown on plastic Petri dishes or coverslips, they grow as predominantly mono- and dinucleate cells, presumably because, when attached to a substratum, the cells are able to pull apart by amoeboid movement (traction-mediated cytofission; Fukui et al., 1990; Fig. 5E,F).

To examine the effect of switching cells from growth in suspension to growth on Petri dishes, we used time lapse video microscopy. *ddrasgap1* null 'log-phase' cells grown in suspension were plated on plastic Petri dishes in standard HL5 axenic growth medium and changes in the cells were continuously recorded under phase contrast microscopy. Within 10 minutes of adhering to the plates, very large and medium sized cells started to divide into smaller sized cells with the smaller sized cells 'pulling' off from the sides of the larger cells (data not shown). In addition, the very large cells underwent periodic contractions during which they rounded up. They then reflattened and at this point a number of smaller cells simultaneously broke off from the peripheries of the larger cells. For very large cells, this process repeated itself several times. Smaller sized cells often split into two 'sister' cells. During this process, one could clearly observe the two sister cells pulling apart from each other by traction-mediated cytofission. All the cells had completed the process of 'dividing' into mono/dinu-

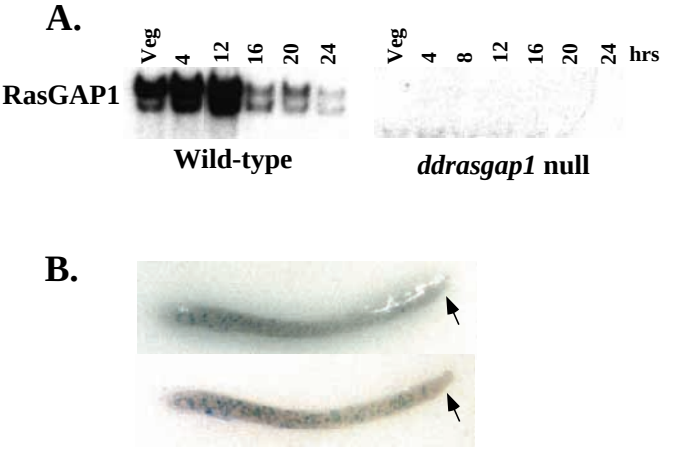

Fig. 4. Temporal and spatial expression of *DdRasGAP1*.
(A) Developmental RNA blot of wild-type and *ddrasgap1* null cells probed with *DdRasGAP1* cDNA (labelled 'GAP1').
(B) *DdRasGAP1/lacZ* expression at the slug stage in wild-type organisms. An arrow points to the anterior of the slug.

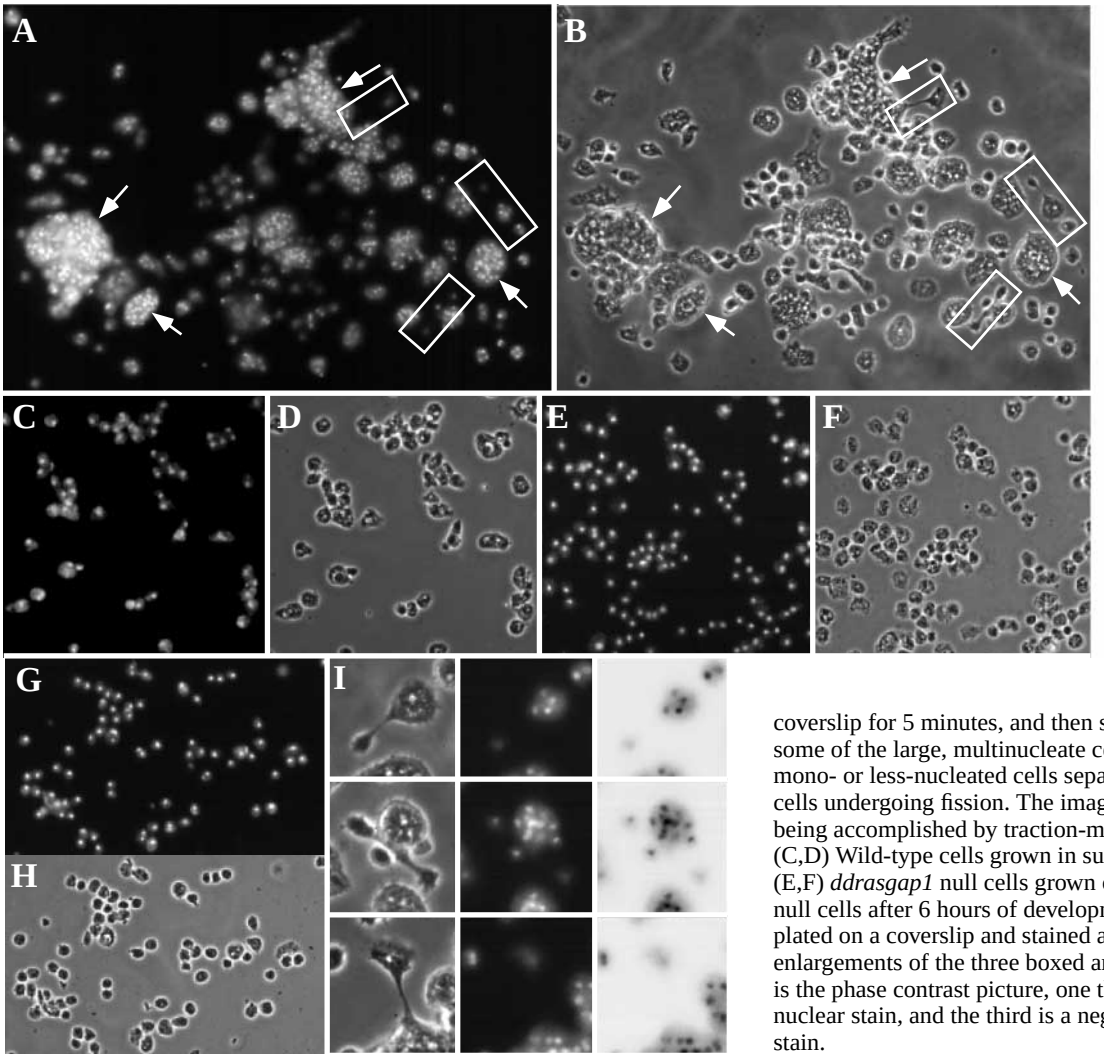

Fig. 5. Growth and morphological defects of *ddrasgap1* null cells. Morphology of vegetative cells. Alternate panels showing cells stained with the nuclear fluorescent dye Hoechst 33258, and phase contrast images. (A,B) *ddrasgap1* null cells grown in suspension, adhered to a coverslip for 5 minutes, and then stained. Arrows point to some of the large, multinucleate cells. Boxed areas show mono- or less-nucleated cells separating from large cells or cells undergoing fission. The images suggests that this is being accomplished by traction-mediated fission (see I). (C,D) Wild-type cells grown in suspension, treated as above. (E,F) *ddrasgap1* null cells grown on plastic. (G,H) *ddrasgap1* null cells after 6 hours of development on non-nutrient agar, plated on a coverslip and stained as above. (I) Three sets of enlargements of the three boxed areas in A and B. One panel is the phase contrast picture, one the positive image of the nuclear stain, and the third is a negative image of the nuclear stain.

cleate cells within 3 hours. On plates, these cells have a wild-type generation time of 8-10 hours.

To determine whether the cells grown in suspension remain multinucleate when starved as they enter development, cells were plated on non-nutrient agar and starved for either 4 hours, before they start aggregating, or 6 hours, after the cells have started to aggregate. They were then examined microscopically for the number of nuclei per cell by Hoechst staining. At the 4 hour time point, the cells were still multinucleate (data not shown). However, at the 6 hour time point, when the cells had initiated chemotaxis, they were mono- or dinucleate, similar to the wild-type cells (Fig. 5G,H).

When examined for multicellular development, both *ddrasgap1* null clones showed normal developmental timing and morphology through the formation of the first finger. As with wild-type cells, the *ddrasgap1* null cell finger elongates, falls to the substratum, and forms a slug-like structure; however, *ddrasgap1* null slugs do not migrate but remain 'tethered' at the posterior to the substratum (Fig. 6, compare A and B). The structure of the slugs is not smooth like wild-type slugs and they develop constrictions along their length with time. Many of the slugs form structures containing an elongated finger/thick stalk-like structure with a thickening in the middle and a small protrusion at the apex (Fig. 6C,D) and do not differentiate into fruiting bodies (see Fig. 6E for wild-type structure). A smaller fraction of the *ddrasgap1* null organisms form elongated second fingers that have a very rough-textured surface. This variability in morphology is independent of the clonal isolate and was observed from plating to plating. When the cells are plated on Millipore filters (the method used for *lac*Z expression studies), most of the organisms form elongated fingers, some of which have a small apical protrusion and a more posterior thickening (see below, Fig. 8B), although this structure is often not as symmetrical or the phenotype as consistent as that observed when the organism is developed on agar. However, many of the organisms form terminal structures that are very rough-textured, twisted fingers with a small apical protrusion.

When assayed, the mature *ddrasgap1* null structures formed very few mature spores (0.4% that of wild-type cells, data not shown). When the *ddrasgap1* null cells were mixed with wild-type cells at a ratio of 1:3 (*ddrasgap1*:wild-type cells), they formed coaggregates (see below). In these chimeras, *ddrasgap1* null cells formed spores at a level of 1±0.5% of wild-type cells when corrected for the fraction of *ddrasgap1* null cells in the mixture (data not shown). This level is statis-

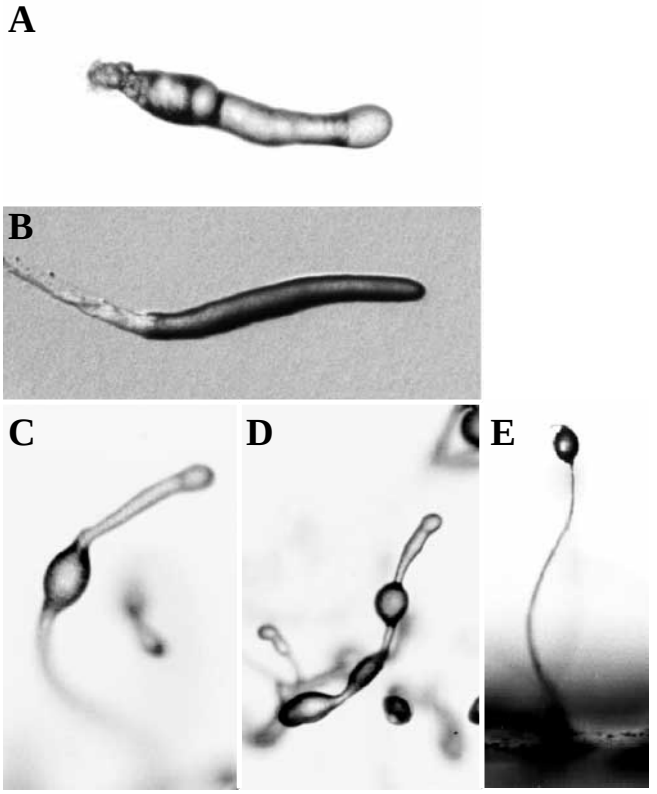

Fig. 6. Developmental morphology of *ddrasgap1* null cells plated on non-nutrient agar. (A) *ddrsasgap1* null slug (16 hours of development); (B) wild-type slug (16 hours of development); (C,D) final structures *ddrsasgap1* null (26 hours of development); (E) wild-type fruiting body (26 hours of development).

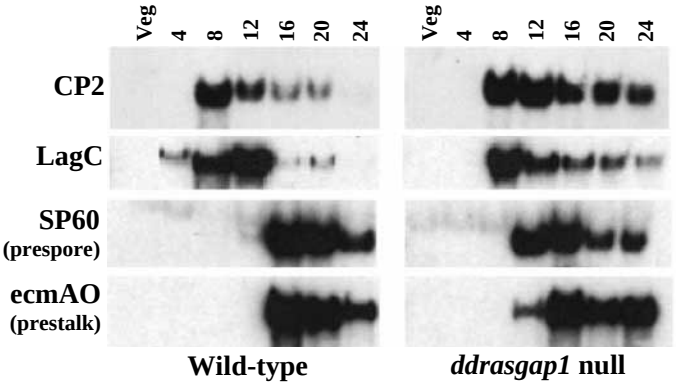

Fig. 7. Developmental RNA blot analysis of postaggregative gene expression in wild-type and *ddrasgap1* null cells. *CP2* and *LagC* are postaggregative genes that are induced by high cAMP at the time of mound formation and lie downstream from the transcription factor GBF, *SP60* is prespore-specific, and *ecmA* is prestalk-specific (Firtel, 1995; Williams and Morrison, 1994). Cells aggregate by 8 hours, tipped aggregates form by 12 hours, and culmination initiates at 18-20 hours. The numbering above the lanes corresponds to the developmental stage.

tically higher than the level of *ddrasgap1* null spores when this strain was plated alone.

Expression of the *DdRasGAP1* cDNA downstream from the *Act15* or *DdRasGAP1* promoters used for the *lac*Z expression studies in *ddrasgap1* null cells complemented the morphological phenotype and the ability to form mature spores (data not shown). At least a 10-fold overexpression of the mRNA from either the *DdRasGAP1* expression vector or the *Act15* promoter in wild-type or *ddrasgap1* null cells showed no abnormal morphology (data not shown).

The timing and level of expression of post-aggregative genes that are induced as the mound forms and prestalk and prespore cell-type-specific genes were assayed by RNA blot hybridization. The analysis shown in Fig. 7 demonstrates that all genes assayed were expressed. The postaggregative gene *CP2*, which is induced at the time of mound formation in response to the transcription factor GBF and rising levels of cAMP, showed a significantly more extended pattern of expression than seen in wild-type cells. The expression pattern of *LagC*, another postaggregative gene, was only slightly more extended, as was that of the prestalk-specific gene *ecmAO*. *ecmAO* and the prespore gene *SP60* showed a slightly precocious timing of expression.

ddrasgap1 null cells show altered cell-type spatial patterning and are blocked in culmination

To examine the spatial patterning of cell types within the *ddrasgap1* null organisms, *ddrasgap1* null cells were transformed with *lac*Z reporter constructs expressed from cell-type-specific promoters and clones were isolated. Cells were developed and then histochemically stained for β-gal activity. As the *ddrasgap1* null finger first forms, prestalk A/O cells, as defined by the expression of the *ecmAO* promoter (Early et al., 1993; Williams and Morrison, 1994), are localized to the anterior of the fingers (Fig. 8Ba), as they are in wild-type fingers (Fig. 8Ab). With time, the pattern changes: fewer *ecmAO/lac*Z-staining cells are observed (Fig. 8Bb) and these are scattered throughout the organism rather than being preferentially localized to the anterior region. In terminal structures containing the anterior protrusion, staining is observed throughout the posterior (Fig. 8Bc; for comparison, a wild-type fruiting body expressing *ecmAO/lac*Z is shown in Fig. 8Ad). In some structures, scattered staining is also seen in the anterior bulbous region (data not shown). Similar staining is observed with the *ecmB* promoter that is expressed in anterior-like cells and another subclass of prestalk cells, prestalk B and AB cells (data not shown). No staining was observed in any part of the organism using the stalk-specific promoter *ecmBΔ89* (Jermyn and Williams, 1991; Williams and Morrison, 1994; data not shown). These findings suggest that stalk-cell differentiation is not induced. Analysis of the structures showed no vacuolated stalk cells. When the structures were stained with calcofluor, which stains cellulose in the stalk and mature spores, only a few staining cells were detected, all of which appeared to be spores (data not shown).

Expression of the prespore-specific reporter *SP60/lac*Z (Haberstroh and Firtel, 1990) was also abnormal. Stained cells were restricted to the posterior 50% of *ddrasgap1* null slugs (Fig. 8Bd), which is distinct from expression seen in wild-type cells in which the posterior approx. 80% of the slug was stained (Fig. 8Aa). As the fingers develop into elongated, twisted slugs, stained cells are found scattered along the entire length of the structure (Fig. 8Be), in contrast to the posteriorly localized staining found in wild-type strains. In final structures that have

an apical protrusion, a concentrated area of stained cells is found in a thickening in the lower third of the structure (Fig. 8Af; for comparison, a wild-type fruiting body expressing *SP60/lac*Z is shown in Fig. 8Ac). Stained cells are also found scattered throughout the *ddrasgap1* null organism, including the apical protrusion. However, only some of the cells in this region stain with *SP60/lac*Z, and no cells within this region stain with the *SpiA/lac*Z spore-specific reporter (see below; Richardson et al., 1994), indicating the apical protrusion does not represent a sorus. The *SpiA* promoter drives *lac*Z expression in cells localized to the thickening described above with no other staining observed. Interestingly, this thickening appears to have at least two lobes as seen in Fig. 8Bg,h. Although *SpiA* expression is induced, very few of these cells form mature spores (see above).

Many mutant strains will form co-aggregates or chimeras with wild-type strains when the cells are mixed. By examining the position of wild-type or mutant cells expressing cell-type markers within the chimera, one can ask questions about cell autonomy of a specific pathway as well as directly examine the effect of wild-type cells on the localization of *ddrasgap1* null cells within aggregates. When *ddrasgap1* null cells were mixed with wild-type cells in a 1:3 ratio and allowed to develop, chimeric fruiting bodies were formed with a sorus that was more elongated and had a bulbous apical protrusion (Fig. 8Ca-c). *ddrasgap1* null cells expressing *SP60/lac*Z were found restricted to the posterior third of chimeric slugs (Fig. 8Cd) rather than throughout the posterior approx. 80% as is seen with wild-type cells (Fig. 8Aa). In culminants, *SP60/lac*Z and *SpiA/lac*Z staining was observed within the sorus with a few staining cells

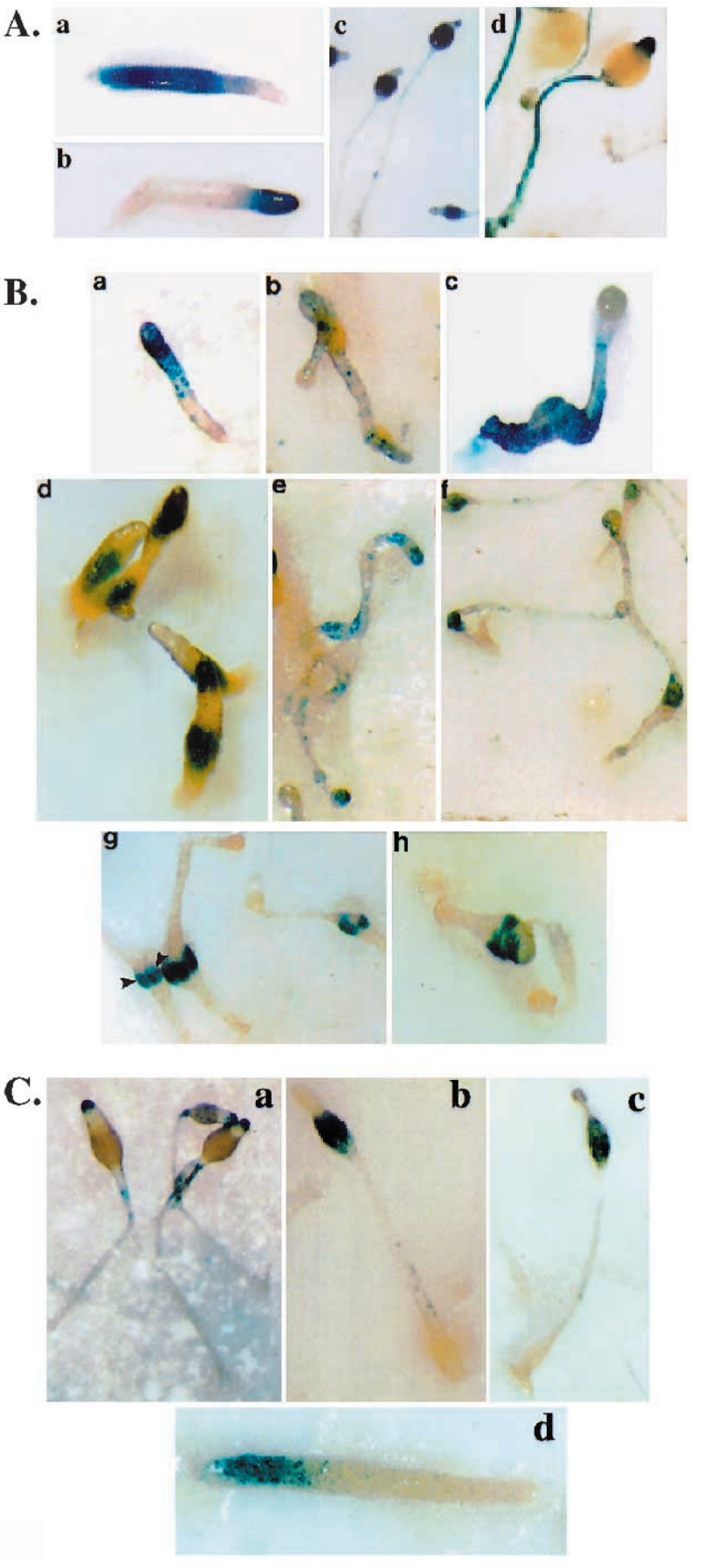

Fig. 8. *lac*Z expression studies of *DdRasGAP1*. (A) Expression of the prespore-specific reporter *SP60/lac*Z (a,c) and the prestalk-specific reporter *ecmAO/lac*Z (b,d) in wild-type cells in migrating slugs (a,b; 16 hours of development) and mature fruiting bodies (c,d; 26 hours of development). (B) All photographs are of *ddrasgap1* null cells expressing cell-type-specific *lac*Z constructs. *ecmA* is prestalk-specific; *SP60* is prespore-specific; *SpiA* is spore-specific. (a) *ecmA*, 14 hours; (b) *ecmAO/lac*Z, 18 hours; (c) *ecmAO/lac*Z, 26 hours; (d) *SP60/lac*Z, 14 hours; (e) *SP60/lac*Z, 18 hours; (f) *SP60/lac*Z, 26 hours; (g,h) *SpiA/lac*Z, 26 hours. In Bg, arrowheads point to the two 'lobes' of the stained region. (C) *lac*Z expression studies of *DdRasGAP1*. All photographs are of chimeras containing 3 parts wild-type cells and 1 part *ddrasgap1* null cells expressing cell-type-specific *lac*Z constructs. Histochemical staining of *lac*Z expression localizes *ddrasgap1* null cells expressing the cell-type-specific marker. (a) Mature fruiting body (26 hours), *ecmAO/lac*Z; (b) mature fruiting body, *SP60/lac*Z; (c) mature fruiting body, *SpiA*; (d) migrating slug, *SP60/lac*Z (16 hours). Cells are at the slug stage.

scattered throughout the stalk structure (Fig. 8Cb,c). *ecmAO/lac*Z-expressing *ddrasgap1* null cells showed normal localization to the anterior prestalk A/O region of the chimeras (data not shown). In fruiting bodies, some staining was observed in the very apex and a band of cells localized to just below the lower cup (Fig. 8Ca). However, little staining was found in the stalk region, consistent with the inability of these cells to form stalk cells. When the *ddrasgap1:ecmBΔ89/lac*Z cells were examined in chimeras, no staining was observed (data not shown), also consistent with the inability of these cells to form stalk cells.

Interaction of RasGAP1 and PKA controlled signaling pathways leading to multiple tips and branched fruiting bodies: possible regulation of the anterior 'oscillator' by RasGAP1 and PKA

Multicellular development in *Dictyostelium* is regulated by an anterior 'organizer' that functions to control morphogenesis (Siegert and Weijer, 1992, 1995). The organizer is a cAMP oscillator that initiates waves of extracellular cAMP that move downward (posteriorly) from the tip. PKA is known to play essential roles in regulating aggregation, induction of cell-type differentiation, and culmination, in which it is thought to be the inductive signal for both the stalk and spore pathways (Simon et al., 1989, 1992; Mann and Firtel, 1991, 1993; Harwood et al., 1992a,b; Hopper et al., 1993; Mann et al., 1994b; Firtel, 1995). *pkacat* null cells are aggregation-deficient due to a lack of adenylyl cyclase and other components essential for aggregation (Mann and Firtel, 1991; Schulkes and Schaap, 1995; S. Mann et al., 1997). Overexpression in wild-type cells of the PKA catalytic subunit (PKAcat), specifically in prespore cells from the *SP60* prespore promoter, leads to very precocious induction of prespore to spore differentiation that initiates within 1 hour of prespore cell determination (Kay, 1989; Mann et al., 1994b). Such strains produce a mound composed mostly of spore cells and an elongated finger that develops into a stalk lacking a sorus. When these cells are developed on filters, the finger extended from the mound produces a slug-like structure in which the differentiating prespore cells immediately develop into spores.

To determine if overexpression of PKA could bypass the block to reduced spore formation in *ddrasgap1* null cells and the possible interaction of PKA and the pathway regulated by DdRasGAP1, we expressed PKAcat from the *SP60* promoter in *ddrasgap1* null cells. At the mound stage (Fig. 9F), up to five independent tips arise from a single mound in contrast to a single tip in wild-type cells, and the organism often develops into a multibranched fruiting body with each branch topped by a sorus-like structure. In some cases, a tree-like structure with multiple branches arises from a single stalk-like structure (Fig. 9B,D,H), in some cases, a two- or multibranched structure gives rise to further branched structures (Fig. 9C,E), and in others multiple single structures arise from a single mound (Fig. 9I). Fig. 9A and G show additional structures. Such structures were never observed for either *ddrasgap1* strains or PKAcat overexpressors strains previously described, nor have they ever been seen in our laboratory for any strain (unpub. obser.). The results suggest a strong interaction between pathways controlled by RasGAP and PKA (see Discussion).

As referenced above, overexpression of PKAcat in prespore cells or the addition of the membrane permeable cAMP analog, 8-Br-cAMP, results in the rapid activation of spore cell differentiation. We therefore assayed the ability of *ddrasgap1/SP60:PKAcat* cells to form mature spores. Although many of the branched structures have an apical bulb that appears to be a sorus, the level of mature spores that were produced was indistinguishable from that of *ddrasgap1* null cells and no spores could be detected in these structures (data not shown). Moreover, we stained the cells with calcofluor, a stain for cellulose, a component of the stalk cell walls and spore coats (see above). Only a few scattered cells were stained, which appeared to represent the few mature spores when examined under the microscope. When the structures were stained with calcofluor, there was no difference between the *ddrasgap1* null and the *ddrasgap1*:SP60/PKAcat cells (data not shown).

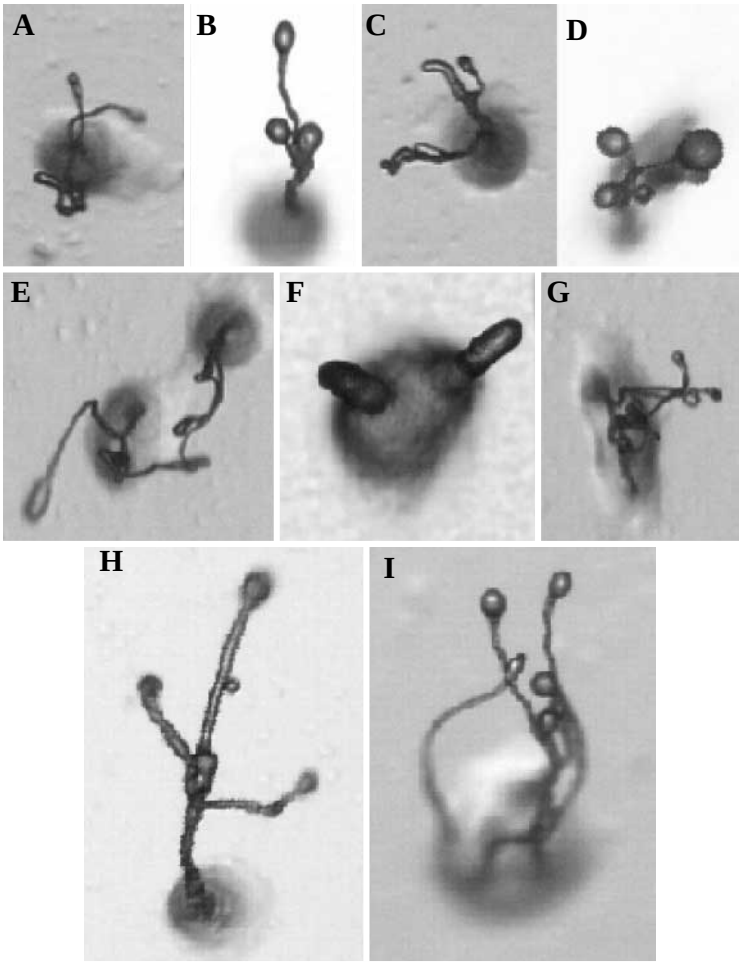

Fig. 9. Phenotype of *ddrasgap1* null cells expressing PKA from the *SP60* prespore promoter. Images were taken at 26 hours except F, which was taken at 12 hours. B,D and H show clear branching of the aerial structure. F shows two tips arising from a single mound. A,C,E,G and I show multiple structures arising from a single mound.

DISCUSSION

Using two-hybrid screening, we have identified genes that preferentially interact with Ras in the GTP-bound state. Because the proteins interact with Ras(G12V) but not Ras(G15A) in the two-hybrid system, we assume that the proteins interact with Ras·GTP but not Ras·GDP. One of these, *DdRasGAP1*, encodes a Ras GAP as defined by the ability of recombinant DdRasGAP1 protein to stimulate Ras GTPase activity *in vitro*. The protein has strongest homology to a human IQGAP and the *S. pombe sar1* gene, which encodes a Ras GAP. DdRasGAP1 does not show homology to known Rho or Rac GAPs, suggesting that it probably does not mediate the GTPase activity of these small G proteins. Because *DdRasGAP1* appears to encode a Ras GAP, a protein whose function is to modulate Ras GTPase activity, we infer that the phenotypes we observe in *ddrasgap1* null cells may be the result of an increase in the level of Ras·GTP or the inability of the cells to cycle Ras between the GTP- and GDP-bound forms (see below) However, we cannot exclude that some or all of the phenotypes exhibited by the *ddrasgap1* null cells result from other, presently not identified, biochemical properties of the DdRasGAP1 protein.

Our analysis of *ddrasgap1* null cells has shown that aspects of *Dictyostelium* growth and development are altered in *ddrasgap1* null cells. Because of differences in the observed phenotypes, we expect that DdRasGAP1, and thus possibly Ras, regulates distinct pathways at different stages during growth and development, including cytokinesis, spatial patterning, and stalk and spore formation.

DdRasGAP1 is essential for cytokinesis: possible requirement of Ras cycling for cell division

Vegetatively growing *ddrasgap1* null cells are very large and multinucleate when grown in suspension, suggesting that karyokinesis is normal and that *ddrasgap1* null cells have a defect in cytokinesis. Reported *Dictyostelium* mutants known to produce multinucleate vegetatively growing cells include myosin II null or antisense mutants (de Lozanne, 1987; Knecht and Loomis, 1987), the null mutation of a novel Rac protein (RacE) (Larochelle et al., 1996), and mutants of a member of the TAT-binding protein family (Cao and Firtel, 1995), some members of which are known components of 26S proteosomes (see also below). The phenotype of the *ddrasgap1* null cells suggests that Ras protein may be involved in mediating cytokinesis, possibly by controlling aspects of cleavage plane formation. This suggestion is supported by the findings of Insall and collaborators (R. Insall, personal communication) that disruption of the *Dictyostelium RasG* gene also results in a cytokinesis defect. If DdRasGAP1 functions as a RasGAP in vivo in controlling cytokinesis, we expect that *ddrasgap1* null cells would have higher levels of Ras·GTP than wild-type cells or would be unable to down-regulate Ras function by converting Ras to the GDP-bound form. If these assumptions are correct, then the findings of Insall and collaborators that the lack of RasG expression results in a cytokinesis defect, combined with our observations, suggest that Ras may need to cycle between GTP- and GDP-bound forms to mediate a particular process, as do small GTPases involved in protein sorting. This latter hypothesis is supported by observations that vegetative cells overexpressing RasG(Q61L), a growth-stage-specific Ras protein, result in only a slight increase (1.7-fold) in the number of nuclei per cell (Khosla et al., 1996; G. Weeks, personal commununication), while overexpression of RasD(Q61L), which is expressed during both growth and development, shows no cytokinesis phenotypes (S. L. and R. A. F., unpublished observation). In wild-type cells expressing an activated Ras, the endogenous Ras proteins would be free to cycle normally, which suggests that having Ras in the GTP-bound state does not, in itself, lead to a cytokinesis defect. This model is distinct from known Ras function in metazoans in which Ras·GTP acts as an activator of MAP kinase pathways and activated Ras and RasGAP mutations have similar phenotypes. Our results do not exclude the possibility that DdRasGAP1 directly regulates cytokinesis by interacting with a component of this pathway in addition to Ras.

When the cells are grown on plastic, they are predominantly mono- or dinucleate. We expect that the cells are able to divide by pulling themselves apart, as myosin II null cells do when grown on plastic (de Lozanne, 1987). Moreover, when *ddrasgap1* null cells are plated for development, they remain multinucleate until aggregation, when they become mono- and dinucleate. This also appears to occur by cells pulling themselves apart (traction-mediated cell fission) in the early stages of aggregation when they start to move. The phenotype of the myosin II null cells is significantly more severe than the *ddrasgap1* null cells, since myosin II null cells are still multinucleate on plastic and die in suspension. The growth phenotype is distinct from that of mutants of the TAT-binding protein homolog (Cao and Firtel, 1995), which behave differently under the same conditions. Our data are consistent with DdRasGAP1 being required for proper cleavage plan formation.

The *DdRasGAP1* probe identifies two transcripts that are lacking in null cells and thus both transcripts are presumably derived from *DdRasGAP1*. Both transcripts are present at moderate levels during growth, at maximal levels from aggregation through the tipped aggregate stage, and at significantly lower levels later in development when morphological abnormalities are first detected in *ddrasgap1* null cells. A morphologically distinguishable phenotype in *ddrasgap1* null cells was not detected until the time when the level of *DdRasGAP1* transcripts starts to decline in wild-type cells. We expect that DdRasGAP1 protein is still at high levels in cells at the slug stage. We expect that RasGAP is required throughout this period, as *ddrasgap1* null cells, expressing SP60/PKA, exhibit specific phenotypes from mound formation through later development. DdRasGAP1 may function at an earlier developmental stage to regulate Ras function but its activity may not be essential for development or there may be another RasGAP that is stage-specific.

DdRasGAP1 is essential for proper differentiation of the stalk pathway and spatial patterning of prespore and prestalk cells

DdRasGAP1 protein is essential for proper morphogenesis, culmination, mature stalk and spore formation, and proper spatial patterning of the cell types. *ddrasgap1* null cells showed aberrant morphogenesis starting after first finger formation. Since there are at least five genes encoding distinct Ras/Ras-related proteins in *Dictyostelium*, it is not clear which or how many of these may interact in vivo with DdRasGAP1, which

we have shown functions as a Ras GAP for RasD in vitro. *lac*Z reporter studies using the *ecmA*, *ecmB* and *SP60* cell-type-specific promoters suggest that the prestalk and prespore cells show a wild-type pattern of spatial localization at the onset of finger/slug formation, except for a possible more posterior localization of the prespore cells. However, cell-type patterning becomes quite abnormal with time. Unexpectedly, the prespore (*SP60*-expressing) and prestalk (*ecmA*-, and *ecmB*-expressing) cells are found scattered throughout the organism at the later stages. The stalk-specific marker *ecmBΔ89* was not expressed and no mature stalk cells were observed. These latter defects associated with stalk cell differentiation were cell autonomous and were not complemented in chimeras with wild-type cells. Sporulation appeared to be induced as evidenced by the localized group of cells that induce *SpiA* expression, but few mature spores were formed. This phenotype was minimally affected in chimeras.

At the present time, we have not identified the specific pathway that is being regulated by DdRasGAP1 during these later stages of development. Since the inability to maintain spatial localization of the cell types is associated with a DdRasGAP1 loss-of-function mutation, activated Ras may function as an inhibitor of these pathways. Presumably, disrupting *DdRasGAP1* resulted in an accumulation of activated (GTP-bound) Ras in those cells in which Ras is activated by upstream signaling pathways. This is distinct from the experimental scenario in which constitutively active Ras proteins are overexpressed from constitutive or developmentally regulated promoters. When the activated RasD(Q61L) is expressed from the cloned *RasD, ecmAO, ecmB, Act15*, or *SP60* promoters, the resulting morphological phenotypes are distinct from those observed for the *ddrasgap1* null cells (unpublished observation). In the case of overexpression of the RasD(Q61L) proteins, the activated Ras protein is expressed at high levels in all cells in which the promoter is active, independent of upstream activating signals. Our *ddrasgap1* null mutant may thus give a better insight into the role of Ras during later development.

The absolute requirement of DdRasGAP1 for stalk cell differentiation suggests that induction of prestalk to stalk may be a prerequisite for the formation of mature spores during culmination under normal developmental conditions, as has been previously suggested (Harwood et al., 1992a,b). The relatively small increase in the number of mature *ddrasgap1* null spores that are formed in chimeras with wild-type cells suggests that *ddrasgap1* null cells have a predominantly cell autonomous phenotype for spore formation. Rises in intracellular cAMP are thought to be essential for stalk cell differentiation (Harwood et al., 1992b; Williams et al., 1993). It is possible that, if DdRasGAP1 functions through Ras, Ras may be a negative regulator of this pathway and the absence of DdRasGAP1 function may lead to constitutive inhibition. Alternatively, many signaling pathways in *Dictyostelium* also have adaptation pathways and the inability to cycle Ras between GTP- and GDP-bound forms in the *ddrasgap1* null cells may lead to adaptation of downstream pathways (Devreotes, 1994; Drayer and van Haastert, 1994; Firtel, 1995). For example, expression of a constitutively active Gα2 subunit (the Gα subunit coupled to cAMP receptors during aggregation) results in a constitutive adaptation of the aggregation-stage pathways rather than a constitutive stimulation (Okaichi et al., 1992). The morphology of the final structure and the distribution of *ecmAO/lac*Z-tagged prestalk and *SP60/lac*Z-tagged prespore cells showed some degree of variability. This variability appears to be an intrinsic part of the *ddrasgap1* null phenotype and independent of which *ddrasgap1* null clones were examined. The analysis strongly suggests that DdRasGAP1 function is required for culmination and the proper maintenance of cell-type patterning in later stages of development. It appears to be essential for stalk cell differentiation. Lastly, we cannot exclude, but do not favor, a model in which Ras functions to activate prestalk and prespore pathways.

Many of the overall spatial patterning defects of the *ddrasgap1* null mutation are complemented in chimeras containing *ddrasgap1* null and wild-type cells. Prestalk and prespore cell types are properly localized and the overall morphology of the chimeras is similar to that of wild-type fruiting bodies with the exception that the sorus of the mature fruiting body is very elongated and has an apical extension. Using *lac*Z reporter constructs, we observed that while the *ddrasgap1* null cells are present in the prespore zone, they are predominantly found in the posterior of this region (see below). From these experiments alone, it is difficult to determine whether the wild-type cells are supplying a soluble morphogen not synthesized in *ddrasgap1* null cells and/or possibly a cell surface signaling molecule that is required to properly regulate the expression of genes controlling slug movement and culmination. It is also possible that *ddrasgap1* null cells may be unable to properly regulate morphogenetic movements or signaling pathways controlling morphogenetic movements. This regulation may be direct or indirect, possibly through other small G proteins such as Rac and Rho, which are known to regulate cell movement in metazoans (Nobes and Hall, 1995, 1996). Our data suggest that RasGAP-mediated pathways are required for maintaining the proper spatial patterning in the slug. The absence of DdRasGAP1 function may prevent cells from exhibiting the proper directional movement necessary to maintain proper patterning. For example, it is known that prestalk cells show a higher rate of cell movement than do prespore cells and their localization in the anterior may be a function of their preferential sorting to this region of the aggregate (Abe et al., 1994; Mee et al., 1986). In *ddrasgap1* null strains, differences in the relative mobility of prespore and prestalk cells may be minimized, resulting in more random movements and a shuffling of the cells within the organism. In chimeras, wild-type cells may provide the necessary cell types and/or signals that allow the *ddrasgap1* null cells to respond. Further insight into the specific function of Ras in the later stages of development should be obtained from the analysis of other proteins that interact with Ras.

Interaction of RasGAP and PKA, possible regulators of organizer formation

In *Dictyostelium*, the anterior or apical tip serves as an organizer to control the morphogenesis of the organism (Raper, 1940) by acting as a cAMP oscillator from which cAMP waves are initiated and propagated posteriorly (Siegert and Weijer, 1991, 1992, 1995; Steinbock et al., 1993; Bretschneider et al., 1995). Our observed phenotypes of PKA overexpression from a prespore promoter show a significant interaction between RasGAP- and PKA-regulated pathways. PKA in *Dictyostelium*, as in metazoans (Perrimon, 1995), regulates

multiple steps during development, including aggregation, cell-type induction and culmination (see above). During aggregation, PKA is required for the expression of adenylyl cyclase and other unidentified signaling components (S. Mann, J. Brown, C. Briscoe, C. Parent, G. Pitt, P. Devreotes, and R. A. F., unpublished data). As *pkacat* null cells or cells expressing a dominant negative regulator subunit do not aggregate (Harwood et al., 1992; Mann and Firtel, 1991; Schulkes and Schaap, 1995), it is difficult to directly evaluate the effect of RasGAP overexpression on morphogenesis during the multicellular stages in *pkacat* null cells. Overexpression of PKA inhibits the adaptation pathway controlling the down-regulation of the MAP kinase ERK2, which is activated by cAMP and required for the activation of adenylyl cyclase, prespore gene expression, and proper morphogenesis during later development (Gaskins et al., 1996; L. Aubry, M. Maeda, R. Insall, P. Devreotes, and R. A. F., unpublished data). During multicellular development, overexpression of PKA in prespore cells is sufficient to induce spore differentiation (see above). Here we have shown that overexpression of PKA via a prespore promoter in *ddrasgap1* null cells results in multiple branched structures. This occurs in the mound, thus being similar to the phenotype of overexpression of an activated RasD [RasD(G12T)] (Reymond et al., 1986), and during later development when multiple branched structures are seen. This differs from the phenotype of RasD(G12T) cells in that RasD(G12T) cell development arrests after tip formation. Multiple tips or branched structures are not observed in *ddrasgap1* null cells or in any other PKAcat overexpression strain that has been examined (see Results for references). After mound formation multiple tips are thought to arise from the initiation of multiple apical cAMP oscillators that act to organize the multicellular structure with each oscillator regulating a structure. Presumably the strength of the oscillator determines the number of cells that can be organized; that is, a weaker oscillator cannot organize and maintain a slug with a large number of cells. In wild-type organisms, the oscillator not only mediates spatial patterning but also suppresses the formation of new oscillators. Treatment of slugs with caffeine, known to inhibit adenylyl cyclase signaling (Brenner and Thoms, 1984), leads to the formation of adventitious tips along the slug that give rise to independent slugs (Siegert and Weijer, 1993). We imagine that the inhibition of the apical oscillator results in a reduction in the level of cAMP from the tip, thus allowing other tips to form.

RasD(G12T) cells have a reduced number of cAMP receptors during aggregation and an altered adaptation of guanylyl cyclase, which is coupled to chemotactic movement (Luderus et al., 1992; Van Haastert et al., 1987). Moreover, during aggregation, cells overexpressing constitutively active RasD(Q61L) show a significantly reduced cAMP stimulated ERK2 activity, whereas overexpressing the dominant negative RasD(S17N) results in an enhanced activation (Aubry et al., 1997). The morphological phenotypes we observe in *ddrasgap1* null cells suggest that constitutive PKA expressed from the *SP60* promoter in a *ddrasgap1* null background causes an increase in the number of apical organizers at different stages of development. We expect that each branch results from the formation of a new oscillator at that position in the organism. When this occurs in the mound, it gives rise to multiple tips, and during later development, it gives rise to branched structures. Interestingly, in some cases, multiple branches appear to arise from the same point, as if the organizer subdivides into multiple organizers simultaneously. If the oscillator functions to inhibit the formation of new organizers, it must be sufficiently unstable in *ddrasgap1* null cells, especially in the presence of excess PKA activity. Our data suggest that PKA potentiates this new tip formation.

Why we observe multiple tips with PKA expressed from a prespore promoter is not known; overexpression of PKAcat from the *ecmA* promoter in wild-type cells leads to a developmental arrest at the mound stage (see above for references). However, *Dictyostelium* cells can transdifferentiate with prespore or prestalk cells dedifferentiating and differentiating into another cell type under specific conditions. In wild-type strains, the organizer and *ecmAO*-expressing prestalk AO and AB cells co-localize to the anterior tip/apex. In *ddrasgap1* null cells, the *ecmA* cells are found more scattered in the organism, possibly increasing the chance of adventitious tip formation. Unexpectedly, overexpression of PKA in prespore cells does not result in spore formation as it does in wild-type cells. This suggests that the pathway(s) regulated by DdRasGAP1 are downstream from PKA or that the DdRasGAP1 regulates parallel pathways that are also essential for spore cell differentiation.

In conclusion, we suggest that DdRasGAP1 is an effector that regulates the Ras signaling pathway in *Dictyostelium* during growth and development. *Dictyostelium* expresses at least five distinct Ras proteins during different stages in the life cycle and we expect that the phenotypes we observe are the result of abnormal regulation of more than one of the Ras gene products. Some of these pathways, such as the role of Ras in cytokinesis, have not been previously defined in mammalian cells or yeast. Our analysis provides further insight into the functions of this evolutionarily conserved signaling component.

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