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

Susan Lee, Ricardo Escalante and Richard A. Firtel*

Department of Biology, Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0634, USA

*Author for correspondence (e-mail: rafirtel@ucsd.edu)

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. lacZ 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 adenyl 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; Raymond 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 (Raymond et al., 1984; Raymond 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 Ras [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 (Raymond 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 (Thierry et al., 1992; Khosla et al., 1996). Recently, a putative Ras GEF has been identified by insertional mutagenesis (Install 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, andlacZ 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 1.5×10⁸ 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) (Raymond et al., 1984) and GST-DdrasGAP1 proteins were expressed inE. 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 Heps, pH 7.3, 50 mM NaCl, 2 mM MgCl₂, 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-³P]GTP (6000 Ci/mmol), 1 mM EDTA, 20 mM Heps, pH 7.3, and 2 mM DTT. After incubation for 5 minutes at 25°C, this was added to 20 mM Heps, pH 7.3, 2 mM MgCl₂, and either 2 mM DTT and GAP was added or the Ras/GTP was allowed to incubate alone. Released [³P]PO₄ 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 1.5x10^8 initial yeast transformants. All 16 clones gave a strong response in the lacZ 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 lacZ 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 sar1 (Imai et al., 1991; Wang et al., 1991) as having exceptionally high scores (smallest sum probabilities of 3.0e^-83 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 NdeI site and the DNA digested with BglII and SaI before transformation into KAx-3 wild-type cells. The EcoRI and XhoI sites on the cDNA clone are from the polylinker insertion points in the λZap library (Schnitzler et al., 1994). RI, EcoRI; Bg, BglII; Nd, NdeI; Sa, SaI; Xh, XhoI.
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-GAP 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 lacZ reporter vector (see Materials and Methods, Fig. 1). Stable transformants carrying the DdrasGAP1/lacZ reporter were allowed to develop and histochromically stained for β-gal expression. Staining of clonal isolates during the multicellular stage 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.
Hydrolysis of details on purification. (B) Ras GAP assay. GST-S-GAP. See Materials and Methods for assay. Affinity purified GST-RasD, GST-GAP, and 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; Jermy 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') (Sutoh, 1993; Fig. 1) was transformed into wild-type cells. Stable Bs' 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' 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- or 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 refatted 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. 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 [$\gamma$-$^{32}$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.

**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.
nucleate 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 lacZ 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) ddrasgap1 null slug (16 hours of development); (B) wild-type slug (16 hours of development); (C,D) final structures ddrasgap1 null (26 hours of development); (E) wild-type fruiting body (26 hours of development).

dd rasgap1 null cells show altered cell-type spatial patterning and are blocked in culmination

To examine the spatial patterning of cell types within the

tically higher than the level of dd rasgap1 null spores when this strain was plated alone.

Expression of the DdRasGAP1 cDNA downstream from the Act15 or DdRasGAP1 promoters used for the lacZ expression studies in dd rasgap1 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 dd rasgap1 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.

Fig. 7. Developmental RNA blot analysis of postaggregative gene expression in wild-type and dd rasgap1 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 ecmAO 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.

dd rasgap1 null organisms, dd rasgap1 null cells were transformed with lacZ 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 dd rasgap1 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. 8A), as they are in wild-type fingers (Fig. 8A). With time, the pattern changes: fewer ecmAO/lacZ-staining cells are observed (Fig. 8B) 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. 8C; for comparison, a wild-type fruiting body expressing ecmAO/lacZ is shown in Fig. 8B). 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 ecmB89 (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 calcifiior, 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/lacZ (Haberstroh and Firtel, 1990) was also abnormal. Stained cells were restricted to the posterior 50% of dd rasgap1 null slugs (Fig. 8D), which is distinct from expression seen in wild-type cells in which the posterior approx. 80% of the slug was stained (Fig. 8B). As the fingers develop into elongated, twisted slugs, stained cells are found scattered along the entire length of the structure (Fig. 8E), 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/lacZ 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/lacZ, and no cells within this region stain with the SpiA/lacZ spore-specific reporter (see below; Richardson et al., 1994), indicating the apical protrusion does not represent a sorus. The SpiA promoter drives lacZ 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/lacZ 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/lacZ and SpiA/lacZ staining was observed within the sorus with a few staining cells

---

Fig. 8. lacZ expression studies of DdRasGAP1.
(A) Expression of the prespore-specific reporter SP60/lacZ (a,c) and the prestalk-specific reporter ecmA0/lacZ (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 lacZ constructs. ecmA is prestalk-specific; SP60 is prespore-specific; SpiA is spore-specific. (a) ecmA, 14 hours; (b) ecmA0/lacZ, 18 hours; (c) ecmA0/lacZ, 26 hours; (d) SP60/lacZ, 14 hours; (e) SP60/lacZ, 18 hours; (f) SP60/lacZ, 26 hours; (g,h) SpiA/lacZ, 26 hours. In Bg, arrowheads point to the two ‘lobes’ of the stained region. (C) lacZ 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 lacZ constructs. Histochemical staining of lacZ expression localizes ddrasgap1 null cells expressing the cell-type-specific marker. (a) Mature fruiting body (26 hours), ecmA0/lacZ; (b) mature fruiting body, SP60/lacZ; (c) mature fruiting body, SpiA; (d) migrating slug, SP60/lacZ (16 hours). Cells are at the slug stage.
scattered throughout the stalk structure (Fig. 8Cb,c). ecmA0/lacZ-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:ecmA89/lacZ 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 (Siegenthaler 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 adenyl cyclase and other components essential for aggregation (Mann and Firtel, 1991; Schulkes and Schaal, 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 multibranching 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 multibranching 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. obs.). 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).
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 sar 1 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 communication), 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. lacZ 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 patterned 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 ecmBA89 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/lacZ-tagged prestalk and SP60/lacZ-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 lacZ 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 metazoa (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 (Siegent 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 metazoa (Perrimon, 1995), regulates
multiple steps during development, including aggregation, cell-
type induction and culmination (see above). During aggrega-
tion, PKA is required for the expression of adenyl cyclase
and other unidentified signaling components (S. Mann, J.
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; Schulske and
Schaap, 1995), it is difficult to directly evaluate the effect of
RasGAP overexpression on morphogenesis during the multi-
cellular stages in pkacat null cells. Overexpression of PKA
inhibits the adaptation pathway controlling the down-regula-
tion of the MAP kinase ERK2, which is activated by cAMP
and required for the activation of adenyl cyclase, prespore
gene expression, and proper morphogenesis during later de-
velopment (Gaskins et al., 1996; L. Aubry, M. Maeda, R. Insall,
P. Devreotes, and R. A. F., unpublished data). During multi-
cellular 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 devel-
oment 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 regu-
late a structure. Presumably the strength of the oscillator
determines the number of cells that can be organized; that is,
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 adenyl cyclase signaling (Brenner and
Thoms, 1984), leads to the formation of adventitious tips along
the slug that give rise to independent slugs (Sieger 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 oscil-
lator 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 develop-
mental 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 differ-
entiation.

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.

We would like to thank A. Vojtek for supplying the mammalian
Ras(G12V) and Ras(G15A) constructs and members of the Firtel lab-
atory for helpful suggestions during the progress of this work. R. E.
was supported by an MEC/Fulbright Scholarship. This work was
supported by USPHS GM37830 and HD30892.

REFERENCES

Abe, T., Early, A., Siegert, F., Weijer, C. and Williams, J. (1994). Patterns of
cell movement within the Dictyostelium slug revealed by cell type-specific,

The Dictyostelium MAP kinase ERK2 is regulated by Ras and cAMP-
dependent protein kinase (PKA) and mediates PKA function. J. Biol. Chem.
in press)

relatives. Nature 366, 643-654


Phil. Trans. R. Soc. Lond. B. Biol. Sci. 349, 283-289


scroll waves of cAMP could direct cell movement and gene expression in

human immunodeficiency virus Tat-binding protein/26S protease subunit

Characterization of a third ras gene, rasB, that is expressed throughout the growth and development of Dictyostelium discoideum. Oncogene 8, 1041-1047.


The aimless RasGEF is required for processing of chemotactic signals through G-protein coupled receptors in Dictyostelium. Curr. Biol. 6, 719-729.


(Accepted 4 December 1996)