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RasC is required for optimal activation of adenylyl cyclase and Akt/PKB during aggregation

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Disruption of *Dictyostelium rasC*, encoding a Ras subfamily protein, generated cells incapable of aggregation. While *rasC* expression is enriched in a cell type-specific manner during post-aggregative development, the defect in *rasC*$^-$ cells is restricted to aggregation and fully corrected by application of exogenous cAMP pulses. cAMP is not produced in *rasC*$^-$ cells stimulated by 2′-deoxy-cAMP, but is produced in response to GTPγS in cell lysates, indicating that G-protein-coupled cAMP receptor activation of adenylyl cyclase is regulated by RasC. However, cAMP-induced ERK2 phosphorylation is unaffected in *rasC*$^-$ cells, indicating that RasC is not an upstream activator of the mitogen-activated protein kinase required for cAMP relay. *rasC*$^-$ cells also exhibit reduced chemotaxis to cAMP during early development and delayed response to periodic cAMP stimuli produced by wild-type cells in chimeric mixtures. Furthermore, cAMP-induced Akt/PKB phosphorylation through a phosphatidylinositide 3-kinase (PI3K)-dependent pathway is dramatically reduced in *rasC*$^-$ cells, suggesting that G-protein-coupled serpentine receptor activation of PI3K is regulated by RasC. Cells lacking the RasGEF, AleA, exhibit similar defects as *rasC*$^-$ cells, suggesting that AleA may activate RasC.

Keywords: adenylyl cyclase/Akt-PKB/cAMP relay/chemotaxis/*Dictyostelium*

Introduction

The *ras* subfamily genes encode monomeric GTPases that function as molecular switches in cellular signal transduction by cycling between an active GTP-bound or an inactive GDP-bound state (Bourne *et al*., 1991). A variety of extracellular stimuli potentiate the activation of Ras by exchanging GDP for GTP (Campbell *et al*., 1998; Gutkind, 1998), a process catalyzed by guanine nucleotide exchange factors (GEFs) (Boguski and McCormick, 1993). GTPase activating proteins increase the intrinsic GTPase activity of Ras, hydrolyzing the bound GTP to GDP (Boguski and McCormick, 1993). In the active state, Ras proteins activate multiple cellular signaling pathways including mitogen-activated protein kinase (MAPK) cascades, the phosphatidylinositide 3-kinase (PI3K)-regulated pathways and RalGDS-dependent activation of Ral (Campbell *et al*., 1998). These Ras-mediated responses to membrane receptor stimuli regulate a wide range of cellular processes, including proliferation, cytoskeletal functions and differentiation.

The discovery of a large number of Ras subfamily homologs in mammals and in the model organisms *Drosophila melanogaster*, *Caenorhabditis elegans* and *Dictyostelium discoideum* (Reuther and Der, 2000; Wilkins and Insall, 2001) has raised important questions regarding the specific functions of individual Ras proteins that cannot be readily resolved by biochemical analysis. To understand Ras function in a multicellular context, it is necessary to analyze organisms that are genetically disrupted in the *ras* gene of interest. The tractability of the *Dictyostelium* haploid genome facilitates the functional characterization of strains with specifically targeted gene disruptions and, furthermore, its unique biology allows studies of growth and differentiation as distinct processes (Parent and Devreotes, 1996; Aubry and Firtel, 1999). Nutrient deprivation triggers the developmental program whereby the secretion and chemotactic response to the chemoattractant cAMP result in the aggregation of up to 10^5 cells. The aggregate then elongates to become a phototactic and thermotactic migrating slug. Cells within the slug differentiate and sort into prestalk or prespore cells that segregate into spatially separated populations, which, upon culmination, form a stalk consisting of dead vacuolated cells supporting a sorus of spores. Underlying this deceptively simple developmental program is a complex of cellular signaling pathways that are highly conserved relative to mammalian systems.

Six *Dictyostelium* Ras subfamily proteins with at least 50% amino acid identity to the mammalian H-, N- and K-Ras proteins have been described (Reymond *et al*., 1984; Daniel *et al*., 1995). Several lines of evidence have indicated that a Ras signaling pathway is involved in the regulation of the cAMP relay and in the chemotactic response to cAMP during aggregation. Disruption of *aleA*, the gene encoding a putative Ras GEF (Insall *et al*., 1996), and *rip3*, encoding a Ras interacting protein (Lee *et al*., 1999), resulted in cells that cannot aggregate due to defects in both the cAMP relay and chemotaxis. Furthermore, disruption of genes homologous to components of metazoan Ras-activated effector pathways also resulted in cells incapable of aggregation, suggesting a possible upstream role for a Ras protein. For example, the MAPK homolog ERK2 was shown to be essential for cAMP relay, but not necessary for chemotaxis (Segall *et al*., 1995), while cells lacking both PI3K1 and PI3K2, and cells lacking Akt/PKB, a downstream effector of PI3K activity, exhibit defects in chemotaxis but not in cAMP relay (Zhou *et al*., 1998; Meili *et al*., 1999).

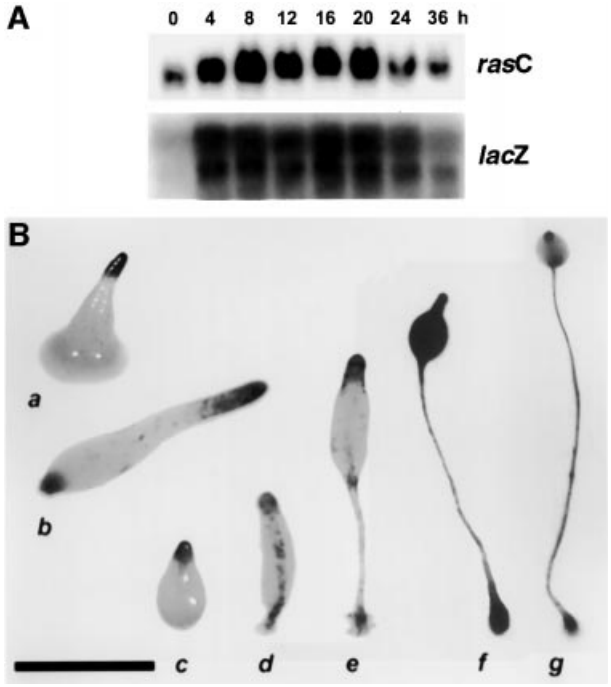


Fig. 1. (**A**) *rasC* and *lacZ* gene expression during development. *rasC::*^{his}*lacZ*-transformed AX2 cells were plated for development on nitrocellulose filters and total RNA harvested at the times indicated for northern blot analysis. Duplicate blots were probed with either *rasC* or *lacZ* cDNA. (**B**) Spatial expression of *lacZ* driven from the *rasC* promoter during development. *rasC::*^{his}*lacZ*-transformed AX2 cells were plated for development on nitrocellulose filters, fixed at various developmental stages and stained *in situ* for β-galactosidase activity. Shown are representative stained structures from the following developmental stages: first finger (a); slug (b); early to mid culmination (c–e); late culmination (f); and terminal fruiting body (g). All were stained for 2 h (a–f), except (g), which was stained overnight. Bar = 0.5 mm.

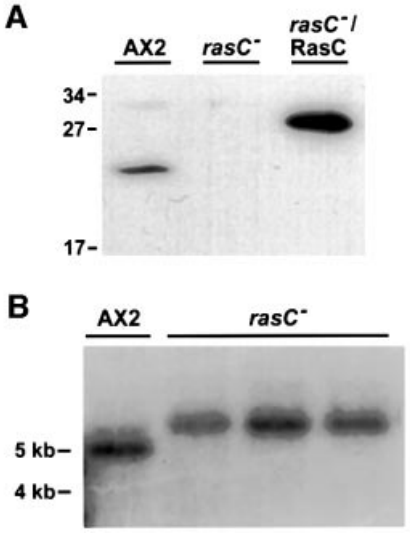


Fig. 2. Disruption of the *rasC* gene. (**A**) Western blot of cell lysates from the parental AX2, *rasC*$^{-}$ and *rasC*$^{-}$/RasC, a *rasC*$^{-}$ transformant expressing RasC from the *rasC* promoter, probed with a highly specific RasC antibody. The higher molecular weight of the RasC protein in the *rasC*$^{-}$/RasC strain is due to the fact that the expression construct encoded a RasC protein with an additional 17 amino acids at the N-terminus. Molecular weight markers in kilodaltons are as indicated. (**B**) Southern blot of genomic DNA from parental AX2 and three independent *rasC*$^{-}$ isolates. Genomic DNA was digested with *Dde*I, separated in 0.7% agarose gel, blotted onto nylon and probed with *rasC* cDNA. *Dde*I restriction sites flank the entire *rasC* genomic locus and are not present within the disruption construct used for homologous recombination. Approximate sizes in kilobases are as indicated.

Three of the *Dictyostelium ras* genes, *rasS, rasG* and *rasD*, have been disrupted, but these disruptions were found to have negligible effects on the aggregation process (Tuxworth *et al*., 1997; Chubb *et al*., 2000; Wilkins *et al*., 2000). In the studies described here, we present genetic and biochemical evidence that RasC is the Ras protein that had been previously implicated in the aggregation processes. *Dictyostelium* cells in which the *rasC* gene had been disrupted by targeted gene replacement failed to aggregate. RasC appears to be a central regulatory molecule acting downstream of serpentine receptor stimulation by cAMP that is required for two distinct effector pathways: the activation of Akt/PKB through PI3K and the activation of adenylyl cyclase. The discovery that the RasC protein is essential for aggregation provides evidence for a novel role for a Ras subfamily protein in the sensing of and response to chemotactic signals.

Results

Growth and developmental expression of rasC

In order to analyze the spatial expression of *rasC* during development, the 0.55 kb DNA fragment upstream of the 5′ *rasC* coding region (Daniel *et al*., 1994) was isolated by PCR and ligated to the ^{his}*lacZ* gene, a variant of *lacZ* that encodes a short half-life β-galactosidase (Detterbeck *et al*., 1994). The resulting *rasC::*^{his}*lacZ* fusion construct was transformed into the wild-type AX2 strain. A northern blot of RNA isolated from different developmental stages of this transformant showed a *lacZ* expression pattern that was almost identical to that for the *rasC* transcript (Figure 1A), indicating that the 0.55 kb DNA fragment encodes sufficient promoter sequence to reproduce the normal regulation of the *rasC* gene.

When the *rasC::*^{his}*lacZ* transformant was developed on nitrocellulose filters, staining for β-galactosidase activity was observed predominantly in the tip during the transition from tipped mound to slug, and by the slug stage there was also some staining in the posterior (Figure 1B). These results indicate that *rasC* expression is enriched in the prestalk cell population, and this pattern of expression was maintained through early and mid culmination, as demonstrated by staining in the tip, stalk tube and basal disk. The whole population became equally stained during late culmination; however, in terminally differentiated fruiting bodies, staining was predominantly in the stalk tube, the upper cup and the lower cup, indicating a reversion to the enriched prestalk pattern of expression (Figure 1B). The transient change in *rasC* spatial expression during late culmination correlated with the peak of mRNA at 20 h of development (Figure 1A). This spatial and temporal expression pattern is suggestive of functional roles for RasC during slug formation and culmination.

Generation of a rasC null strain

In order to generate a *rasC*$^{-}$ strain, a *rasC* disruption vector was transformed into AX2 cells, and blasticidin S-resistant colonies were screened by western blotting using the RasC

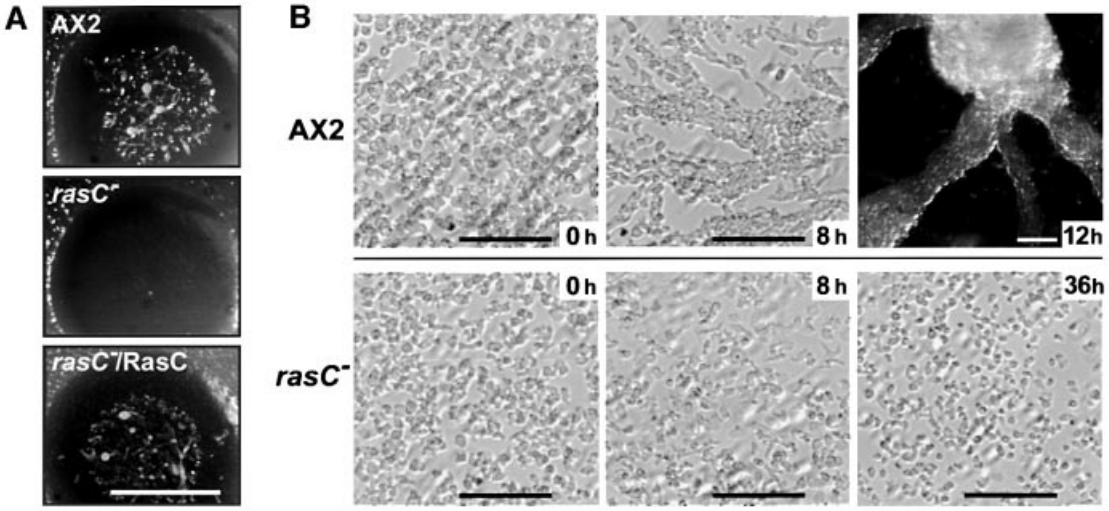


Fig. 3. Developmental phenotypes of parental AX2 and *rasC*$^-$ strains. (**A**) Clonal plaques of parental AX2, *rasC*$^-$ and *rasC*$^-$/RasC strains after 5 days growth on a bacterial lawn. Bar = 5 mm. (**B**) AX2 and *rasC*$^-$ cells were seeded as monolayers at a density of 5×10^5 cells/cm^2 in Nunc tissue culture dishes and submerged under Bonner's salts solution. Images were taken at the times indicated following plating. Bars = 100 μm.

antibody. Out of 70 independently isolated clones analyzed, three exhibited no detectable RasC protein (one of the three is shown in Figure 2A). Southern blotting, using a *rasC* cDNA probe, verified that all three transformants contained a simple targeted disruption at the *rasC* locus (Figure 2B). Subsequent rehybridization with the *bsr* gene probe revealed a single insertion of *bsr* into the genome for all three isolates (data not shown), indicating that the null strains were the result of *rasC* disruption and not attributable to secondary effects resulting from random integration. All three *rasC*$^-$ cell lines exhibited identical phenotypes under all assay conditions and, therefore, all subsequent data presented are for one of the three transformants.

Phenotype of the rasC$^-$ *cells*

When plated on a lawn of bacteria, *Dictyostelium* cells grow by ingesting the bacteria, resulting in plaque formation. Cells at the plaque periphery continue to feed, expanding plaque size, while starving cells toward the center initiate multicellular development and eventually form fruiting bodies. When *rasC*$^-$ cells were clonally grown on a bacterial lawn, initial plaque formation was indistinguishable from that of the parental AX2, indicating normal growth and phagocytosis. However, *rasC*$^-$ cells failed to aggregate, appearing as clear plaques 5 days after initial plating (Figure 3A). At that time, all phases of multicellular development were observed in the AX2 plaques. Ectopic expression of the RasC protein from the *rasC* promoter restored multicellular development in the null strain (Figures 2A and 3A), confirming that the observed phenotype was a functional consequence of *rasC* gene disruption.

The aggregation process can also be observed by incubating cell monolayers on plastic surfaces submerged under non-nutrient buffer. Under these conditions, aggregation streams were observed for AX2 after 8 h, and distinct aggregates formed by 12 h (Figure 3B, top panels). *rasC*$^-$ cells did not form aggregation streams or centers, even after prolonged starvation for 36 h (Figure 3B, bottom panels). The aggregation defects observed in the *rasC*$^-$ cells were similar to those reported for the *aleA*$^-$ strain (Insall *et al*., 1996), suggesting the possibility that AleA is the GEF responsible for regulating RasC activation.

Exogenous cAMP pulses circumvent the block in aggregation of rasC$^-$ *cells*

Dictyostelium cells are unable to aggregate if they are deficient in the cAMP relay, a process whereby cells synthesize and secrete cAMP in response to an extracellular cAMP stimulus. When cells in suspension were pulsed every 6 min for 5 h with 50 nM cAMP (herein referred to as 'cAMP-pulsed cells') and then plated on nitrocellulose filters, development of *rasC*$^-$ cells was indistinguishable from that of AX2 cells (data not shown). cAMP-pulsed *rasC*$^-$ cells produced spores in equal numbers to cAMP-pulsed AX2 cells, and these spores exhibited the same viability (data not shown). Furthermore, when developed in chimeric mixtures with equal numbers of AX2 cells, *rasC*$^-$ cells completed development, producing equal numbers of spores (data not shown). The *rasC*$^-$ spores germinated to form aggregation-negative plaques when plated on bacterial lawns (data not shown). Contrary to the spatial expression results suggesting functional roles for RasC during mid to late development, the development of *rasC*$^-$ cells is fully restored if the block in aggregation is circumvented by exogenous application of cAMP pulses, an indication that RasC function is necessary for the cAMP relay.

cAMP receptor and heterotrimeric G-protein-dependent activation of adenylyl cyclase is mediated by RasC

To confirm that the *rasC*$^-$ cells were defective in the cAMP relay, cAMP-pulsed cells were stimulated *in vivo* with 2′-deoxy-cAMP, and cell lysates assayed for cAMP accumulation at various time points following stimulation. There was negligible accumulation of cAMP in the *rasC*$^-$ cells relative to that observed for AX2 cells (Figure 4A),

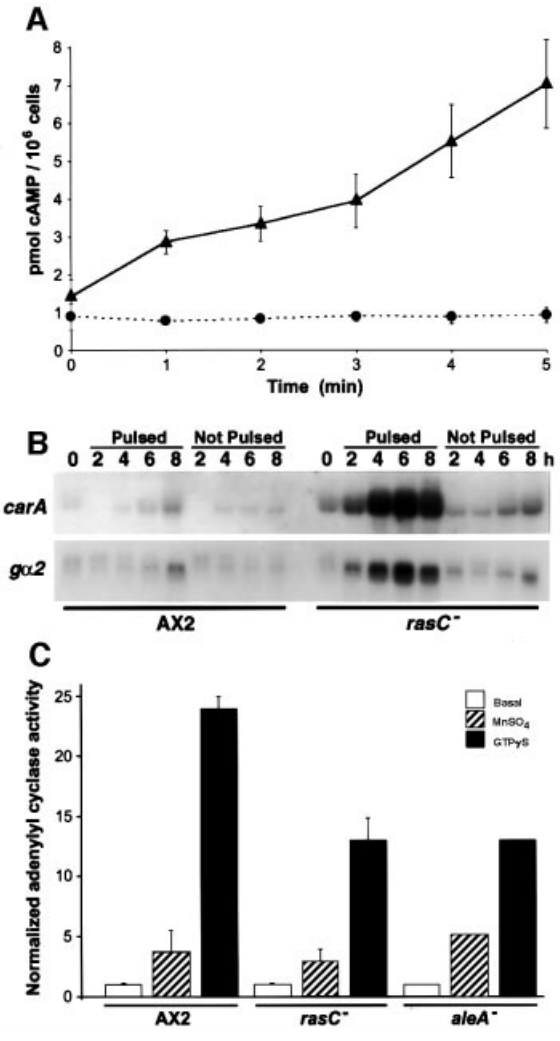


Fig. 4. Adenylyl cyclase assays and expression of aggregation phase genes. (**A**) cAMP-pulsed AX2 (triangles) and *rasC*⁻ (circles) cells were stimulated with 10 μM 2′-deoxy-cAMP, and cell lysates assayed for total cAMP accumulation at the indicated times. Plotted values are the means ± SD for three independent experiments. (**B**) AX2 and *rasC*⁻ cells were shaken in suspension in KK2, with or without the application of 50 nM cAMP pulses every 6 min. Total RNA was prepared from cells harvested at the times indicated and duplicate northern blots probed with either *carA* or *gα2* cDNA. (**C**) cAMP-pulsed AX2, *rasC*⁻ and *aleA*⁻ cell-free lysates were assayed for adenylyl cyclase activity in the presence of either 5 mM $MnSO_4$ (hatched bar), 40 μM GTPγS (black bar) or no additional component (white bar) (see Materials and methods). Plotted values are normalized relative to the unstimulated activity obtained in the absence of $MnSO_4$ or GTPγS. Values for AX2 and *rasC*⁻ cell lysates are the means ± SD for three independent experiments. Values for *aleA*⁻ cell lysates are from a single experiment.

indicating that RasC is required for cAMP-stimulated activation of adenylyl cyclase A (ACA), the predominant adenylyl cyclase present during early *Dictyostelium* development (Parent and Devreotes, 1996). A northern blot of mRNA isolated from cAMP-pulsed cells showed that the *rasC*⁻ cells overexpress the cAMP receptor *carA* and the heterotrimeric G-protein *gα2*, relative to AX2 cells (Figure 4B). While the significance of this increased expression is not apparent, it is clear that the inability of the *rasC*⁻ cells to activate ACA is not due to insufficient

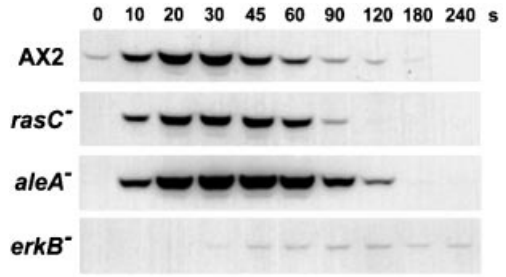


Fig. 5. cAMP-induced stimulation of ERK2 phosphorylation. cAMP-pulsed AX2, *rasC*⁻, *aleA*⁻ and *erkB*⁻ cells were stimulated with cAMP to a final concentration of 100 nM, and cells lysed directly in SDS gel loading buffer at the indicated times. Protein samples of 10 μg were fractionated by SDS–PAGE, and western blots probed with a phospho-MAPK-specific antibody to assay for ERK2 phosphorylation. Results shown are representative of at least three independent experiments for each strain.

expression of required signaling components such as cAR1 and $G\alpha_2$ (Parent and Devreotes, 1996).

To further assess the nature of the signaling defect, GTPγS-mediated activation of ACA was assayed *in vitro* using extracts prepared from cAMP-pulsed cells. Since *aleA*⁻ cells also exhibit negligible cAMP synthesis when stimulated *in vivo* with cAMP (Insall *et al*., 1996), we included *aleA*⁻ cell-free extracts in this assay for comparison. GTPγS is thought to stimulate ACA activity by uncoupling the Gβγ subunit from the heterotrimeric G-protein, thus bypassing the need for receptor activation in cell-free lysates (Parent and Devreotes, 1996). The addition of GTPγS stimulated the ACA activity of AX2 lysates ~24-fold over the basal level, and the ACA activity of *rasC*⁻ and *aleA*⁻ cell lysates ~12-fold (Figure 4C). Thus, cell lysates lacking RasC or AleA were capable of generating appreciable amounts of cAMP in the presence of GTPγS, although the levels were not as high as those obtained with wild-type cells. The lower levels of *in vitro* cAMP synthesis in the *rasC*⁻ and *aleA*⁻ cell lysates were not due to reduced expression of ACA, since the Mn^{2+}-activated levels were similar for AX2, *rasC*⁻ and *aleA*⁻ cell lysates (Figure 4C).

RasC is not necessary for activation of ERK2

Dictyostelium ERK2 is transiently activated in response to a cAMP stimulus (Maeda *et al*., 1996) and cells lacking ERK2 are unable to aggregate due to a defective cAMP relay (Segall *et al*., 1995). Since ERK proteins are important downstream effectors for Ras in metazoan signaling, the possibility that RasC is an upstream activator of ERK2 was tested. cAMP-pulsed cells were stimulated with cAMP and cell lysates were analyzed for ERK2 phosphorylation in western blots using a phospho-MAPK-specific antibody. Following cAMP stimulation, a 42 kDa protein corresponding to the predicted molecular weight of ERK2 was transiently phosphorylated in wild-type cells (Figure 5) with kinetics similar to that reported previously for ERK2 activation (Maeda *et al*., 1996). An *erkB*⁻ strain assayed similarly showed only a very faint phosphorylation of the 42 kDa component (Figure 5). Since this particular *erkB*⁻ strain (HS174) expresses low levels of ERK2 due to insertional disruption of a plasmid in the 3′ untranslated region of the gene (Segall *et al*.,

1995), our results verify that the phosphorylated protein detected in the immunoblots is indeed ERK2. When *rasC*$^-$ cells were stimulated with cAMP, ERK2 was phosphorylated to similar levels and with similar kinetics to those seen for wild-type cells. This demonstrates that the activation of ERK2 is not downstream of RasC in the classical Ras–MAPK signaling cascade observed in metazoan systems.

Since there had been a correlation between the phenotypes of the *rasC*$^-$ and *aleA*$^-$ cells, we repeated the ERK2 activation assay with the *aleA*$^-$ cells. Phosphorylation of ERK2 in *aleA*$^-$ cells reached a higher level, peaked at a later time point and was more persistent compared with wild-type cells (Figure 5), confirming the results of an earlier experiment using an in-gel kinase assay to measure ERK2 activity (Aubry *et al*., 1997). The results indicate that AleA may activate another Ras (Aubry *et al*., 1997; Kosaka *et al*., 1998), in addition to RasC.

rasC$^-$ *cells exhibit an altered chemotactic response to cAMP*

Dictyostelium aggregation is also dependent on the cell's ability to sense and respond chemotactically to cAMP. To examine the chemotactic behavior of cells lacking RasC, green fluorescent protein (GFP)-labeled *rasC*$^-$ cells were mixed with unlabeled AX2 cells and allowed to aggregate on plastic submerged under buffer. The fluorescent label allowed tracking of individual *rasC*$^-$ cells in response to the natural cAMP oscillations produced by the wild-type cells. At 8 h of development, there were very few labeled *rasC*$^-$ cells in the small aggregates that had formed, while, in contrast, there were numerous labeled *rasC*$^-$ cells in the aggregates and aggregation streams by 12 h (Figure 6A, left panels). When GFP-labeled AX2 cells were mixed with unlabeled AX2 cells as a control, numerous labeled cells were observed both within the small aggregates at 8 h and within the aggregation streams by 12 h (Figure 6A, right panels), indicating that the GFP label had no deleterious effect on chemotactic behavior. Further analysis of the mixed population at 6 h following starvation by time-lapse microscopy revealed that synchronous, pulsatile movements of AX2 cells could be observed, whereas GFP-labeled *rasC*$^-$ cells exhibited no such response (data not shown). In contrast, by 12 h, the *rasC*$^-$ cells within the aggregation streams exhibited the same pulsatile responses as the AX2 cells, while the *rasC*$^-$ cells that remained outside the streams remained unresponsive and non-polarized.

AX2 cells that had been starved for 6 h without exogenous cAMP pulsing responded within 20 min to an artificial cAMP gradient released from a micropipet (Figure 6B, top panels). In contrast, under these conditions, *rasC*$^-$ cells underwent chemotaxis poorly and did not exhibit the characteristic clustering of cells around the micropipet tip even after 40 min (Figure 6B, bottom panels). AX2 cells that had been pulsed for 5 h with cAMP polarized and underwent chemotaxis toward the cAMP source within 20 min of micropipet tip application (Figure 6C, top panels). However, equivalently treated *rasC*$^-$ cells polarized and underwent chemotaxis toward the tip within 6 min of application of the micropipet (Figure 6C, bottom panels). This enhanced chemotaxis of the *rasC*$^-$ cells might be the result of overexpression of *carA*, *gα2* (Figure 4B) and possibly other components involved in mediating chemotaxis. Thus, *rasC*$^-$ cells are initially slow in undergoing chemotaxis to cAMP, but underwent chemotaxis rapidly after being pulsed. These results are consistent with the behavior of the *rasC*$^-$ cells in mixtures with AX2 described above.

Akt/PKB phosphorylation through a PI3K-dependent pathway requires RasC

pi3k1$^-$*/pi3k2*$^-$ and *pkbA*$^-$ cells exhibit aggregation defects despite having normal cAMP relays, suggesting that the chemotactic response to cAMP requires a PI3K-mediated signaling pathway (Zhou *et al*., 1998; Meili *et al*., 1999). Consistent with this model, cAMP-stimulated activation of Akt/PKB does not occur in *pi3k1*$^-$*/pi3k2*$^-$ cells, but is fully restored in *pi3k1*$^-$*/pi3k2*$^-$ cells constitutively expressing PI3K1 (Meili *et al*., 1999). *Dictyostelium* Akt/PKB activation requires phosphorylation at conserved threonine residues in the kinase domain and at the C-terminus (Meili *et al*., 1999). When cell lysates of cAMP-stimulated AX2 cells were analyzed by western blotting using a phospho-threonine-specific antibody, a protein with the predicted molecular weight of *Dictyostelium* Akt/PKB (51 kDa) was transiently phosphorylated following cAMP stimulation (Figure 7A, left panels). When the blot was stripped and re-probed with an Akt/PKB-specific antibody, a 51 kDa protein was also detected (Figure 7A, right panels), consistent with the idea that the phospho-threonine-specific antibody had detected the transient phosphorylation of Akt/PKB. Pre-treatment of AX2 cells with 12.5 μM LY294002, a PI3K inhibitor (Vlahos *et al*., 1995), caused an ~90% reduction in threonine phosphorylation of the 51 kDa protein, indicating that the observed phosphorylation was PI3K dependent (Figure 7A). Finally, the phosphorylation kinetics of the 51 kDa protein observed here was consistent with the activation of Akt/PKB reported previously (Meili *et al*., 1999). We are therefore confident that the transiently phosphorylated 51 kDa component is the Akt/PKB protein encoded by *pkbA*.

cAMP-pulsed *rasC*$^-$ cells exhibit dramatically reduced levels of phosphorylated Akt/PKB at 10 s following cAMP stimulation (Figure 7B), indicating that RasC is a major upstream effector of Akt/PKB phosphorylation. cAMP-stimulated Akt/PKB phosphorylation in *aleA*$^-$ cells was also reduced to levels similar to those observed for *rasC*$^-$ cells (Figure 7B), indicating that AleA also functions upstream of Akt/PKB, consistent with the possibility that it is a GEF for RasC. The trace of Akt/PKB phosphorylation at 10 s observed in the *rasC*$^-$ and *aleA*$^-$ cells suggests that low levels of activation not involving RasC or AleA can occur. The lower levels of Akt/PKB phosphorylation were not due to reduced expression of Akt/PKB in *rasC*$^-$ and *aleA*$^-$ cells, since western blots probed with an Akt/PKB-specific antibody revealed identical levels to those in AX2 (Figure 7C).

Discussion

A role for a Ras protein in aggregation has been suggested by several lines of evidence (Segall *et al*., 1995; Insall *et al*., 1996; Aubry *et al*., 1997; Lee *et al*., 1999; Meili *et al*., 1999; Firtel and Chung, 2000). Gene disruption of *rasG* produced cells that exhibited reduced motility and

cytokinesis defects (Tuxworth *et al*., 1997); however, their ability to aggregate was only slightly delayed (our unpublished observations). Similarly, there is also no evidence for an involvement of RasD or RasS since disruption of the respective genes generated cells that aggregated normally (Chubb *et al*., 2000; Wilkins *et al*., 2000). In this report, we have presented results which clearly demonstrated a role for RasC in aggregation. We have generated a *rasC*$^-$ strain in which expression of RasC is completely abolished, and have shown that *rasC*$^-$ cells fail to aggregate. RasC appears to function as a regulatory molecule for both the cAMP relay and the chemotactic response to cAMP.

rasC$^-$ cells exhibited no accumulation of cAMP following *in vivo* stimulation by a cAMP analog, indicating that RasC is necessary for G-protein-coupled cAMP receptor-mediated activation of ACA. This process requires the release of the Gβγ subunit from the trimeric $G\alpha_2\beta\gamma$ complex (Wu *et al*., 1995). In mammalian cells, Ras is activated downstream of Gβγ in G-protein-coupled serpentine receptor activation (Gutkind, 1998). In order to determine whether RasC activation is also downstream of Gβγ in *Dictyostelium*, we measured the effect of GTPγS on ACA activity in cell-free extracts. The addition of GTPγS releases Gβγ subunits from G-proteins, thus bypassing the need for receptor activation (Parent and Devreotes, 1996). Since ACA activity was stimulated in *rasC*$^-$ cell lysates, it is clear that RasC is not essential for ACA activation in the same manner that Gβ (Wu *et al*., 1995) and the cytosolic regulatory proteins CRAC and Pia are required (Insall *et al*., 1994; Chen *et al*., 1997). The block to ACA activation in *rasC*$^-$ cells must lie upstream of $G\alpha_2\beta\gamma$. A possible explanation of our results is that RasC is essential for the dissociation of the $G\alpha_2\beta\gamma$ complex both *in vivo* and *in vitro*, and that the partial activity observed in the *rasC*$^-$ cell-free extracts *in vitro* was provided by release of Gβγ from the remaining G-complexes ($G\alpha_{1,3-8}\beta\gamma$) (Parent and Devreotes, 1996). This would be similar to the situation for $g\alpha_2^-$ cells, where ACA activation by GTPγS occurs *in vitro*, but not in intact cells stimulated through cAMP receptors (Pupillo *et al*., 1992).

Dictyostelium ERK2 is essential for ACA activity (Segall *et al*., 1995) and is transiently activated following receptor stimulation by cAMP (Maeda *et al*., 1996). Although both *erkB*$^-$ and *rasC*$^-$ cells exhibited a similar defect in cAMP relay, cAMP stimulation induced normal ERK2 phosphorylation in *rasC*$^-$ cells (Figure 5), indicating that RasC is not involved in ERK2 activation. Since ERK2 activation is cAR1-dependent but $G\alpha_2$ and Gβ independent (Maeda *et al*., 1996), our results are consistent with the contention that RasC signaling is specifically routed through the $G\alpha_2\beta\gamma$-dependent pathway. Despite the fact that ERKs are downstream signaling components of Ras activation in metazoans, there is still no evidence that Ras-mediated signaling is required for ERK2 activation in *Dictyostelium*. In fact, studies using strains overexpressing activated or dominant-negative forms of RasG or RasD suggest a negative regulatory role for a Ras protein in ERK2 activation (Aubry *et al*., 1997; Kosaka *et al*., 1998). It has been postulated that ERK2 function is important for the adaptation response, inhibiting degradation of intracellular cAMP when activated (Loomis, 1998; Aubry and Firtel, 1999). Cells lacking AleA exhibit increased and prolonged ERK2 phosphorylation when stimulated with cAMP (Figure 5) (Aubry *et al*., 1997), suggesting that AleA may regulate two Ras-mediated responses during cAMP relay: the activation of RasC for synthesis of cAMP and the activation of another Ras for down-regulation of ERK2 during adaptation, allowing the degradation of intracellular cAMP.

It has been shown that PI3K activity is necessary for the activation of *Dictyostelium* Akt/PKB, since Akt/PKB is not activated in *pi3k1*$^-$*/pi3k2*$^-$ cells stimulated with cAMP (Meili *et al*., 1999). Akt/PKB activation is greatly reduced in *rasC*$^-$ and *aleA*$^-$ cells, strongly suggesting that both RasC and AleA play a role in activating PI3K upon cAMP stimulation. The mechanism for this RasC-dependent activation of PI3K remains to be determined, but since both PI3K1 and PI3K2 appear to possess putative Ras binding domains in their N-terminal regions (Zhou *et al*., 1995), RasC might be directly involved in the activation of either protein. Since RasC also appears to be required for $G\alpha_2\beta\gamma$ dissociation, and $G\alpha_2$ and Gβ are necessary for Akt/PKB activation (Meili *et al*., 1999), RasC could be required for both direct and G-protein-dependent activation of the PI3Ks. While it is evident that Akt/PKB is necessary for efficient chemotaxis (Meili *et al*., 1999), our results indicate that this process does not require full Akt/PKB phosphorylation. The dramatic reduction in Akt/PKB phosphorylation in the *rasC*$^-$ cells produces only a slight deleterious effect on cAMP-mediated chemotaxis. It is likely that the trace of Akt/PKB phosphorylation observed in cAMP-stimulated *rasC*$^-$ cells, which had been cAMP-pulsed for 5 h, is sufficient to achieve efficient chemotaxis.

Several lines of evidence point to AleA as the GEF that activates RasC during aggregation. Genetic disruption of *aleA* was the only one of five *ras gef* gene disruptions that resulted in a clear aggregation-negative phenotype similar to that of *rasC*$^-$ cells (Wilkins and Insall, 2001; R.Insall, personal communication), and both *rasC*$^-$ and *aleA*$^-$ cells are defective in the cAMP relay (Insall *et al*., 1996). GTPγS was shown to be capable of partially activating ACA in cell-free extracts of *aleA*$^-$ and *rip3*$^-$ (Figure 4C; Lee *et al*., 1999). These results are consistent with the idea that AleA is the GEF that activates RasC during aggregation, and suggest the possibility that RasC, AleA and RIP3 form part of a complex that is necessary for optimum activation of ACA. *aleA*$^-$ cells also exhibited reduced phosphorylation of Akt/PKB in response to cAMP stimulation, indicating that AleA acts upstream of PI3K activity in a manner similar to RasC.

Despite the enriched expression of *rasC* in prestalk cells of slugs and the transient expression of *rasC* in prespore cells during late culmination, there is no evidence for a requirement for RasC beyond the aggregation stage of development. *rasC*$^-$ cells that had been pulsed with cAMP to circumvent the aggregation defect proceeded to complete development to form normally proportioned slugs and fruiting bodies. Furthermore, the fact that *rasC*$^-$ cells in chimeric mixtures were initially non-responsive to pulsatile cAMP signals from surrounding wild-type cells, but eventually became chemotactically competent after prolonged association, suggests that RasC is important during early aggregation, but is not necessary for later

developmental events. Late in aggregation, cAMP signaling is coupled through cAR2 and cAR4 receptors (Parent and Devreotes, 1996), possibly leading to activation of PKB-R1, an Akt/PKB-related kinase that is cAMP stimulated in a PI3K-independent manner (Meili *et al*., 2000). Thus, while cells during early aggregation require RasC-mediated activation of PI3K for maximum phosphorylation of Akt/PKB, cAMP receptor stimulation during late aggregation could activate PKB-R1 directly.

It is possible that an important role for a Ras protein during late development is masked by functional redundancy due to the presence of one or more Ras homologs. The spatial expression of *rasC* throughout most of development is remarkably similar to that described previously for *rasD* (Esch and Firtel, 1991), suggesting the possibility that RasC and RasD might perform similar functions. We generated *rasC*$^-$/*rasD*$^-$ double disruptant strains and found that these cells had exactly the same phenotype as *rasC*$^-$ single disruptants, including an aggregation defect that could be circumvented by cAMP pulsing (data not shown). Furthermore, the fruiting bodies that formed as a result of cAMP pulsing contained spores that exhibited no apparent defects. *rasC*$^-$/*rasD*$^-$ cells also exhibited the same wild-type-like transient ERK2 phosphorylation in response to cAMP stimulation as did *rasC*$^-$ or *rasD*$^-$ single disruptants (data not shown). It had previously been shown that *rasD*$^-$ strains had no developmental phenotype other than an inability to perform slug phototaxis or thermotaxis (Wilkins *et al*., 2000). The available evidence therefore suggests that the role of RasC in development is restricted to the early aggregation stage.

Our data suggest that RasC regulates cAMP synthesis during aggregation and is important for optimum chemotactic cell movement during early development. There is striking conservation in the signaling components required for chemosensing and chemotaxis between *Dictyostelium* and human neutrophils. In both systems, chemoattractant signals are transmitted through G-protein-coupled serpentine receptors, resulting in transient activation of PI3K, and spatial activation and translocation of Akt/PKB to the membrane (Firtel and Chung, 2000; Rickert *et al*., 2000). Our results provide the first direct link between a Ras protein and PI3K in cells undergoing chemotaxis. The recent demonstration that human PI3Kγ activity is stimulated when co-expressed with activated H-Ras and G$\beta\gamma$ in COS cells (Pacold *et al*., 2000) suggests the possibility that the G$\beta\gamma$-dependent activation of PI3K during neutrophil chemotaxis will also involve a Ras protein.

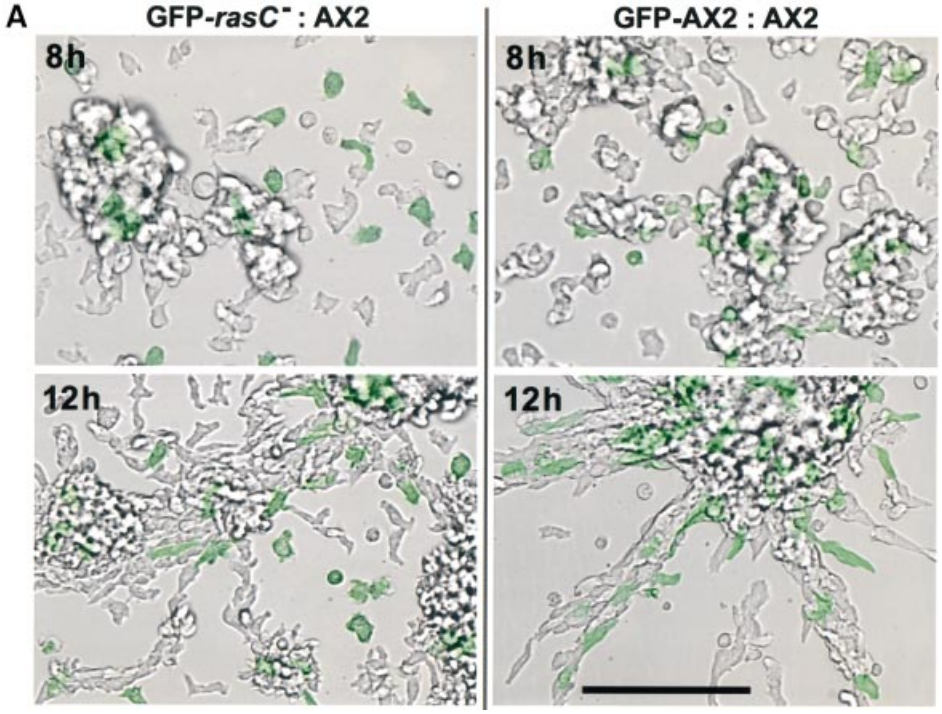
Materials and methods

Cell culture and development

Dictyostelium discoideum AX2 and transformed strains were grown in HL5 medium supplemented with 10 μg/ml blasticidin S, 25 μg/ml hygromycin (Calbiochem) or 10 μg/ml G418 (Gibco-BRL), as appropriate. To prepare cAMP-pulsed cells, vegetative cells were washed twice and resuspended to 5.0×10^6 cells/ml in KK2 (20 mM potassium phosphate pH 6.1), shaken at 160 r.p.m. for 1 h and pulsed every 6 min for 5 h with 50 nM cAMP. Washed vegetative or cAMP-pulsed cells were spread on nitrocellulose filters (Millipore) resting on KK2-saturated support pads to observe multicellular development. To observe aggregation streams, washed vegetative cells were seeded at ~5×10^5 cells/cm^2 in Nunclon tissue culture dishes submerged under Bonner's salts (10 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$). For growth and development on bacterial lawns, *Dictyostelium* cells were clonally plated in association with *Klebsiella oxytoca* on rich nutrient agar plates. Spores were treated in 1% Triton X-100 in KK2 prior to plating on bacterial lawns.

Transformations

Twenty micrograms of the *rasC* disruption vector were linearized with *Pvu*II, and electroporated into AX2 cells as described in Tuxworth *et al*. (1997), except that the $MgCl_2$, $CaCl_2$ healing step was omitted. All other transformations utilized the $CaPO_4$ DNA precipitation method as described previously (Nellen *et al*., 1987). Following 7–14 days of selection, transformants were subcultured and clonally re-isolated as plaques on bacterial lawns.



Molecular cloning and vectors

The *rasC* promoter was isolated using a PCR-based method of chromosome walking from the known *rasC* coding region towards the non-coding 5′ flanking region, as described previously (Min and Powell, 1998). Based on sequences derived from the chromosomal walk, the nested primers 5′-AAAACCTCAAACACACATATTTAC-3′ (RC10) and 5′-AAGATCTTAATAATTTTGACATTGTGTATTTTC-3′ were used to PCR amplify a 0.55 kb DNA fragment from AX2 genomic DNA, which was ligated into pGEM-T Easy (Promega) to generate pJLW12. The *rasC::*^{his}*lacZ* reporter plasmid was generated by directionally ligating the 0.55 kb *Spe*I–*Bgl*II *rasC* promoter fragment from pJLW12 into the *Xba*I–*Bgl*II sites of the *psa::*^{his}*lacZ* plasmid (Detterbeck *et al*., 1994), replacing the *psa* promoter. The *rasC::rasC* expression plasmid was generated by directionally ligating the 0.67 kb *Bam*HI–*Xho*I full-length *rasC* cDNA into the *Bgl*II–*Xho*I sites of the *rasC::*^{his}*lacZ* plasmid, replacing the *lacZ* gene.

The *rasC* disruption vector, containing both genomic and cDNA *rasC* sequences flanking a blasticidin S resistance (*bsr*) selectable marker, was constructed as follows. First, a 1.6 kb genomic fragment encompassing the promoter and the complete *rasC* coding region was PCR amplified from AX2 DNA using RC10 and 5′-TTACAATATAATACATCCCC-TTTTCTTTG-3′, and ligated into pGEM-T Easy to generate pJLW15. The terminal 5′ 0.84 kb *Afl*III (blunted)–*Eco*RI fragment, encompassing the promoter to the *Afl*III site within the second *rasC* exon, was excised from pJLW15 and directionally ligated into the *Sma*I–*Eco*RI sites of the *bsr* expression vector, pRHI119, to generate pJLW24. A 0.46 kb *Afl*III (blunted)–*Not*I fragment from the 3′ terminal end of a *rasC* cDNA plasmid was then directionally ligated into the *Spe*I (blunted)–*Not*I sites of pJLW24. This construct selectively omits use of the second *rasC* intron, which contains repetitive sequences, since earlier attempts to generate homologous recombinants using the full-length 1.6 kb genomic sequence were not successful. All plasmid constructions were verified by restriction mapping and DNA sequencing.

The pDHGABD(S65T) plasmid, which expresses a fusion protein comprising GFP and the actin binding domain from ABP-120, was provided by Dr D.A.Knecht. This is a variant that carries a hygromycin resistance selectable marker of the one described previously (Pang *et al*., 1998).

In situ β-galactosidase assays

AX2 cells transformed with the *rasC::*^{his}*lacZ* vector were developed on nitrocellulose filters, fixed *in situ* and stained for β-galactosidase activity as described previously (Detterbeck *et al*., 1994). Cells transformed with the more labile ^{ile}*lacZ* vector provided poor expression; therefore, the less

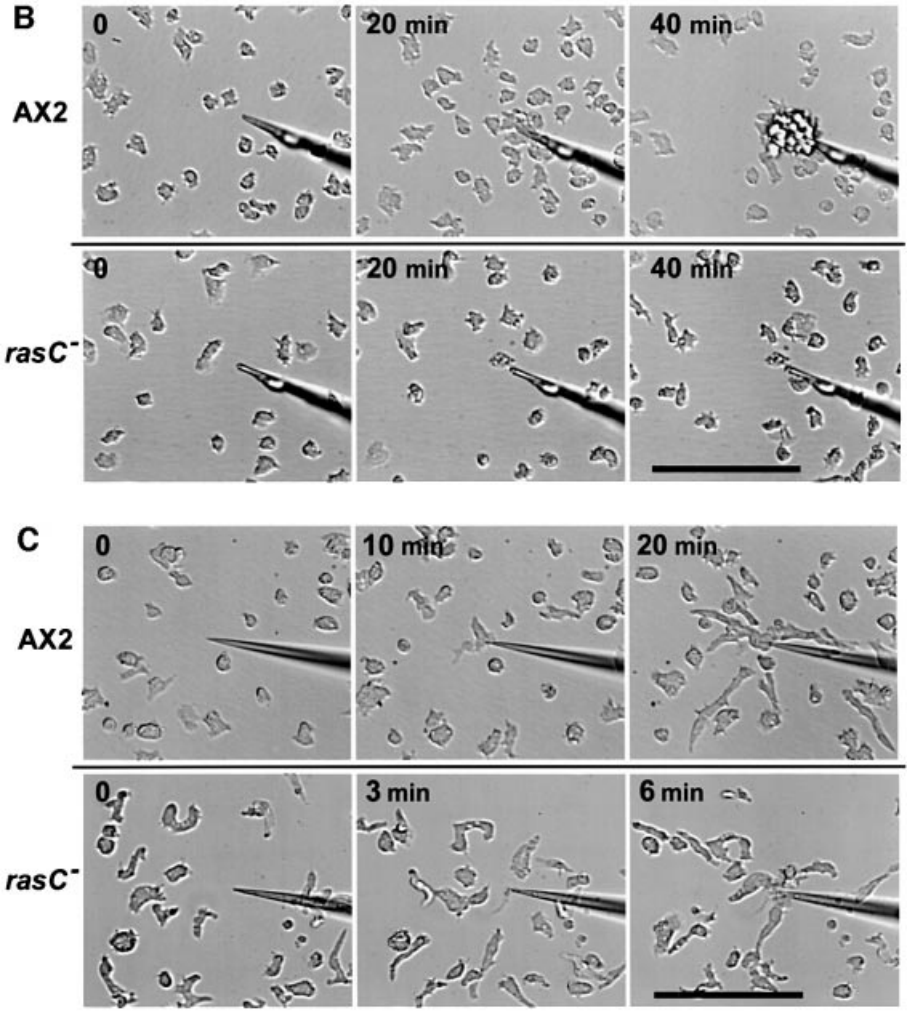


Fig. 6. cAMP-mediated chemotaxis. (**A**) Aggregation of *rasC*⁻ cells in chimeric mixtures with AX2 cells. GFP-labeled *rasC*⁻ or GFP-labeled AX2 cells were mixed with unlabeled AX2 cells in a ratio of 1:4 and seeded at 5×10^5 cells/cm^2 in Nunc tissue culture dishes submerged under Bonner's salts solution. Shown are the images of GFP-labeled fluorescent cells (in green) that have been overlayed on phase-contrast images of the total aggregating population, at 8 and 12 h of development. (**B**) AX2 and *rasC*⁻ cells seeded on Nunc dishes at 10^5 cells/cm^2 submerged under Bonner's salts were starved for 6 h and subjected to a micropipet filled with 100 μM cAMP at $T = 0$. Shown are phase-contrast images of the cells in the vicinity of the micropipet tip at the indicated times. (**C**) AX2 and *rasC*⁻ cells that had been pulsed with cAMP for 5 h were seeded onto Nunc dishes and subjected to a cAMP filled micropipet as described in (B). Bars = 100 μm.

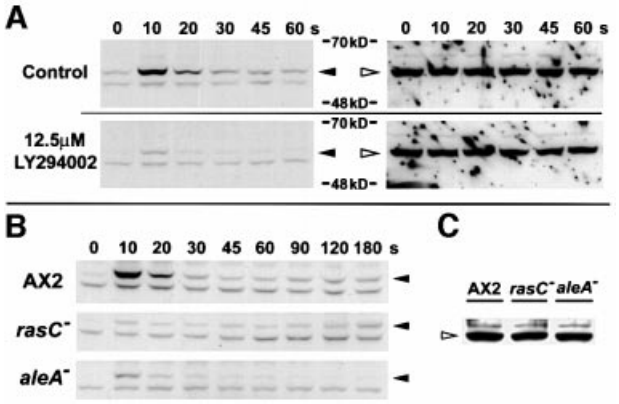


Fig. 7. cAMP-induced stimulation of Akt/PKB phosphorylation. (**A**) cAMP-pulsed AX2 cells were either not treated (Control) or pre-treated with 12.5 μM LY294002 for 1 min, before stimulation with 100 nM cAMP. At the indicated times, cells were lysed directly in SDS gel loading buffer, 10 μg of protein fractionated by SDS–PAGE and western blots probed with a phospho-threonine-specific antibody (left panels). The blots were subsequently stripped of bound antibodies and re-probed with an Akt/PKB-specific antibody (right panels). Molecular weight markers in kilodaltons are as indicated beside each blot. (**B**) cAMP-pulsed AX2, *rasC*– and *aleA*– cells were stimulated with 100 nM cAMP and analyzed as described in (A). Results shown are representative of at least three independent experiments for each strain. (**C**) Ten micrograms of protein from cAMP-pulsed AX2, *rasC*– and *aleA*– cells were analyzed by western blotting with the Akt/PKB specific-antibody to demonstrate equal Akt/PKB expression levels in all three strains. Closed arrowheads indicate phosphorylated Akt/PKB and open arrowheads indicate total Akt/PKB protein.

labile ^{his}*lacZ* variant was used. Staining reactions were conducted for 2 h or overnight at 22°C, and terminated by washing with Z-buffer.

Adenylyl cyclase assays

cAMP-pulsed cells were washed twice and resuspended to 5.0×10^7 cells/ml in KK2, and gently vortexed to maintain a uniform cell suspension. Cells were stimulated with 10 μM 2′-deoxy-cAMP in the presence of 5 mM dithiothreitol (DTT), and at the indicated time points 100 μl samples were lysed in 100 μl of 3.5% perchloric acid. The samples were then neutralized with 50 μl of 50% saturated $KHCO_3$, and cAMP levels in the supernatants were measured using a cAMP binding protein assay kit (Amersham TRK432).

The *in vitro* adenylyl cyclase assay has been described elsewhere (Pupillo *et al*., 1992; Chen *et al*., 1997). Briefly, cAMP-pulsed cells were treated with 2 mM caffeine for 30 min and washed three times with ice-cold KK2. Cells were then resuspended to 5×10^7 cells/ml in ice-cold 2 mM $MgSO_4$/KK2, mixed in equal volumes with lysis buffer (20 mM Tris–HCl pH 8.0, 2 mM $MgSO_4$) and immediately lysed by filtration through two 5.0 μm TMTP membranes (Millipore). For stimulation by GTPγS, 80 μM GTPγS and 2 μM cAMP were included in the lysis buffer. Lysates were kept chilled on ice for 5 min, 200 μl samples were then incubated for 2.0 min at 22°C in a reaction mix containing 10 mM Tris–HCl pH 8.0, 0.1 mM ATP, 1.0 mM cAMP, 10 mM DTT and 10^6 c.p.m. [α^{32}P]ATP (Amersham). Mn^{2+}-stimulated activity was measured by inclusion of 5 mM $MnSO_4$ in the reaction mix. Reactions were terminated with 100 μl of 1% SDS containing 1 mM cAMP and 9 mM ATP. cAMP was purified by sequential chromatography through Dowex-50 and alumina, as previously described (Salomon, 1979), and the eluted [^{32}P]cAMP measured by scintillation counting.

ERK2 and Akt/PKB phosphorylation assays

cAMP-pulsed cells were washed twice and resuspended to 5.0×10^7 cells/ml in KK2, and vortexed gently. Before and after addition of cAMP to a final concentration of 100 nM,100 μl aliquots were removed at the indicated time points and immediately mixed with 20 μl of 6× SDS gel loading buffer (Sambrook *et al*., 1989) supplemented with 300 mM NaF, 1.2 mM Na_3VO_4, 12 mM EDTA and protease inhibitors (Roche Complete). For the PI3K inhibitor experiment, AX2 cell suspensions were treated for 1 min with 12.5 μM LY294002 (Cell Signaling Technologies) prior to cAMP addition. Protein samples of 10 μg were fractionated by SDS–PAGE, blotted onto nitrocellulose (Amersham), blocked with non-fat milk and probed with the appropriate antibody following the manufacturer's instructions. Equal sample loading was verified by staining a duplicate gel with Coomassie Blue. To account for possible variations between multiple blots, standard cell extracts were included in each blot and the ECL autoradiogram signals verified by densitometric analysis (GeneQuant; Molecular Dynamics).

Chemotaxis assays

For the chimeric aggregation experiments, *rasC*– and AX2 cells transformed with pDHGABD(S65T) were mixed with non-labeled AX2 cells in a 1:4 ratio and seeded in Nunclon tissue culture dishes at ~5×10^5 cells/cm^2 submerged under Bonner's salts. Micropipet assays were carried out using cells starved for 6 h under Bonner's salts or 5 h cAMP-pulsed cells that were dispersed and seeded in Nunclon dishes. At $T = 0$, a micropipet (Eppendorf Femtotip) filled with 100 μM cAMP was positioned in the field of view and cell movements monitored by time-lapse microscopy. Phase-contrast and epifluorescent images were captured through an Olympus IX-70 inverted microscope equipped with a DAGE CCD camera using a Scion frame grabber and Scion Image 4.0.

Antibodies

The C-terminal 168- to 185-amino acid peptide of RasC (Daniel *et al*., 1994) was synthesized by the NAPS facility at the University of British Columbia. Rabbit polyclonal antiserum was raised against the peptide conjugated to keyhole limpet hemocyanin (Pierce). RasC antibodies were further purified by affinity chromatography against the peptide covalently coupled to AffiGel10 (Bio-Rad) according to the manufacturer's protocols. The purified antibody exhibited no cross-reactivity when tested in western blots against bacterially expressed glutathione *S*-transferase fusion proteins of *Dictyostelium* RasG, RasD, RasS, RasB and Rap1.

The Akt/PKB-specific antibody was a gift from F.Jiang and Dr R.Dottin (Hunter College, New York). Phospho-MAPK antibody (Cat#9101) and phospho-threonine antibody (Cat#9381) were from Cell Signaling Technologies.

Southern and northern hybridization analysis

Fifteen micrograms of total RNA extracted using TRIzol reagent (Gibco BRL) were size fractionated in 1.25% agarose–formaldehyde gels. Equal loading of samples was checked by observing the intensities of ethidium bromide-stained rRNA bands. Genomic DNA was salt/ethanol precipitated from the nuclear fraction of cells that had been lysed in 40 mM Tris–HCl pH 7.8, 1.5% sucrose, 0.1 mM EDTA, 6 mM $MgCl_2$, 40 mM KCl, 5 mM DTT and 0.4% NP-40. Ten micrograms of DNA were digested with the indicated restriction enzyme, size fractionated in 0.7% agarose/TBE gel, blotted onto Hybond N+ (Amersham) and hybridized to random primed ^{32}P-labeled probes as described previously (Sambrook *et al*., 1989). Probes used were: (i) 0.6 kb complete *rasC* cDNA fragment; (ii) 2.1 kb *Cla*I–*Nde*I *lacZ* gene fragment; (iii) 0.5 kb *Nco*I–*Nde*I *carA* gene fragment; and (iv) 1.1 kb complete *gα2* cDNA fragment.

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