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The *aimless* RasGEF is required for processing of chemotactic signals through G-protein-coupled receptors in *Dictyostelium*

Robert H. Insall*, Jane Borleis† and Peter N. Devreotes†

Background: Ras proteins are small GTP-binding proteins that play an essential role in a wide range of processes, particularly in mammalian growth control. They act as molecular switches, being inactive when GDP is bound, and active when associated with GTP. Activation is accomplished by guanine nucleotide exchange factors (RasGEFs); when RasGEFs interact with Ras proteins, GDP is allowed to escape, and is replaced by GTP. *Dictyostelium* responds to chemoattractants through typical seven transmembrane domain receptors and heterotrimeric G proteins. There are at least five different *Dictyostelium Ras* genes, whose functions are not yet known.

Results: We have isolated the *aimless* gene, which encodes the *Dictyostelium* homologue of RasGEFs, during a screen for insertional mutants that fail to aggregate. We found that *aimless* null mutants grew at a normal rate, but were severely impaired in both chemotaxis and activation of adenylyl cyclase, both of which are critical for the early stages of development. Although coupling between receptors and their G proteins is unaffected, and several cyclic AMP (cAMP)-mediated responses appear normal, activation of adenylyl cyclase by receptors and GTPγS (a non-hydrolyzable GTP analogue) is reduced by up to 95 %. The motility of mutant cells appears normal, suggesting a true defect in gradient sensing.

Conclusions: The discovery of the *aimless* gene adds an interesting new member to the family of RasGEFs. Our data suggest an unforeseen role for a RasGEF, and therefore presumably a complete Ras pathway, in the processing of chemotactic signals through G-protein-coupled receptors.

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Introduction

Chemotaxis, the process by which cells move towards sources of diffusible chemicals, is crucial to a range of processes. Mammalian leukocytes locate sites of infection by chemotaxis towards bacterial byproducts and host signals such as interleukins. Developing nerves locate their targets using diffusible messages such as netrins [1], among other cues. Amoebae like *Dictyostelium discoideum* hunt their bacterial food source by chemotaxis towards folates, and aggregate using chemotaxis towards secreted cyclic AMP (cAMP). Despite its importance, little is known about the mechanisms by which cells detect and respond to gradients. Chemotactic signals are generally detected by seven transmembrane domain receptors and G proteins, but the pathways through which activated G proteins control cell movement are not understood. Several proteins are implicated in the control of movement, in particular a range of small GTP-binding proteins related to Ras. In mammalian cells, the Ras-related proteins Cdc42 and Rac appear to regulate the formation of filopods and ruffles in response to growth factors [2], whereas Rho proteins are implicated in the control of focal adhesions and stress fibres [3]. Similarly, in *Saccharomyces cerevisiae*, the small GTP-binding proteins Bud1p and Cdc42p combine to control the actin cytoskeleton during the formation of new buds [4]. Activation of Ras proteins themselves causes complex and variable changes in the motility of mammalian cells, in addition to the more typical effects on the control of growth and differentiation. Presumably the changes in motility are mediated in part by activation of the Cdc42 and Rac pathways. However, the pathways that link seven transmembrane receptors, small GTP-binding proteins and motility remain poorly understood.

The connections between receptors and movement are more complex than those between receptors and other processes, because of a requirement for an additional level of information processing. Eukaryotic cells can read a static gradient across its length. For example, a stationary amoeba can detect a gradient and respond to it with localized actin polymerization [5]; in contrast, bacteria can only read gradients by detecting a rising concentration while moving. In *D. discoideum*, this effect may be demonstrated by challenging cells with a local source of chemoattractant in a micropipette; pseudopods are only extended towards the micropipette [6], even though receptors all over their

surfaces become occupied. The ability to read a stationary gradient implies that cells can compare receptor occupancy over their surfaces, and only respond where chemoattractant levels are higher than average. The receptors and components of the information processing system are unknown.

Dictyostelium is an increasingly popular experimental organism for studying the connections between signalling and motility [7]. Cells grow as individual amoebas, but upon starvation they move together to form a multicellular aggregate. This process is coordinated by cAMP, in an unusual role as an extracellular mediator. A small, random proportion of cells begin intermittently producing and secreting cAMP. The surrounding cells move chemotactically towards them, and secrete additional cAMP, in a process called cAMP relay [8]. This process reinforces and amplifies the initial cAMP signal and allows it to travel over a distance of several centimetres. Repeated stimulation by cAMP also induces the expression of genes required for aggregation, including an autocatalytic induction of several components of the cAMP signalling system [9,10].

Extracellular signals like cAMP are detected and processed by a conventional G-protein-coupled receptor system. Many of the proteins involved have been identified and their genes cloned. These proteins include two partially redundant seven transmembrane domain receptors, cAR1 and cAR3 [11,12], the α and β subunits of heterotrimeric G proteins [9,13], phospholipase C [14], and an adenylyl cyclase [15]. Eight Gα subunits are known [16–18], of which only one (Gα2) is known to be required for signalling through cAMP receptors [9]; each apparently couples to the single Gβ subunit [13]. We recently described the isolation of a novel cytosolic protein, CRAC [19,20], which is required for coupling of activated G proteins to adenylyl cyclase.

The mechanisms that underlie chemotaxis in *Dictyostelium* closely resemble those used in other types of crawling eukaryotic cells. *Dictyostelium* amoebae and polymorphonuclear leukocytes, for example, respond to chemoattractants with similar behaviour, and similar changes in levels of second messengers (reviewed in [21]). For example, chemoattractant stimulation causes calcium influx and phospholipase C activation in both cell types [22,23], as well as several transient changes in the cytoskeleton, including phosphorylation of myosin II [24] and a rapid polymerization of actin [25,26]. These changes correlate with a pattern of changes in cell shape.

In this paper we report the isolation of a *Dictyostelium* gene, *aimless*, which is required for normal activation of adenylyl cyclase and chemotaxis. Unexpectedly, the predicted protein shows considerable homology to Ras guanine nucleotide exchange factors (GEFs), a family of Ras-activating proteins which includes *Drosophila* and human Son-of-sevenless proteins (Sos and hSos1/2, respectively) and *S. cerevisiae* Cdc25p (reviewed in [27]). GEFs activate GTP-binding proteins by allowing bound GDP to escape and be replaced by GTP, in an analogous fashion to the activation of heterotrimeric G proteins by seven transmembrane receptors. The Sos and hSos proteins appear to mediate the effects of a range of signals, in particular those transduced by receptor tyrosine kinases. Receptor phosphorylation leads to recruitment of adaptor proteins, such as Grb2 and Sem5 [28]; these in turn recruit RasGEFs to the membrane. Membrane localization itself seems to be sufficient to cause activation of Ras. GEFs for other small GTP-binding proteins have been shown to play key roles in the control of cell movement. The Cdc24p and Bud5p GEFs — which activate the Cdc42p and Bud1p GTPases, respectively — cooperate to control the initiation and position of yeast budding. GEFs are implicated in metastasis; the gene encoding mammalian RhoGEF, *Tiam-1*, stimulates the invasiveness of tumour cells by increasing their motility. However, little is known about how molecules other than receptor tyrosine kinases can control the activity of GEFs.

Our results suggest that a pathway involving Ras is required for the processing of chemotactic signals from seven transmembrane domain receptors, and that the same pathway couples G proteins to chemotaxis and the activation of adenylyl cyclase.

Results

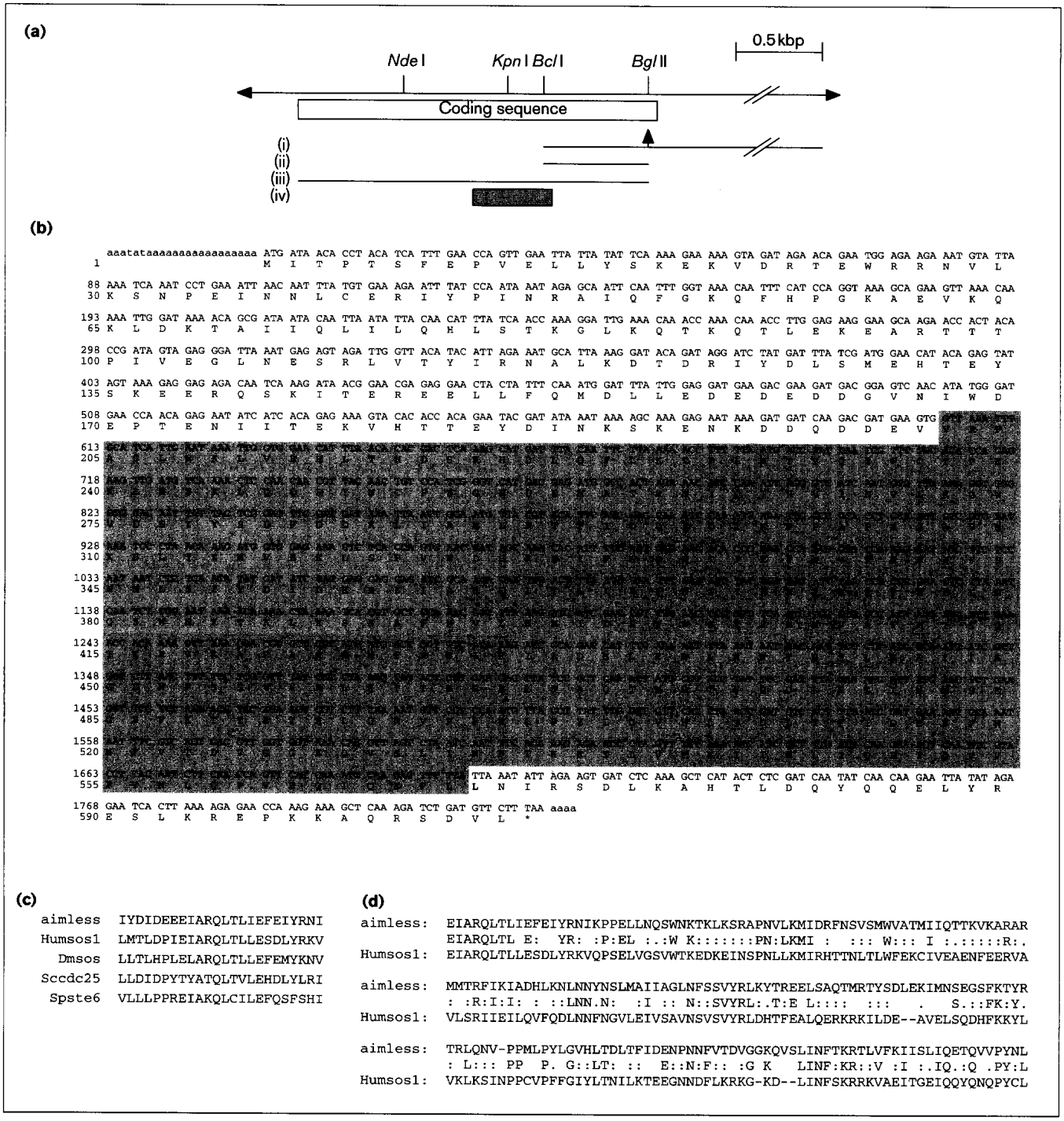
Mutagenesis and cloning of the *aimless* gene

The *aimless* (*aleA*) gene was identified during a screen for aggregation-deficient mutants. Cells were mutagenized using restriction enzyme mediated integration (REMI), in which the random insertion of a linear plasmid containing a *pyr5-6* marker gene into the genome is stimulated using a restriction enzyme [29], in this case *Bgl*II. Transformants were selected in minimal medium, then plated clonally on lawns of bacteria. The *aimless* mutant JB10 was isolated as a colony that showed no aggregation or development. The mutant cells were unable to migrate up gradients of cAMP, despite apparently normal motility in the absence of stimulus, hence the name *aimless*.

The region surrounding the insertion was cloned using standard REMI methods. Genomic DNA was digested with *Bcl*I, which does not cut within the inserted plasmid. The fragments were circularized, then used to transform *Escherichia coli*. A 7.5 kbp plasmid was rescued, containing the original plasmid plus genomic fragments of 0.6 kbp and 1.7 kbp, 5′ and 3′, respectively, to the insertion site. As shown in Figure 1a, analysis of this plasmid revealed that the REMI insertion had occurred just within the 3′ end of an open reading frame.

We obtained several isolates of the same 2.0 kbp *aimless* cDNA (see Fig. 1a) by screening a cDNA library prepared

Figure 1



Identification and cloning of *aimless*. **(a)** Schematic diagram of *aimless* clones. Shown below the restriction map of the genomic region are (i) the original REMI rescue; (ii) the probe used for library and Southern screens; (iii) the cDNA; (iv) the region deleted in the genome of RI-19. The arrowhead marks the site of the vector integration in JB-10. **(b)** cDNA sequence of the *aimless* gene and predicted coding sequence. The region of high homology to other known RasGEFs is shaded. The GenBank accession number for this sequence is U53884. **(c)** An example of the conservation between the predicted amino-acid sequence of the aimless protein and RasGEFs: human Sos1 (Humsos1), *S. cerevisiae* Cdc25p (Sccdc25), *Schizosaccharomyces pombe* Ste6 (Spste6). The sequence shown starts at amino-acid 349 in (b). **(d)** Extended alignment of the catalytic domains from the predicted aimless (amino acids 356–567) and human Sos1 (amino-acids 781–988) protein sequences. Identical amino acids are shown between the sequences; conservative substitutions are designated by a colon; a dot indicates a neutrally related amino acid.

from AX3 (wild-type) cells at the 3-hour stage with the 0.6 kbp coding fragment from the rescued plasmid. Sequence analysis (Fig. 1b) revealed an open reading frame of 605 amino acids which encodes a predicted 70.8 kDa protein. The carboxy-terminal half of the protein shows strong homology to various RasGEFs, in particular the *Drosophila* Sos [30] and its human homologue hSos1 [31]. A region of 210 amino acids around the presumed RasGEF catalytic domain of aimless was 34 % identical and 66 % similar to the equivalent region of Sos. The similarity between conserved domains of aimless and other RasGEFs was very high. Examples are shown in Figure 1c, which shows structurally conserved region 1 [27] from several RasGEFs, and Figure 1d, which shows an alignment between *aimless* and hSos1 covering the predicted catalytic domain. Outside the conserved catalytic domain, RasGEFs in general are divergent; many contain features such as PH domains and SH3 domains, but are otherwise different. The predicted carboxyl terminus of the aimless protein contained no common motifs, and showed no significant homology to any other proteins.

The insertion in the original mutant had occurred three codons from the end of the open reading frame. The phenotype could therefore have resulted from changes in RNA stability, a partially active protein, or a gene fusion which dominantly interferes with signalling. We therefore used homologous recombination to construct a mutant, RI-19, in which most of the region of the *aimless* gene that encoded the catalytic domain was replaced by *pyr5-6* (Fig. 1a). These cells had a similar phenotype to the REMI mutant in most respects; because they were less complex genetically, they were used in the work described in this paper.

RI-19 cells plated on non-nutrient agar did not show any signs of development or aggregation, even after several days of starvation (Fig. 2a). Under similar conditions, wild-type cells aggregated to form mounds within about 8 hours; these went on to form fruiting bodies within 24 hours. When the *aimless* cDNA was expressed in RI-19 cells under the control of an actin promoter, they aggregated (Fig. 2b) and developed (Fig. 2c,d) normally. Overexpression of the cDNA in wild-type cells, which caused 10–20-fold increase in mRNA levels, had no perceptible effect on development (data not shown).

Unexpectedly, if mutant cells were starved in suspension and provided with exogenous cAMP pulses to mimic normal development, then plated on non-nutrient agar, they could form functional but aberrant slugs (Fig. 2e,f); unlike wild-type slugs, which are normally fairly straight, these could be corkscrew-shaped or bent back upon themselves. The mutant slugs went on to form apparently normal fruiting bodies with heat-resistant spores (data not shown).

aimless expression during growth and differentiation

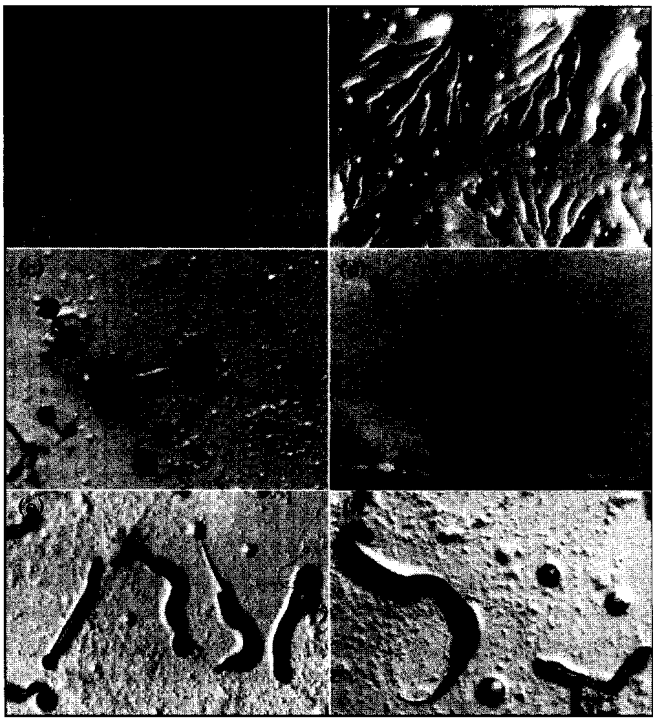
Growing cells expressed two different *aimless* transcripts of 3.0 and 3.5 kb (Fig. 3). When development in shaking suspension was initiated, the level of each transcript initially decreased by about 80 %. Each started to increase again after 2 hours of development, returning to greater than growth-stage levels at 4 hours and 6 hours (for the smaller and larger transcripts, respectively).

To ensure that this complex pattern of expression was not caused by non-stringent detection of other GEFs, we also probed *aimless* null (RI-19) cells (Fig. 3). A small quantity of a larger transcript was just visible, which represents a longer mRNA from the *aimless* gene with a *pyr5-6* insertion. Although aimless was expressed during growth, the deletion had no apparent effects on the appearance of the cells, or their growth rate on bacteria or in liquid culture (data not shown).

aimless is required for a subset of responses to cAMP

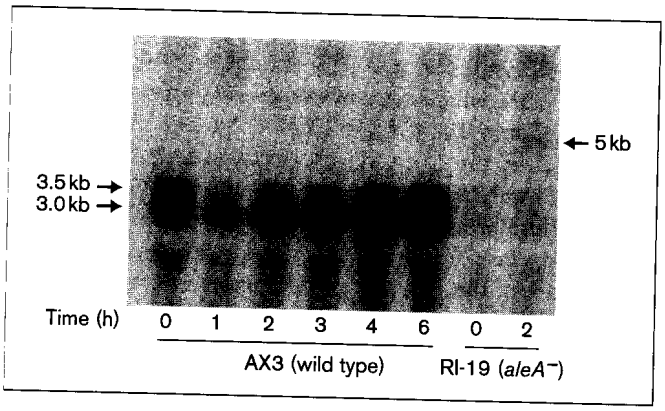
Pathways centred around Ras proteins have typically been associated with growth control and cellular development. Because the growth rates of *aimless*-null mutant and overexpresser cells, either on bacterial lawns or in liquid medium,

Figure 2



Phenotypes and rescue of *aleA*$^-$ mutants. **(a)** RI-19 (*aleA*$^-$) cells developed on non-nutrient agar for 36 h. **(b)** RI-19 cells constitutively expressing an *aimless* cDNA, developed on non-nutrient agar for 10 h. **(c,d)** The same cells, developed on non-nutrient agar for 36 h. **(e,f)** Unusual slugs formed after a suspension of RI-19 cells was treated with cAMP pulses for 8 h, then plated on non-nutrient agar.

Figure 3

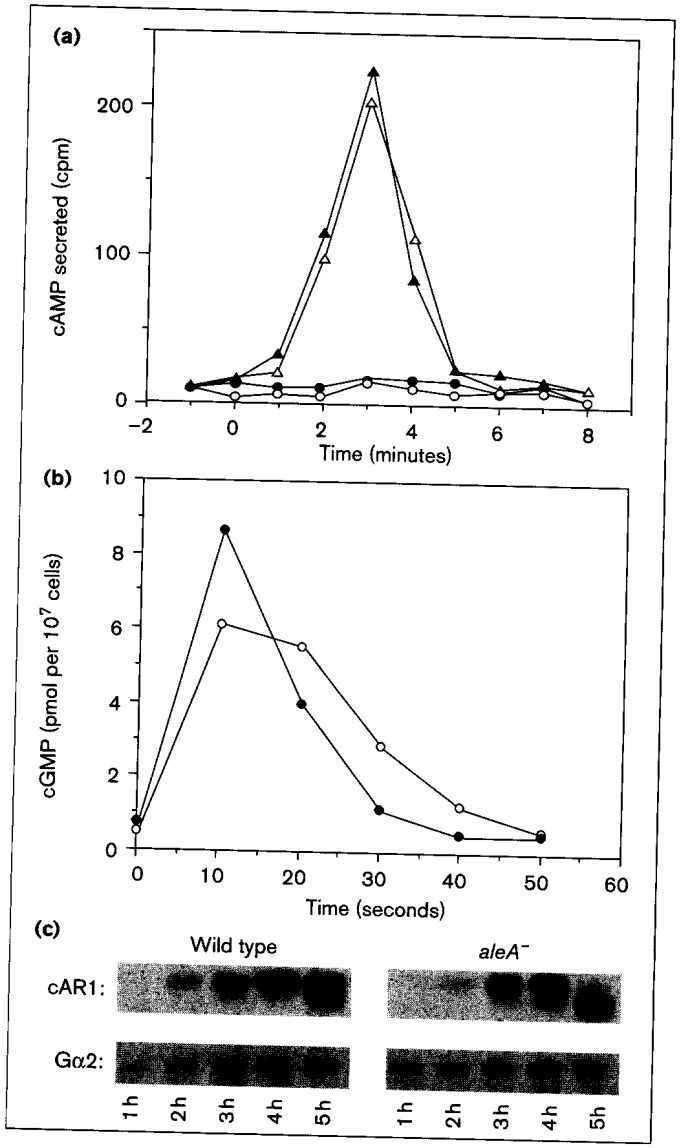


Expression of *aimless* mRNA during growth and early development. Wild-type (AX3) and *aimless* (RI-19) cells were developed in shaken suspension with addition of 100 nM cAMP pulses every 6 minutes. Samples were taken every hour. RNA was prepared using Catrimox-14, separated on a 1 % agarose/formaldehyde gel, blotted onto nylon, and probed with a full-length *aimless* cDNA. The bands visible in wild-type RNA are 3.0 and 3.5 kb; a very faint band of around 5 kb is visible in RNA from RI-19 cells.

were indistinguishable from controls (data not shown), it appears that this RasGEF is not required for growth control or cell-cycle progression. One possible explanation for the lack of aggregation of *aleA*⁻ cells could be a failure to express an essential, developmentally regulated component of the normal chemoattractant receptor signalling pathway. We therefore examined the expression of several components of cAMP signalling pathways whose levels are regulated during normal development, for example the cAMP receptor, cAR1. The levels of cAR1 rise following starvation, and are subsequently further induced by spontaneous cAMP oscillations [11]. In *aleA*⁻ mutants, cAR1 levels were induced normally, as long as the cells were repeatedly stimulated with exogenous cAMP (Fig. 4c). This suggests that the deficiency in *aleA*⁻ mutants is caused by an inability to generate cAMP pulses, and is not a developmental problem. Other proteins, including the α and β subunits of the G protein G2 and adenylyl cyclase (Fig. 4c and data not shown), were expressed at normal levels. Thus, the phenotype does not appear to be the result of either a failure to induce genes involved in aggregation, or a lack of any known signal-transduction component.

We next analysed the responses of second messengers to chemoattractant stimulation. Stimulation of cells by cAMP normally activates both guanylyl and adenylyl cyclases, with peaks in activity at about 20 and 120 seconds after stimulation, respectively [8,32]. We monitored adenylyl cyclase activity by labelling *aleA*⁻ mutant and control (*aimless* nulls rescued with *aimless* cDNA) cells with tritiated adenosine and perfusing them with buffer. As shown in Figure 4a, the rescued cells responded normally to

Figure 4



Second messenger responses in wild-type (AX3) and *aimless* null mutant (RI-19) cells. **(a)** cAMP-induced cAMP secretion. RI-19 cells (circles) and RI-19 cells rescued with a constitutively expressed *aimless* cDNA (triangles) were labelled with [³H]adenosine, developed on non-nutrient agar, then perfused with buffer. At time zero, the buffer was supplemented with 100 nM cAMP. cAMP was purified from the effluent, and tritiated cAMP was counted. Open and closed symbols represent duplicate cell samples. **(b)** cAMP-induced cGMP synthesis. Wild-type (AX3) cells (open circles) and *aimless* mutant (RI-19) cells (closed circles) were developed in shaken suspension for 5 h, then washed and resuspended at 2×10^7 cells per ml in DB (see Materials and methods). 100nM cAMP was added, samples were taken every 10 sec and the reaction stopped with perchloric acid. After neutralization, cGMP was measured using a radioimmunoassay kit. **(c)** Normal expression of signalling components in RI-19 (*aleA*⁻) cells. Wild-type (AX3) and *aimless* mutant (RI-19) cells were developed in shaken suspension with addition of 100 nM cAMP pulses every 6 minutes. Samples were taken every hour, dissolved in sample buffer, separated on a 10 % polyacrylamide protein gel, and electroblotted onto nitrocellulose. The blot was cut in two, and the appropriate parts were probed with polyclonal antisera directed against the Gα2 and cAR1 proteins. Bands were visualized using enhanced chemiluminescence.

persistent cAMP stimulation, by briefly secreting cAMP and then adapting. Similar results are seen when wild-type cells are used for the control (data not shown). The *aleA*$^-$ mutants, on the other hand, barely secreted any cAMP at all; a cAMP pulse of the normal duration but less than a tenth of the intensity of the rescued cells is detected.

Guanylyl cyclase, on the other hand, behaved normally in *aleA*$^-$ cells. A peak in cGMP levels occurred at the same time and to the same level as in wild-type cells (Fig. 4b). The aimless protein is therefore required for a subset of second messenger responses, and not a necessary component for signal perception.

Coupling between receptors and G proteins

The *aimless* null mutants expressed normal levels of adenylyl cyclase, but could barely activate it when stimulated. This could be caused by poor coupling between receptors and G proteins, or inefficient activation of the pathway that connects activated G proteins to adenylyl cyclase. To distinguish these possibilities, we performed two tests. Firstly, we assayed the affinity of receptors for cAMP in isolated membranes in the presence or absence of GTP. High-affinity cAMP sites reflect the binding of receptors to G proteins that are unoccupied by guanine nucleotides [33]. Addition of GTP to wild-type membranes causes G protein subunits to be occupied, and therefore to release the receptors; the affinity of the uncoupled receptors is lower, so they bind less ligand at subsaturating concentrations. When membranes from wild-type and *aleA*$^-$ cells were incubated with 2 nM tritiated cAMP, the addition of 50 μM GTP caused a 40.4 % and 42.3 % decrease in cAMP binding, respectively (Table 1a). There is therefore no obvious defect in receptor coupling to or activation of G proteins in the *aleA*$^-$ cells. Secondly, we bypassed G protein–receptor interactions by treating cell lysates with GTPγS (a non-hydrolyzable analogue of GTP), which directly activates G proteins [34]. As shown in Table 1b, GTPγS caused greater than 10-fold stimulation in the adenylyl cyclase activity of wild-type lysates, but only a 1.6-fold increase in lysates from *aimless* nulls. It therefore seems likely that the weak activation of adenylyl cyclase in *aleA*$^-$ mutants is caused by a defect downstream of the receptor–G protein interaction.

Because CRAC is required to connect G proteins to adenylyl cyclase [19,20], a deficiency in CRAC activity might underlie the phenotype of *aleA*$^-$ mutants. It has been demonstrated that *dagA* null mutants (*dagA* is the gene which encodes CRAC) show no activation of adenylyl cyclase by chemoattractants *in vivo* or by GTPγS *in vitro* [19]. If purified CRAC or cytosol from wild-type cells was added to mutant lysates, the ability of GTPγS to activate adenylyl cyclase was reconstituted. To test whether CRAC activity was normal in *aimless* null cells, we performed reciprocal reconstitution assays. Cytosols from wild-type and *aleA*$^-$ cells reconstituted *dagA*$^-$ lysates equally well (Fig. 5), which demonstrates that CRAC levels are normal in *aimless* nulls. Conversely, addition of wild-type CRAC did not rescue the *aimless*

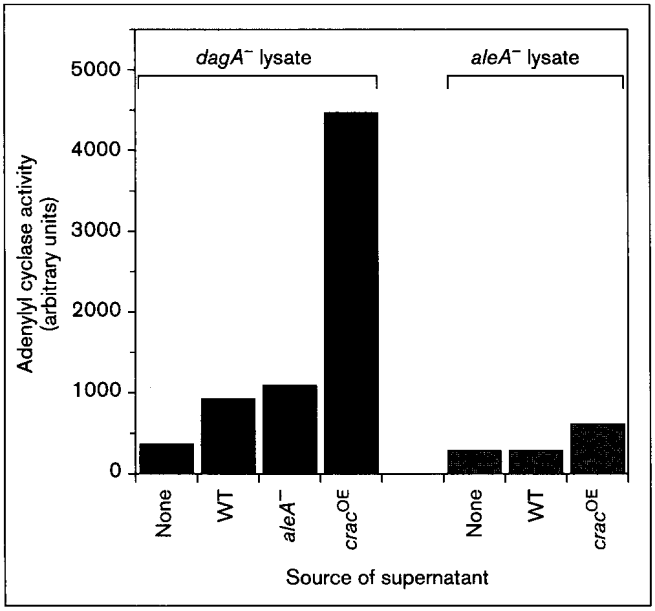
Table 1

Coupling between receptors and intracellular signalling.

(a)	Strain	cpm bound (−GTP)	cpm bound (+GTP)	% Inhibition
	AX3	7585	4517	41.4 %
	RI-19	4673	2557	45.3 %
(b)	**Strain**	**Unstimulated ACA activity (pmol min^{-1} mg^{-1})**	**Stimulated ACA activity (pmol min^{-1} mg^{-1})**	**Fold stimulation**
	AX3	1.3	18.5	14.3
	RI-19	1.5	2.4	1.6

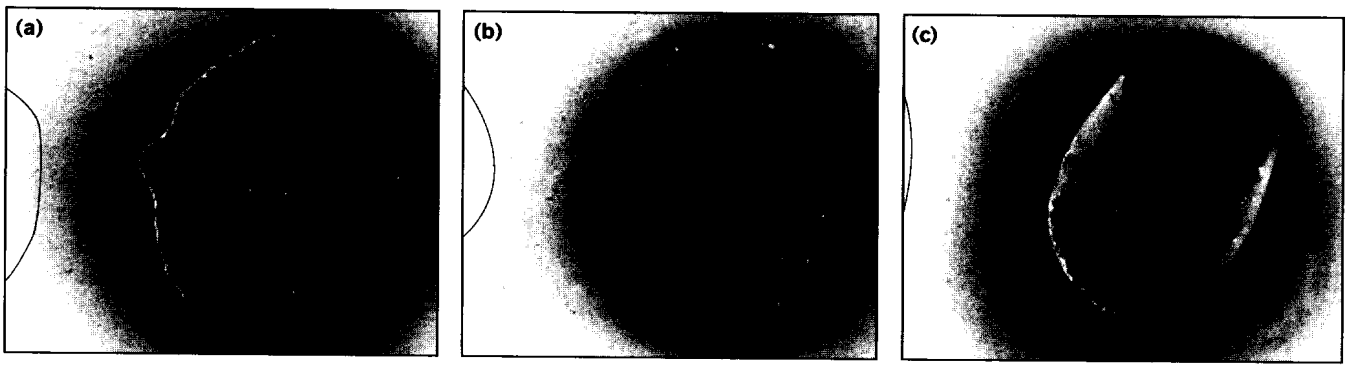
(a) Inhibition of binding of cAMP to its receptor by GTP. Wild-type (AX3) and *aimless* mutant (RI-19) cells were developed in shaken suspension for 5 h. Membranes were prepared by filter lysis, washed, and incubated with 2 nM [^{3}H]cAMP in the presence or absence of 50 μM GTP. Bound label was separated by centrifugation through silicone oil. Non-specific binding, measured by adding 1 mM unlabelled cAMP, was subtracted from all points. **(b)** Activation of adenylyl cyclase (ACA) activity in lysates by GTPγS. Wild-type (AX3) and *aimless* mutant (RI-19) cells were developed in shaken suspension for 5 h, then lysed in the presence or absence of GTPγS and assayed for ACA activity exactly as described in [12].

Figure 5



Reconstitution of CRAC activity. Activated lysates from *dagA*$^-$ (BW4, black bars) and *aleA*$^-$ (RI-19, grey bars) cells were mixed with cytoplasmic fractions from wild-type, AX3, cells (WT), RI-19 *aimless* mutant cells (*aleA*$^-$), and CRAC-overexpressing, RI-8, cells (*crac*^{OE}). The resulting mixtures were assayed for CRAC-dependent adenylyl cyclase activity as described in [20].

Figure 6



Chemotaxis assays. Small drops of **(a)** wild-type (AX3), **(b)** *aleA*$^-$ (RI-19), and **(c)** *aca*$^-$ (CAP1) cells were assayed for chemotaxis to a small drop of 100 nM cAMP by a variant of the method in [35]. Cells were developed for 6 h in shaken suspension with addition of 100 nM cAMP pulses every 6 minutes, then washed once in DB just before dropping. The positions of the cAMP drops are marked by fine lines.

phenotype. The addition of cytosol from wild-type cells reconstituted adenylyl cyclase activity in *dagA*$^-$ lysates, but did not affect *aleA*$^-$ lysates (Fig. 5). Similarly, cytosol from a CRAC overexpresser caused hyperstimulation of adenylyl cyclase in *dagA*$^-$ lysates, but had a relatively slight effect on *aleA*$^-$ lysates. The requirement for aimless protein for normal adenylyl cyclase activity cannot therefore be bypassed by increasing CRAC levels, and the *aimless* phenotype is not caused by a shortage of CRAC activity.

Chemotaxis and actin dynamics in *aimless* cells

Further observation of the behaviour of *aimless* null cells revealed a second defect, in chemotaxis. Figure 6 shows a small-drop assay [35], in which 0.1 μl of a cAMP solution is spotted near a 0.1 μl drop of cells. After 25 minutes, most wild-type cells had moved towards the drop of chemoattractant (Fig. 6a), whereas the *aleA*$^-$ cells showed almost no response (Fig. 6b). In keeping with the results for adenylyl cyclase, a weak chemotactic response was occasionally seen after a prolonged observation (2 hours after spotting, very occasionally less). The defect in adenylyl cyclase activation did not itself cause poor chemotaxis; mutants with defects in *dagA*$^-$ or *aca*$^-$ (the adenylyl cyclase expressed during aggregation) could produce an efficient chemotactic response under these conditions (Fig. 6c and data not shown). In addition, the *aimless* mutation did not cause a problem in normal cell motility; unstimulated wild-type and *aleA*$^-$ cells moved at approximately the same speed (8.2 ± 1.6 μm min^{-1} *versus* 9.5 ± 1.9 μm min^{-1}; data not shown), and their movement appeared similar when developed cells were viewed in a chemotaxis chamber.

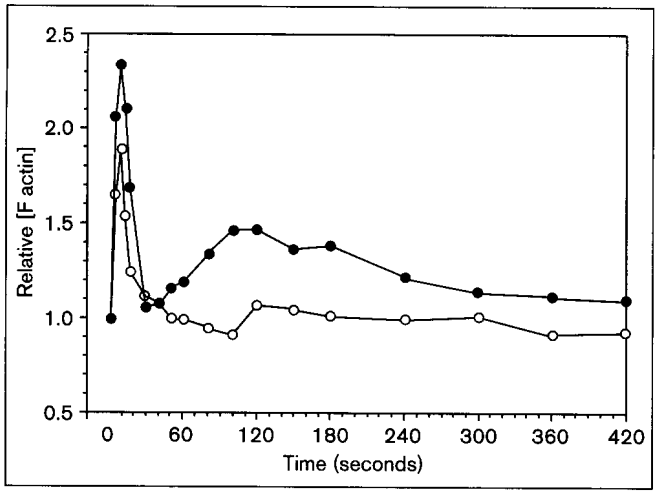
Stimulation of *Dictyostelium* or neutrophils with chemoattractants causes a rapid and complex polymerization of actin, which has been correlated with the extension of new pseudopods during chemotaxis [36]. We therefore investigated the levels of F actin in *aleA*$^-$ cells following treatment with cAMP. Wild-type cells showed the expected biphasic response, with a peak in F actin about 10 seconds after stimulation (which corresponds with the 'cringe' response) and a second peak at about 1 minute (Fig. 7). In *aleA*$^-$ cells, the time course of the response was similar, but levels of F actin were diminished after stimulation, especially at later times. Thus, the first peak was almost normal, but the later peak was diminished; F actin levels could sometimes be seen to drop down below resting levels following stimulation (Fig. 7).

Discussion

The *Dictyostelium aimless* gene encodes a protein with considerable homology to the family of Ras guanine nucleotide exchange factors. Many of the proteins in this family have been shown to catalyze exchange of nucleotides bound to Ras proteins, and to be required for normal Ras function [27]. The family members whose products do not affect Ras proteins (for example, *S. cerevisiae* Bud5p and Lte1p) are considerably more diverged from the consensus than *aimless*. It therefore seems likely that the signalling and chemotaxis phenotypes of *aimless* mutations are caused by a lack of normal activation of a Ras protein. This was an unexpected discovery; Ras proteins are more usually associated with control of cell growth and differentiation, rather than short-term processes like signalling and chemotaxis. Cell growth (under laboratory conditions at least) is unaffected by null mutations, so it would appear that the *aimless* pathway in *Dictyostelium* has other roles, including coupling chemoattractant receptors to chemotaxis and adenylyl cyclase.

In the recent cases where Ras proteins have been associated with short-term signalling and chemotaxis, for example towards platelet-derived growth factor [37], they operate in connection with tyrosine kinase receptors rather than G-protein linked receptors. Receptor tyrosine kinases are known to require Ras for activity, but the

Figure 7



Chemoattractant-induced alterations in actin polymerization. Wild-type (AX3, closed circles) and *aleA*$^-$ (RI-19, open circles) cells were developed for 6 h in shaken suspension with addition of 100 nM cAMP pulses every 6 minutes, then washed in DB, resuspended at 2×10^7 cells ml^{-1}, and shaken. 100 μl samples were taken, and F actin measured by a modification of the method of Hall *et al.* [36]. At time zero, 100 nM cAMP was added; the amount of F actin at different times was measured and is expressed relative to the amount in resting cells.

involvement of Ras in signalling mediated by G-protein-coupled receptors was surprising. Most physiologically relevant examples of chemotaxis, in particular movement of leukocytes to sites of infection, are similarly mediated by G-protein-coupled receptors rather than receptor tyrosine kinases. Recent reports demonstrate that the βγ subunits of G proteins can activate RasGEFs, apparently by recruitment of Grb2 following the phosphorylation of Shc adaptor protein [38]. The amino-acid sequence of the aimless protein has no obvious homology to sequences that are found in other RasGEFs, such as Grb2-binding sequences, PH domains or SH3 domains; such sequences are thought to mediate protein–protein interactions and could be involved in recruiting proteins to the membrane. We are trying to determine whether or not the aimless protein is recruited to the membrane or otherwise activated during cAMP signalling. If so, recruitment must presumably occur through the amino terminus, as the entire carboxy-terminal end is taken up with the presumptive catalytic domain.

A complex connection between adenylyl cyclase and chemotaxis

Certain mammalian adenylyl cyclases can be controlled by a direct interaction with activated G proteins [39]. The pathway now emerging from genetic analysis in *Dictyostelium*, in which genes such as *aimless*, *erkB* and *dagA* are required for normal function, therefore seems particularly complex. CRAC behaves like a simple adaptor, connecting activated G proteins to adenylyl cyclase [19], and *dagA* and *aca* mutants have very similar phenotypes [15,19]. Mutation of *erk2*, the gene encoding a recently described MAP kinase homologue, causes a more complex phenotype, including a failure to activate adenylyl cyclase and a block in later development [40]. The *aimless* gene is not required for development or several signalling pathways, but is necessary for adenylyl cyclase activation and chemotaxis. Both gene products seem to operate upstream of CRAC, perhaps by modulating the function of the G protein G2, and therefore they have additional phenotypic effects. This interplay may be a means of coordinating the different signalling pathways required during development.

Activation of G proteins generates two independent species, activated α subunits and free βγ subunits, which have been shown to act separately in several cases [41–43]. The simplest way of generating two counterbalancing signals from receptor occupancy would therefore be for the pathways downstream of α and βγ subunits to oppose one another. If this is the case, *aimless* would appear to be required for the normal effects of βγ subunits (for example, activation of adenylyl cyclase [13]) and not for functions that have been proposed to be mediated by α subunits (such as activation of guanylyl cyclase [44]).

Guanylyl cyclase is stimulated to a normal level by chemoattractants in *aleA*$^-$ cells. This could suggest that an attenuated signal from G proteins is sufficient to cause normal guanylyl cyclase activation. However, expression of dominant-mutant Gα2 subunits in wild-type cells causes a considerable drop in guanylyl cyclase activation, while affecting other pathways less severely [44]. It therefore seems more likely that there are at least two separate routes by which activated G proteins affect cellular processes, and that the pathway which leads to guanylyl cyclase is controlled exclusively by a branch of the pathway that does not involve *aimless* and that appears to be that mediated by Gα subunits.

Which Ras protein interacts with aimless?

There are several different Ras proteins that are potential targets for the putative GDP–GTP exchange activity of the aimless protein. In *Dictyostelium*, five genes are known to encode Ras proteins (as opposed to any other family of small GTP-binding proteins) [45–48]. In addition, *Dictyostelium* has other Ras-related genes, including one that encodes Rap [49]. RasD is the most likely candidate to play a role during developmental chemotaxis and, like aimless, its sequence is highly similar to its mammalian counterparts, and its expression peaks during the height of aggregation [45]. The similarity between RasD and RasG, both of which are expressed during growth, makes it possible that they share redundant functions [46]. Another gene, *RasS*, is also expressed during aggregation, but its sequence is far less similar to the mammalian family than

that of *RasD* [48]. Expression of activated RasD mutant proteins causes a subtle phenotypic effect [50]: although changes in cAMP signalling are seen, they are much less obvious than in *aleA*$^-$ mutants. This could suggest that RasD does not mediate the effects of aimless, but might also reflect insufficient expression of mutant protein to overcome normal controls. The identity of the target (or targets) of the aimless protein is currently being determined experimentally.

Other RasGEFs in *Dictyostelium*

Several lines of evidence suggest that there are other RasGEFs in *Dictyostelium*. Growth, development and phagocytosis are normal in *aleA*$^-$ mutants, yet expression of dominant-negative Ras mutants affects cell growth [50] (G. Weeks, personal communication). This suggests that another RasGEF functions early in development. Similarly, *aimless* null mutants can form slugs; their abnormal appearance suggests that *aimless* has a role in normal slug formation. Because cells must perform chemotaxis to make slugs, however, and the residual chemotaxis in *aleA*$^-$ mutants is very poor, it seems likely that another RasGEF allows chemotaxis late in development. The presence of at least six different Ras proteins in *Dictyostelium* suggests that a fairly large number of independent RasGEFs must be needed.

Chemotaxis, actin and Ras proteins

As discussed earlier, the ability of a cell to detect stationary gradients requires a mechanism for comparing the occupancy of receptors on different parts of its surface. Most receptor-mediated signalling is normal in *aleA*$^-$ mutants, and they move at a normal rate when not stimulated by chemoattractants. It is therefore possible that a ras pathway involving *aimless* is involved in the extra level of signal processing used during chemotaxis, and that *aleA*$^-$ mutants are unable to convert local receptor activation into localized actin polymerization and/or movement. It remains to be seen whether the slight difference in chemoattracant-induced actin polymerization is strong enough to account for the clear chemotaxis defects of *aleA*$^-$ cells. If not, it may be a secondary consequence of some other fundamental defect.

Conclusions

Our results show that the *aimless* RasGEF is required for normal activation of adenylyl cyclase by G proteins, and for chemotaxis to cAMP through seven transmembrane receptors. It appears that *aimless* is somehow involved in the additional processing of a subset of responses to G-protein activation. This implies that existence of a complete Ras pathway linking G proteins to motility and signalling. Identification of the other members of the pathway, the molecular connections between cAMP receptors and Ras, and the precise nature of the defects in the *aimless* mutants promise to illuminate basic mechanisms of chemotaxis.

Materials and methods

Cell growth, development and transformation

Cells were grown axenically in HL5 medium [51], except when selecting uracil prototrophs, when FM medium was used [52]. REMI transformation was performed essentially as in [29], using pRHI30 vector and DH1 cells. pRHI30 contains the *pyr5-6* gene with the 5′ untranslated region truncated 630 bp upstream of the start site by partial *Nde*I digestion, and the 3′ untranslated region trimmed to 100 bp using a PCR primer. DH1 (kindly provided by D. Hereld) is an AX3-derived line in which the entire *pyr5-6* sequence present in pRHI30 has been deleted. It differs from the HL330 used in [29] in that sequences 3′ to *pyr5-6* are retained. REMI mutagenesis, selection of aggregation-deficient mutants, and isolation of plasmid containing pRHI30 with fragments from the *aimless* gene either side of the integration site, were performed exactly as in [29].

For development on a solid substratum, cells were washed and allowed to starve on non-nutrient 1.5 % agar plates. For development in liquid suspension, cells were harvested, washed twice in DB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$ and 0.2 mM $CaCl_2$), then resuspended and shaken in DB at 2×10^7 cells ml^{-1}. After 1 h of starvation, pulses of 100 nM cAMP were applied every 6 minutes to mimic normal developmental signalling [53].

DNA and RNA preparation, blotting and library screening

Dictyostelium genomic DNA was prepared as described in [54], digested with the appropriate restriction enzymes, then electrophoretically separated on a 0.8 % agarose gel. Blotting and hybridization were performed according to [55] using random-primed cDNA probes. *Aimless* cDNAs were obtained from an amplification of a commercially prepared λgt11 library (Clontech) from 3 hour-developed cells, and were sequenced manually, using sequenase (USBC), and by machine (Johns Hopkins core facility).

RNA was prepared using catrimox-14 [56] (Iowa Biotech Corp., Iowa). Cells were developed in liquid suspension with pulses of cAMP for 0–6 hours, then 20 μl catrimox-14 was added to 100 μl samples of cells; the pellet was washed in 2 M LiCl, followed by 96 % ethanol, then air-dried. RNA was resuspended in 20 μl diethyl pyrocarbonate-treated water, quantitated, and equal amounts separated on a 1.0 % agarose gel in the presence of formaldehyde [57]. Blotting and probing, using a full-length randomly primed cDNA probe, were performed according to [57].

Accession number

The GenBank accession number for the sequence of the *aimless* gene is U53884.

Western immunoblotting

Membranes were prepared using ammonium sulphate lysis as described in Klein *et al.* [57], taken up in SDS sample buffer, separated by SDS–PAGE on 8 % polyacrylamide gels, and blotted onto nitrocellulose. cAR1, Gα2, Gβ and adenylyl cyclase were probed with the polyclonal antibodies described in [11,20,58,59]. Bands were visualized using an enhanced chemiluminescence kit (Amersham)

GTP inhibition of binding

The binding of cAMP to isolated membranes and its inhibition by GTP were measured as described in [33]. Briefly, membranes were prepared by filter lysis of developed cells, washed, and incubated in the presence of 2 nM [^{3}H]cAMP, with or without 100 μM GTP. Bound label was separated by centrifugation through silicone oil. Following centrifugation, the tubes were frozen in dry ice, the part of the tube containing the pellet was cut off, and the pellet thawed, dissolved in scintillant, and counted in a liquid scintillation counter. Non-specific binding was measured by adding 1 mM unlabelled cAMP and subtracted from all points. All measurements were made in triplicate.

Second messenger and actin assays

cGMP was measured as described using a kit (Amersham) as described in Mato *et al.* [60]. cAMP secretion was measured using a perfusion assay, as described in [61]. Developed cells (10^7 per filter) were perfused with DB at 7 drops per minute; at t = 0 the buffer was replaced with DB/100 nM cAMP. The perfusate was collected, cAMP was purified on Dowex and alumina columns [53], and the amount of secreted label measured by liquid scintillation counting. Adenylyl cyclase activity was measured as described in [53], and the reconstitution of adenylyl cyclase by supernatants containing CRAC as in [20].

F-actin levels were measured by a modification of the method of Hall *et al.* [36]. Instead of fixing the cells for 15 minutes then adding rhodamine-labelled phalloidin, the phalloidin was included in the fixative. This gave consistently higher and more reproducible results than [36]. In other respects the assay was identical. Cells were developed in shaken suspension for 6 hours, then washed and resuspended at 2×10^7 per ml in DB. Duplicate 100 µl samples were taken from the unstimulated suspension, then a final concentration of 100 nM cAMP was added, and 100 µl samples were taken at various times. The precise timing of the F-actin peak varied from experiment to experiment, so results were not averaged. The results of one representative experiment are shown in Figure 7.

Chemotaxis and cell motility

Chemotaxis was assayed by a variant of the method of Konijn [62]. Cells were developed in shaken suspension for 5.5–6 hours, then washed and resuspended at 2×10^6 ml^{-1} in DB containing 2 mM caffeine. Approximately 100 nl spots of cell suspension were then deposited on freshly poured 0.5 % agarose (in DB containing 2 mM caffeine), and 100 nl spots of DB containing 2 mM caffeine containing various concentrations of cAMP were placed adjacently. The plates were kept in a moist box, and examined after 20 minutes (by which time a response was easily visible in wild-type cells) and 60 minutes.

Cell motility was examined in a chamber like that described in Devreotes *et al.* [53]. Cells were developed in shaken suspension for 5.5–6 h, then allowed to adhere to a cover slip. This was sealed into the chamber with silicone grease, and the cells were perfused with DB for 10–20 minutes. Cells were viewed using a phase-contrast microscope connected either to a charge-coupled device video camera or a still camera.

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