A Density-sensing Factor Regulates Signal Transduction in *Dictyostelium*

Ita S. Yuen,* Renu Jain,* John D. Bishop,* David F. Lindsey,*‡ William J. Deery,* Peter J. M. Van Haastert,§ and Richard H. Gomer*‡

*Department of Biochemistry and Cell Biology, and ‡Howard Hughes Medical Institute, Rice University, Houston, Texas 77251-1892; and §Biochemistry Department, University of Groningen, 9747 AG Groningen, The Netherlands

Abstract. *Dictyostelium discoideum* initiates development when cells overgrow their bacterial food source and starve. To coordinate development, the cells monitor the extracellular level of a protein, conditioned medium factor (CMF), secreted by starved cells. When a majority of the cells in a given area have starved, as signaled by CMF secretion, the extracellular level of CMF rises above a threshold value and permits aggregation of the starved cells. The cells aggregate using relayed pulses of cAMP as the chemoattractant. Cells in which CMF accumulation has been blocked by antisense do not aggregate except in the presence of exogenous CMF. We find that these cells are viable but do not chemotax towards cAMP. Videomicroscopy indicates that the inability of CMF antisense cells to chemotax is not due to a gross defect in motility, although both video and scanning electron microscopy indicate that CMF increases the frequency of pseudopod formation. The activations of Ca^{2+} influx, adenylyl cyclase, and guanylyl cyclase in response to a pulse of cAMP are strongly inhibited in cells lacking CMF, but are rescued by as little as 10 s exposure of cells to CMF. The activation of phospholipase C by cAMP is not affected by CMF. Northern blots indicate normal levels of the cAMP receptor mRNA in CMF antisense cells during development, while cAMP binding assays and Scatchard plots indicate that CMF antisense cells contain normal levels of the cAMP receptor. In *Dictyostelium*, both adenylyl and guanylyl cyclases are activated via G proteins. We find that the interaction of the cAMP receptor with G proteins in vitro is not measurably affected by CMF, whereas the activation of adenylyl cyclase by G proteins requires cells to have been exposed to CMF. CMF thus appears to regulate aggregation by regulating an early step of cAMP signal transduction.

THE relative simplicity of the development of *Dictyostelium discoideum* lends itself to the study of fundamental issues such as the generation of cell-type diversity and the regulaton of differentiation. This simple haploid eukaryote normally exists as vegetative amoebae which eat bacteria on soil and decaying leaves and increase in number by fission. When the amoebae are starved for bacteria, they cease dividing and aggregate together using relayed pulses of cyclic AMP as the chemoattractant. The pulses of cAMP also regulate the expression of several developmentally regulated genes. The pulses of cAMP can be first detected approximately two hours after starvation. Aggregation then occurs between 5 and 10 h after starvation. The aggregate, containing typically 10^5 cells, forms a worm-like slug that crawls towards light. When the slug finds itself in a brightly lit, dry open area, a location favorable for spore dispersion, it develops into a fruiting body consisting of a mass of spore cells supported on a ~2-mm-high column of stalk cells (reviewed in Loomis, 1975, 1993; Van Haastert, 1991; Cubitt et al., 1992).

As they overgrow their food supply, the cells in an aggregation field will starve asynchronously. Some cells, unable to find a bacterium, will starve earlier than the others. We postulated that if this first cohort and the subsequent cohorts of cells were to each aggregate and form a fruiting body, the resulting structures would be small and relatively ineffective (Jain et al., 1992; Yuen and Gomer, 1994). Thus, to maximize spore dispersal, the cells in an aggregation field would need to form a single large fruiting body by coordinating their development. We have found that this coordination of development appears to be mediated by a mechanism that senses the density of starved cells and allows aggregation to occur only when there is a sufficiently high density of starved cells.

In submerged monolayer culture, *Dictyostelium* cells at relatively high densities (10^5 cells/cm^2) differentiate, whereas cells at low density (2×10^3 cells/cm^2) do not. However, when the low density cells are starved in buffer in which high density cells had previously been starved (a condi-

Address all correspondence to Richard H. Gomer, Dept. of Biochemistry and Cell Biology, HHMI-Rice University, Houston, TX 77251-1892. Tel.: (713) 527-4872. Fax: (713) 285-5154.

1. *Abbreviations used in this paper*: CMF, conditioned medium factor; CRAC, cytoplasmic regulator of adenylyl cyclase; IP_3, inositol 1,4,5-triphosphate.

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tioned medium), the low density cells then differentiate (Grabel and Loomis, 1978; Kay, 1982; Mehdy and Firtel, 1985). Timelapse videomicroscopy indicated that cell–cell contact is not needed for differentiation (Gomer and Firtel, 1987). *Dictyostelium* cells thus appear to secrete a self-stimulating soluble factor (conditioned medium factor, CMF)[1] during development.

CMF consists of two size fractions which both have CMF activity (Gomer et al., 1991). We purified the larger CMF as an 80-kD glycoprotein, sequenced tryptic peptides and used a degenerate oligonucleotide probe to isolate CMF cDNA (Jain et al., 1992). Database searches show that CMF shows no obvious similarity to any known protein and has no obvious motif content aside from a signal sequence. The small CMF fraction is a family of breakdown products of 80-kD CMF which, like it, are glycosylated (Yuen et al., 1991), although glycosylation is not part of the active site of CMF, which lies within an 88–amino acid region near the NH_2 terminus (Jain and Gomer, 1994). Neither class of CMF is the PFS density sensing factor used by *Dictyostelium* cells in late growth phase (Clarke et al., 1992). We found that CMF is sequestered in vegetative cells and is then secreted upon starvation (Yuen et al., 1991). CMF is sensed by cells via a developmentally regulated cell surface receptor (Jain and Gomer, 1994).

CMF potentiates its own accumulation into starvation buffer (although it is secreted by starved cells regardless of cell density); little else including pH, light, cAMP pulses or cell cycle phase at the time of starvation affects CMF's accumulation rate or activity threshold (Yuen and Gomer, 1994). We used diffusion calculations to show that even after many hours of continuous secretion, the CMF concentration adjacent to an isolated starved cell on a leaf or soil surface would be too low to allow differentiation, whereas an extracellular concentration of CMF sufficiently high to allow differentiation would occur when starved cells are at high densities. There is a close match between the predicted and experimentally observed density necessary for differentiation. To gain insight into the function of CMF, we used antisense transformation to make cells lacking CMF (Jain et al., 1992). When starved, the CMF antisense transformants did not aggregate unless exogenous purified or recombinant CMF was added. The theoretical and observed behavior of cells at different cell densities thus suggested that due to its accumulation rate, diffusion coefficient, and activation threshold, CMF can function as part of a cell density-sensing system which allows *Dictyostelium* cells in the wild to coordinate the onset of the cAMP pulse-mediated aggregation.

When a pulse of cAMP arrives at a starved *Dictyostelium* cell which is in the presence of high levels of CMF, three things happen. The cell moves towards the source of cAMP, a burst of cAMP is released by the cell to relay the signal, and the expression of specific classes of genes is affected (Mann and Firtel, 1989). The incoming cAMP pulse is sensed by cell surface cAMP receptors. There are several cAMP receptor genes, all of which are developmentally regulated (Saxe et al., 1991). The *Dictyostelium* cAMP receptor which mediates chemotaxis, cAR1, contains seven transmembrane domains, typical of receptors which interact with G-proteins (Klein et al., 1988; Saxe et al., 1988). The binding of cAMP to the cAMP receptors causes the receptors to activate G proteins (Van Haastert, 1984; Theibert and Devreotes, 1986; Van Haastert et al., 1987). During development, at least eight different G protein α subunit genes are expressed (Wu and Devreotes, 1991; Cubitt et al., 1992). Gα2 appears to be the subunit which mediates many of the downstream responses to activation of the cAMP receptor, including chemotaxis and gene expression (Kumaga et al., 1989, 1991).

G proteins are involved in transiently activating at least three enzymes in response to a pulse of cAMP: guanylyl cyclase, phospholipase C, and adenylyl cyclase (see Firtel et al., 1989; Dottin et al., 1991; Peters et al., 1991; and Van Haastert et al., 1991 for review). In addition, there is a transient uptake of extracellular Ca^{2+} 5 s after cAMP activation (Bumann et al., 1984), which does not appear to be mediated by G proteins, intracellular cAMP, or cGMP (Milne and Coukell, 1991; Milne and Devreotes, 1993).

Gα2, activated by the cAMP receptor, activates guanylyl cyclase. This causes a transient elevation in intracellular cGMP with a peak at 9–12 s after stimulation of cells with cAMP (Newell et al., 1988; Van Haastert et al., 1989). Gα2 also activates phospholipase C, resulting in a transient rise in inositol 1,4,5-trisphosphate (IP_3) which peaks at ~6 s (Europe-Finner and Newell, 1987*a*,*b*; Van Haastert et al., 1989; Okaichi et al., 1992). Interestingly, the Gα2 which activates phospholipase C is regulated by a cAMP receptor that is not cAR1 (Bominaar and Van Haastert, 1994). Both the cGMP and the IP_3 levels return to baseline ~30 s after their peak. The activation of adenylyl cyclase occurs over several minutes and is mediated by the $\beta\gamma$ heterodimer moiety of an activated G protein (Okaichi et al., 1992). The GTPγS activation of adenylyl cyclase in vitro requires membranes and a cytosolic protein designated cytoplasmic regulator of adenylyl cyclase (CRAC) (Theibert and Devreotes, 1986; Van Haaster et al., 1987; Snaar-Jagalska and Van Haastert, 1988; Insall et al., 1994). A nonaggregating mutant, *Synag 7*, appears to lack the factor (Theibert and Devreotes, 1986).

In this report, we examine how CMF regulates the aggregation of cells. We find that CMF regulates the ability of cAMP to activate Ca^{2+} influx, guanylyl cyclase, and adenylyl cyclase and that CMF has no effect on the activation of phospholipase C by cAMP. This suggests that CMF regulates aggregation by regulating aspects of cAR1-mediated cAMP signal transduction.

Materials and Methods

Cell Culture and CMF Production

Cells were grown, conditioned medium (CM) was prepared, and CMF assays were performed as previously described (Jain et al., 1992; Jain and Gomer, 1994). Ax-4 and K2 control transformant cell lines were recloned and checked for the ability to aggregate monthly. The K3 CMF antisense transformant (Jain and Gomer, 1994) was recloned monthly, and repeatedly checked for the phenotype of no aggregation on filter pads except when starved in the presence of CMF. The 722 axenic *Synap 7* cell line was a gift from Dr. Peter Devreotes. Starvation of cells was initiated by centrifugation at 800 *g* for 5 min, and resuspension and recentrifugation twice in PBM (20 mM KH_2PO_4, 10 μM $CaCl_2$, 1 mM $MgCl_2$, pH 6.1, with KOH). The cells were resuspended in PBM to a final density of 1×10^6 cells/ml, and shaken at 180 rpm. For the Ca^{2+} influx assays, cells were starved on filter pads as described in Jain and Gomer (1994). All growth and development was done at 21°C. To assay for live cells by dye exclu-

sion, saturated trypan blue was added at an equal volume to cells. Recombinant CMF was prepared and assayed for protein concentration and CMF activity as described in Jain and Gomer (1994), and stored as aliquots of 100 ng/ml in PBM at −70°C. 1 μl of thawed CMF (0.1 ng) or PBM were then added to each 100 μl of cells.

Northern Blots

RNA isolation and Northern blots were performed as described (Jain et al., 1992) with the exception that after hybridization, blots were washed three times with 2× SSC, 0.1% SDS at room temperature for 15 min each and then three times for 15 min each with 0.1× SSC, 0.1% SDS at 55°C. The probe was a 1.3-kb EcoRI fragment of the cAR1 cDNA (a gift from Dr. Alan Kimmel, National Institutes of Health, Bethesda, MD). Autoradiography on preflashed Kodak X-omat AR5 film was done at −70°C for one to three weeks. Blots were then stained with methylene blue (Monroy, 1988) to verify RNA transfer.

Chemotaxis Assays

Chemotaxis of cells was determined by the small drop method as described in Konijn (1970). Cells were starved for 6 h in PB (3.20 mM Na_2HPO_4, 6.94 mM KH_2PO_4, pH 6.5) at 1×10^7 cells/ml in shaking culture. Approximately 1 μl of cells were placed on PB/agar plates next to 1-μl droplets of cAMP. Chemotaxis was also determined by punching holes with the back end of a Pasteur pipette in agar/PBM plates. One microliter of cells at a density of 1×10^7 cells/ml in PBM was placed 3 mm away from the edge of the holes. 6 h later, the holes were filled with 1 mM or 0.1 mM cAMP in either water or 5 mM caffeine and the morphology of the spots of cells was examined 4 h later with a Nikon microphot Fx and a 4× objective.

Measurement of Motility

Cell translocation and pseudopod activities were studied by videomicroscopy using a Nikon TMS inverted microscope with an Ikegami ITC-400 TV camera connected to a Sony EVT 801 timelapse videocassette recorder. Cells were starved at low density in submerged monolayer culture in PBM or CM in plastic petri dishes (Fisher, Pittsburgh, PA) following Gomer and Firtel (1987). We observed cells in two modes. In the first, to determine gross translational movements, cells were observed by videomicroscopy continuously for 15 h with a 4× objective, giving a 1-mm field of view. One frame was recorded every 8 s (when played back, events were sped up 480 fold). In the second mode, cells were videotaped, after having been starved for 3.5 to 12 h, at 4 frames/s for 100 min with either a 10× or 40× phase contrast objective.

Scanning Electron Microscopy

Cells were starved in submerged culture at a density of 3×10^5 cells/cm^2. Wild-type cells were also allowed to develop in the presence or absence of gel-purified CMF at a density of 4×10^3 cells/cm^2. At the indicated times, cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 on ice for 1 h, followed by two 10-min washes in 0.1 M cacodylate/5% sucrose, pH 7.2. After dehydration in a series of increasing concentrations of ethanol following Millonig (1976), samples were critical-point dried using an Autosamdri-814 (Tousimis Research Corp., Rockville, MD) and platinum/palladium coated using a Balzers SCD 050 Sputer Coater. Scanning electron microscopy was performed with a JSM-5300 (JEOL U.S.A. Inc., Peabody, MA).

IP_3, cAMP, cGMP Production, and Ca^{2+} Influx

The production of IP_3 in response to a pulse of cAMP was determined following Van Haastert (1989) using a kit (Amersham Corp., Arlington Heights, IL) for the IP_3 assay, with the exception that the assay was performed using 100 μl of supernatant from the neutralized cell extract. Unless indicated otherwise, the uptake of $^{45}Ca^{2+}$ by cells in response to a pulse of cAMP was determined following Milne and Coukell (1991) except that the uptake medium contained 10 μM $CaCl_2$. Ca^{2+} influx assays were also performed in the presence of cobalt following Milne and Devreotes (1993). The production of cAMP in response to a pulse of cAMP was determined following Van Haastert (1984). Cells were stimulated with 10 μM of the functional cAMP analog 2′-deoxy-cAMP in the presence of 10 mM dithiothreitol (an inhibitor of the *Dictyostelium* phosphodiesterase). At 0, 3, and 5 min after stimulation, the cells were lysed and cAMP was measured with an isotope dilution assay kit (Amersham). The production of cGMP in response to 10^{-7} M extracellular cAMP was determined in a similar manner following Kesbeke et al. (1988) using a cGMP assay kit (Amersham).

cAMP Binding Assays

Binding of cAMP to cells was determined following Van Haastert and Kien (1983). Cells were starved by shaking in PBM for 5 h, then washed twice in PB and resuspended to 1×10^8 cells/ml. 45 μl of cells was added to a mixture of 5 μl of 100 mM dithiothreitol containing 20 nCi [^{3}H]cAMP, and 425 μl of 90% saturated ammonium sulphate in PB; 25 μl of 1 mg/ml bovine serum albumin was then added. After incubating for 5 min at 0°C, the cells were collected by centrifugation at 10,000 *g* for 2 min. The pellet was resuspended in 100 μl of 1 M formic acid and dissolved in 1.3 ml of ScintiVerse II scintillation fluid (Fisher, Fair Lawn, NJ). For binding to cells starved at low cell density, Ax-4 cells were starved at 5×10^4 cells/ml in the presence or absence of 1 ng/ml bacterially synthesized CMF. After 5 h, cells were harvested, washed twice in PB, and resuspended at 1×10^8 cells/ml; cAMP binding was immediately measured as described above. Scatchard plots of cAMP binding were done with a variety of [^{3}H]cAMP concentrations in the presence of ammonium sulphate as described above.

G Protein Assays

Preparation of membranes and assays of the ability of GTPγS to inhibit the binding of [^{3}H]cAMP to membranes followed Kesbeke et al. (1988), with the exceptions that cells were starved by shaking for 5 hours at 1×10^7 cells/ml in PBM, and the binding reactions were done in 50 μl of (2.56 mM Na_2HPO_4, 5.55 mM KH_2PO_4, 10 mM dithiothreitol) in the presence or absence of 30 μM GTPγS. After binding for 5 min at 0°C, the membranes were collected by centrifugation and the pellets were resuspended in 100 μl of 1 M formic acid, allowed to sit overnight and dissolved in 1.3 ml of scintillation fluid. The effect of 10 μM cAMP on the binding of [^{35}S]GTPγS to membranes was determined following Kesbeke et al., (1990), except that cells were starved for 5 h by shaking in PBM, and the reactions were done in 50 μl and contained 1 instead of 10 mM $MgCl_2$. The effect of cAMP on the binding of [^{3}H]GTP to membranes were determined following Snaar-Jagalska and Van Haastert (1988). The ability of GTPγS to stimulate adenylyl cyclase was determined following the method used by Theibert and Devreotes (1986) for Ax-3 cells, with the exception that cells were grown to a density of roughly 2×10^6 cells/ml and starved for 5 h at a density of 1×10^6 cells/ml, harvested, and assayed immediately. The reaction buffer contained no cAMP or labeled ATP, and the reaction was stopped by addition of EDTA to 20 mM and heating to 100°C for 2 min. cAMP levels were then determined using the Amersham cAMP assay kit.

Results

CMF Regulates Chemotaxis but not Motility

CMF antisense cells do not aggregate except when starved in the presence of CMF (Jain et al., 1992). To determine if CMF is necessary for viablity, we assayed exclusion of trypan blue and observed that Ax-4, control transformant, or CMF antisense cells starved for 8 h were alive (Table I and data not shown). To determine if CMF regulates aggregation by controlling chemotaxis, we examined the chemotaxis of cells in a gradient of cAMP. Using two different chemotaxis assays, we found that control transformant cells could chemotax towards cAMP whereas CMF antisense transformants did not (Table I). This indicated that cells in the absence of CMF do not respond to a gradient of cAMP. The inability of CMF antisense transformant cells to chemotax could be due to a defect in motility itself or to a defect in the control of motility. We used high magnification, high speed videomicroscopy to determine if the CMF antisense cells could move normally in a 5-min interval. We observed that at both 6 and 12 h after starvation,

Table I. Viability and Chemotaxis of CMF Antisense Cells

Cell line	Trypan blue exclusion at 8 h	Chemotaxis to cAMP						
		Small droplet assay			Stationary gradient assay			
		10^{-8}	10^{-7}	10^{-6}	0.1	0.1 + caffeine	1	1 + caffeine
		M			*mM*			
Control transformant	200/200	–	+	+	+	+	+	+
CMF antisense	200/200	–	–	–	–	–	–	–

Control transformant and CMF antisense cells were starved and tested for viability by trypan blue exclusion; the number of dye-excluding (viable) cells out of 200 cells examined is shown. Cells were also examined for the ability to chemotax towards the indicated concentrations of cAMP.

CMF has no obvious effect on the average speed of cell movement (Table II), although the distribution of speeds was quite large. Continuous timelapse videomicroscopy, at a lower magnification and time resolution, also indicated that CMF does not obviously affect the total distance that cells traveled from 0 to 12 h after starvation (data not shown). At 6 h after starvation, pseudopod extension and retraction occurs ~2 times/min in the absence of CMF and at 4 times/min in the presence of CMF. At 12 h after starvation, the pseudopod extension rate appears to drop slightly but is still higher in the presence of CMF (Table II). Thus CMF, which dramatically affects aggregation, does not strongly affect the speed of cell movement, but does affect pseudopod extension.

To examine in more detail the effect of CMF on the extension of pseudopods from cells, we used scanning electron microscopy to examine the morphology of cells. Ax-4 cells developing as they normally do at high cell density (Fig. 1 *A*), and Ax-4 cells starved at low density in the presence of purified CMF (Figure 1 *C*) show large numbers of ruffles and pseudopods as previously observed (Loomis, 1975). Ax-4 cells starved at low density in buffer alone (Fig. 1 *B*) tend to be much smoother. Vector-alone transformed cells (Fig. 1 *D*) starved at high densities have an appearance similar to untransformed cells starved at high cell densities, while CMF antisense transformants (Fig. 1 *E*) have many fewer pseudopods and ruffles. Together with the observations from high magnification videomicroscopy (Table II), this indicates that CMF increases the frequency of pseudopod formation.

The addition of cAMP to starved cells causes a dramatic change in cell morphology in ~5–15 s (Wessels et al., 1989; Segall, 1992). To determine the rate at which CMF stimulates pseudopod activity, fields of starved cells at cell densities too low to allow cAMP relay were examined by videomicroscopy before and after the addition of CMF. We observed that the frequency of pseudopod formation does not change significantly during the first 50 min of CMF treatment. Approximately 1 h after CMF addition, the frequency of pseudopod formation increases ~2.5-fold for 3.5-h starved cells and also for 4-, 5-, and 6-h starved cells (data not shown). The extent of stimulation varies from cell to cell, although the 1-h time delay does not. CMF alone thus affects cell morphology much more slowly than cAMP.

Table II. The Effect of CMF on Cell Speed and Pseudopod Extension

Cell line	Distance moved in 5 min	Pseudopodia extended in 5 min
	μm	
Ax-4 at 6 h	6 ± 4	9.3 ± 5.1
Ax-4 at 12 h	5 ± 3	6.1 ± 3.4
Ax-4 + CMF at 6 h	8 ± 7	23.0 ± 5.7
Ax-4 + CMF at 12 h	7 ± 3	16.5 ± 5.4

Cells were starved at low density in the absence or presence of 1.2 ng/ml purified CMF and then observed by timelapse videomicroscopy at the times indicated. Ten cells were observed for 5 min each and the distance translocated by the approximate center of the cell, and the number of pseudopods extended during the 5 min was measured. The means ± SD are shown.

CMF Is Required for cAMP Signal Transduction

One of the first observed responses of cells to a pulse of cAMP is an increase in the rate of Ca^{2+} influx (Milne and Coukell, 1991). The amount of Ca^{2+} taken up by stimulated or unstimulated untransformed and control transformed cells (Table III) was very similar to the corresponding amounts measured by Milne and Coukell (1991) at 6 h after starvation. In the absence of cAMP stimulation, CMF antisense transformed cells take up the same amount of Ca^{2+} as untransformed or control transformant cells, but do not take up significant additional Ca^{2+} in response to cAMP. The poor response of the CMF antisense cells to cAMP can be rescued by starving the cells in the presence of recombinant CMF. This suggests that CMF regulates the activation of Ca^{2+} influx by the cAMP receptor. To determine the kinetics of the CMF regulation of cAMP-stimulated Ca^{2+} influx, CMF antisense cells were starved in the absence of CMF. The cells were harvested for a Ca^{2+} influx assay and, at various times before the addition of cAMP, recombinant CMF was added to the cells. As shown in Fig. 2, exposure of cells to CMF for 10 s or more permits cAMP-induced Ca^{2+} influx. CMF had no effect on the cAMP-independent Ca^{2+} influx (data not shown). Ca^{2+} influx was also assayed for control transformant or CMF antisense cells following Milne and Devreotes (1993), which is a modification of the assay used above. As before, CMF antisense cells showed no significant Ca^{2+} influx in response to cAMP, whereas after a 10-s exposure to recombinant CMF they had cAMP-stimulated Ca^{2+} influx similar to that of untransformed Ax-4 cells (data not shown).

Another rapid response of developing cells to a pulse of cAMP is a transient rise in the level of intracellular IP_3, presumably due to an activation of phospholipase C (Europe-Finner and Newell, 1987*a*,*b*; Van Haastert, 1989). In the absence of cAMP stimulation, Ax-4, control transformant and CMF antisense transformant cells all contained

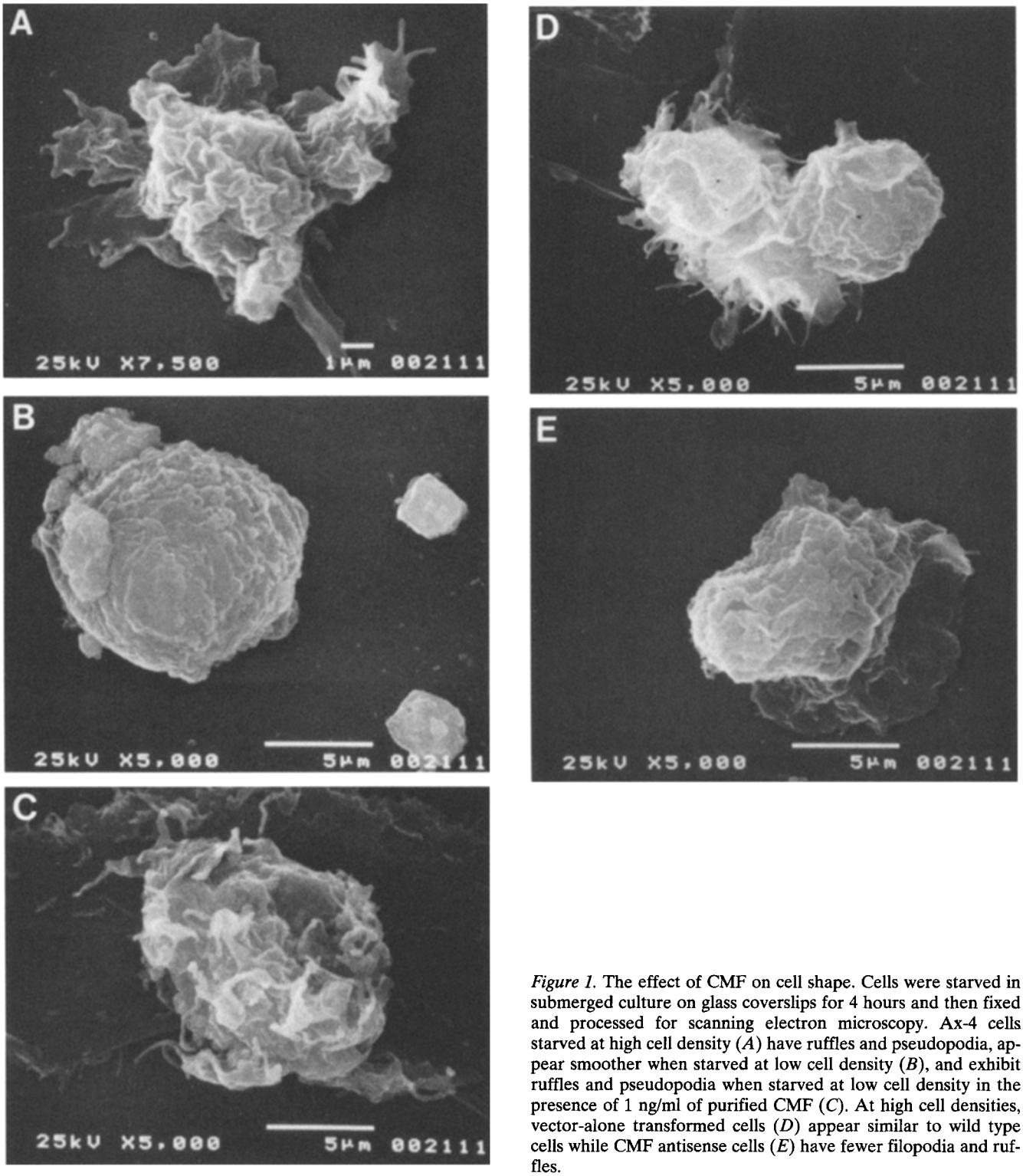

Figure 1. The effect of CMF on cell shape. Cells were starved in submerged culture on glass coverslips for 4 hours and then fixed and processed for scanning electron microscopy. Ax-4 cells starved at high cell density (*A*) have ruffles and pseudopodia, appear smoother when starved at low cell density (*B*), and exhibit ruffles and pseudopodia when starved at low cell density in the presence of 1 ng/ml of purified CMF (*C*). At high cell densities, vector-alone transformed cells (*D*) appear similar to wild type cells while CMF antisense cells (*E*) have fewer filopodia and ruffles.

approximately the same amounts of IP_3 (Table IV); these values are approximately half of the amounts previously reported (van Haastert, 1989). The amount of IP_3 secreted into the medium was also roughly the same for the three cell lines, and in close agreement with the previously observed values. 8 s after stimulation by cAMP, the amount of IP_3 in both the control and CMF antisense cells rose by approximately the same amount (Table IV), to the concentration of IP_3 previously observed in cAMP stimulated cells. The observed increase is probably predominantly intracellular, since IP_3 levels in the medium do not change after cAMP stimulation (Van Haastert, 1989). The similarity of the IP_3 responses in control and CMF antisense cells suggests that CMF is not required for the cAMP activation of IP_3 production in developing cells.

The ability of cells to synthesize a pulse of cGMP in response to a pulse of extracellular cAMP was measured in CMF antisense transformant and control cells in the pres-

Table III. The Effect of CMF on $^{45}Ca^{2+}$ Influx

Cell line	$^{45}Ca^{2+}$ taken up by cells in 30 s		
	−cAMP	+cAMP	Fold increase in Ca^{2+} influx
		nmol/mg protein	
Ax-4	0.097 ± 0.016	0.190 ± 0.021	1.93 ± 0.15
Control transformant	0.108 ± 0.016	0.210 ± 0.024	1.82 ± 0.08
CMF antisense	0.080 ± 0.011	0.081 ± 0.015	1.05 ± 0.01
CMF antisense + CMF	0.093 ± 0.015	0.173 ± 0.005	2.05 ± 0.02

Ax-4, control transformant, or CMF antisense cells were starved for 6 h in the presence (+CMF) or absence of 1 ng/ml recombinant CMF. The amount of $^{45}Ca^{2+}$ taken up by cells in 30 s was determined in triplicate for unstimulated cells, and for cells stimulated with cAMP. The mean ± SD for the averages for three separate experiments is shown. The ratio of stimulated influx to unstimulated influx was calculated separately for each of the three experiments, and the mean ± SD for the three ratios for each cell line is shown. The non-specific binding was 0.007 nmol/mg protein. A value of 1.00 for the fold increase would indicate that cAMP has no effect on the influx of Ca^{2+}.

ence or absence of purified bacterially synthesized CMF. The amount of cGMP in unstimulated cells was approximately the same for both cell types in the presence or absence of CMF (data not shown). The CMF antisense transformants poorly activated guanylyl cyclase in response to a pulse of extracellular cAMP (Table V *A*). The activation of guanylyl cyclase in response to a pulse of extracellular cAMP was increased when the cells were starved in the presence of bacterially synthesized CMF. This indicates that CMF controls the activation of guanylyl cyclase by the cAMP receptor. We found that exposure of CMF antisense cells to CMF for 10 s was sufficient to permit the cAMP stimulation of guanylyl cyclase (Table V *B*). Exposure of cells to CMF for 5 s also permitted cAMP stimulation of guanylyl cyclase, whereas when CMF was added at the same time as cAMP, poor stimulation was observed (data not shown).

The ability of cells to synthesize and secrete cAMP in response to a pulse of extracellular cAMP (the cAMP relay response) was also measured. The amount of cAMP in unstimulated cells was approximately the same for both cell types in the presence or absence of CMF (data not shown). The CMF antisense transformants were unable to activate adenylyl cyclase in response to a pulse of cAMP when examined at either 3 or 5 min after the pulse, but were able to activate adenylyl cyclase when starved in the presence of bacterially synthesized CMF (Table V *A*). To minimize secondary effects, we examined the effect of exogenous CMF only at 3 min after the pulse of cAMP. The addition of CMF to control cells had no obvious effect on cAMP levels. These results indicate that CMF also controls the activation of adenylyl cyclase by the cAMP receptor.

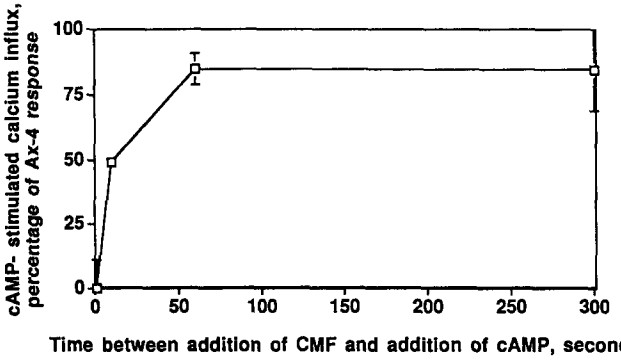

Figure 2. The effect of CMF on Ca^{2+} influx. Untransformed Ax-4 and CMF antisense cells were starved and assayed for cAMP-stimulated Ca^{2+} influx as described in Table III, with the exception that the CMF antisense cells were harvested and treated with 1 ng/ml recombinant CMF for 1, 10, 60, or 300 s before addition of $^{45}Ca^{2+}$ in the presence or absence of cAMP. The ratio of cAMP-stimulated Ca^{2+} influx in the CMF antisense cells to cAMP-stimulated Ca^{2+} influx in the Ax-4 cells was then calculated. Values are the average of five separate determinations; bars show the standard deviation. The error bars for 10-s exposure to CMF are smaller than the plot symbol.

CMF Does Not Affect the Availability of cAMP Receptors

To examine where in the pathway CMF regulates cAMP signal transduction, we first determined whether CMF regulates the availability of cAMP receptors. Binding of cAMP to cells was determined in nearly saturated ammonium sulfate for cells starved in the presence or absence of purified bacterially synthesized CMF. The presence or absence of CMF did not significantly alter the gross amount of cAMP binding (Table VI). As determined by analysis of Scatchard plots, the number and affinity of the two classes of cAMP binding sites in the presence of nearly saturated ammonium sulfate was similar to previous observations for wild-type cells (Van Haastert, 1985), and was not significantly affected by the presence or absence of CMF (Table VI). At low cell densities, where there would be very low levels of CMF, the addition of CMF also did not affect cAMP binding (Table VI). We also examined the expression of the cAMP receptor cAR1 mRNA in developing CMF antisense transformant and control cells. The cAR1 gene encodes the majority of surface receptors responsible for the activation of adenylyl cyclase and guanylyl cyclase (Klein et al., 1988; Sun et al., 1990). This gene produces a 1.9-kb transcript upon starvation; the amount then decreases between 6 and 8 h after starvation, and then increases again (Abe and Maeda, 1994). An additional 2.1-kb cAR1 transcript can be detected starting at approximately 10 h after starvation (Saxe et al., 1991 and Fig. 3 *A*). In the CMF antisense transformants, the 1.9-kb transcript is present in the vegetative and developing cells, while the 2.1-kb message is absent (Fig. 3 *B*). Although

Table IV. The Effect of CMF on IP_3 Production

Substance assayed	IP_3		Fold increase in IP_3 production at 8 s
	−cAMP	+cAMP	
		μM	
Ax-4 cells	1.5	ND	ND
Ax-4 medium	0.127	ND	ND
Control transformant cells	1.65 ± 0.22	5.56 ± 0.75	3.6 ± 0.2
Control transformant medium	0.101 ± 0.007	ND	ND
CMF antisense cells	1.71 ± 0.30	6.08 ± 0.47	3.6 ± 0.3
CMF antisense medium	0.116 ± 0.004	ND	ND

Ax-4, control transformant, or CMF antisense cells were starved for 5 h, collected by centrifugation, resuspended to 1×10^8 cells/ml and aerated for 10 min. For unstimulated cells, the cell suspension was then centrifuged and the amount of IP_3 inside cells or in the supernatant (medium) was determined. For cAMP-stimulated cells, cAMP was added after the 10-min aeration and 8 s later the amount of IP_3 in the entire suspension was determined. The averages from one experiment (Ax-4 cells and buffer) or the mean ± SD for the averages from three separate experiments (all others) is shown. The ratio of the concentration of IP_3 in the stimulated cells to the concentration of IP_3 in the unstimulated cells was also calculated for each of the three experiments, and the mean ± SD for the three ratios is shown. A value of 1.00 for the fold increase would indicate that cAMP has no effect on IP_3 production.

CMF thus appears to affect the expression of the cAMP receptor during late development, the biochemical and Northern blot data both suggest that the absence of CMF does not affect the accumulation of the cAMP receptor during development.

CMF Is Required for G Protein Activity

We next examined whether CMF regulates the interaction between the cAMP receptor and G proteins. A common characteristic of G protein-receptor interactions, including the *Dictyostelium* cAMP receptor, is that the GDP- or GTP-occupied G protein affects the affinity of the receptor for cAMP (reviewed in Gilman, 1987, 1991). In cells starved at high densities, the addition of the nonhydrolyzable GTP analogue GTPγS causes a 50–70% decrease in the binding of cAMP to the membranes (Van Haastert, 1984). To determine if CMF affects this, we starved antisense and control transformant cells in the presence or absence of CMF for 5 h. The binding of [^{3}H]cAMP to crude membrane fractions was measured in the presence or absence of GTPγS. We observed that CMF did not affect the ability of GTPγS to inhibit the binding of [^{3}H]cAMP to the membranes (Table VII). This suggests that CMF does not affect the ability of G proteins to interact with the cAMP receptor in vitro.

Another common characteristic of G protein-receptor interactions, including the *Dictyostelium* cAMP receptor, is that binding of the ligand to the receptor causes an increased binding of GTP to membranes. We thus examined whether CMF affects the ability of cAMP to regulate the exchange of GTP for GDP in plasma membrane-associated G proteins. CMF antisense transformant and control transformant cells were starved in the presence or absence of CMF for 5 h. After adding either cAMP or buffer as a control, the amount of [^{35}S]GTPγS bound to membranes was measured. We found that in the absence of added CMF or cAMP, the binding of [^{35}S]GTPγS to membranes was similar for Ax-4, control transformant, and CMF antisense cells (Table VIII). Conversely, starvation of cells in the presence of added CMF caused a slight but statistically insignificant increase in the amount of [^{35}S]GTPγS bound to membranes (Table VIII). Normally, the addition of cAMP causes a 1.38 ± 0.05-fold increase in the amount of

Table V. The Effect of CMF on cGMP and cAMP Production

A

Cell line	Fold increase in cGMP production at 10 s	Fold increase in cAMP production at 3 min	Fold increase in cAMP production at 5 min
Control transformant	3.90 ± 1.12	3.02 ± 1.33	4.53 ± 0.67
Control transformant + CMF	2.67 ± 0.12	3.04 ± 1.89	ND
CMF antisense	1.13 ± 0.13	0.87 ± 0.14	0.96 ± 0.04
CMF antisense + CMF	2.13 ± 0.98	2.24 ± 0.72	ND

B

Cell line	Fold increase in cGMP production at 10 s
CMF antisense	0.96 ± 0.14
CMF antisense + CMF for 10 s	2.32 ± 0.18

(*A*) Control transformant or CMF antisense cells were starved for 5 h in the presence (*+CMF*) or absence of 1 ng/ml recombinant CMF. 2′-deoxy-cAMP (cAMP response) or cAMP (cGMP response) was added to the cells and the amount of cAMP or cGMP in the cells was determined in triplicate at the times indicated after adding the stimulus. The fold increase in cAMP or cGMP was calculated as the average amount at the time indicated divided by the average amount at 0 min. The means ± SD for three separate experiments (3 min or 10 s determinations) or two experiments (5 min determinations) are shown. (*B*) CMF antisense cells were starved for 5 h, harvested for a cGMP production assay as in *A*, and 10 s before adding the cAMP stimulus either buffer or buffer containing 1 ng/ml recombinant CMF was added to the cells. The means ± SD for four separate experiments are shown. A value of 1.00 for the fold increase would indicate that cAMP has no effect on the cAMP or cGMP production.

Table VI. The Effect of CMF on the Binding of [^{3}H]cAMP to Cells

Cell line	cAMP binding (% of control)	Low affinity sites		High affinity sites	
		number/cell	K_D	number/cell	K_D
			nM		*nM*
Control transformant	100	8.3×10^4	3.1	2.4×10^4	0.37
Control transformant + CMF	155 ± 15	ND	ND	ND	ND
CMF antisense	140 ± 42	7.7×10^4	3.1	2.4×10^4	0.37
CMF antisense + CMF	120 ± 33	8.3×10^4	3.1	2.0×10^4	0.37
Ax-4, low density (control)	100	ND	ND	ND	ND
Ax-4, low density + CMF	96 ± 0.07	ND	ND	ND	ND

Control transformant or CMF antisense cells were starved in the presence or absence of 1 ng/ml recombinant CMF, and the binding of cAMP to the intact cells was measured in the presence of ammonium sulphate. In each experiment, the amount of cAMP bound to cells was determined in quadruplicate, and the average of each quadruplicate was normalized to the average amount of cAMP bound to the control transformant cells in the absence of exogenous CMF. The mean ± SD for the normalized averages from three separate experiments is shown. Binding of cAMP was also determined for untransformed Ax-4 cells starved at low density (where CMF concentrations would be low) in the presence or absence of exogenous bacterially synthesized CMF. In two experiments, binding of cAMP was measured in quadruplicate. Scatchard plots were used to determine the affinity and number of binding sites for cAMP binding to cells in the presence of ammonium sulfate.

[^{35}S]GTPγS bound to membranes (Snaar-Jagalska et al., 1988); this increase was observed regardless of whether CMF was present (Table VIII). We also examined the binding of [^{3}H]GTP to membranes, and observed somewhat higher amounts than previously described (Snaar-Jagalska and Van Haastert, 1988) (Table IX). As previously observed, addition of cAMP to the membranes increases the binding of [^{3}H]GTP, the bnding of [^{3}H]GTP to *synag 7* membranes is lower than the amount bound to control cell membranes, and the binding of [^{3}H]GTP to *synag 7* membranes is increased by stimulation with cAMP. As with the binding of [^{35}S]GTPγS to membranes, we observed no significant difference in the binding of [^{3}H]GTP to control or CMF antisense transformant membranes either in the presence or absence of cAMP stimulation. The results of the GTPγS and the GTP binding experiments therefore suggest that CMF does not strongly affect the ability of cAMP to regulate the binding of GTP to membranes in vitro.

The above data indicate that CMF regulates signal transduction without strongly affecting the interaction between the cAMP receptor and G proteins in vitro. We thus examined whether CMF regulates the interaction between G proteins and adenylyl cyclase. As shown in Table X, using the method of Theibert and Devreotes (1986), we found that at 22°C the adenylyl cyclase activities of Ax-4 and CMF antisense cells were roughly similar, indicating that CMF does not affect the basal level of adenylyl cyclase activity. Stimulation with GTPγS resulted in a substantial increase in adenylyl cyclase activity in the Ax-4 cells regardless of whether cells were exposed to additional CMF. These activities are similar to those previously observed (Theibert and Devreotes, 1986). CMF antisense cells showed poor adenylyl cyclase stimulation by GTPγS; preincubation of these cells with CMF for 10 s before lysis however resulted in a GTPγS stimulation that was stronger than that seen in Ax-4 cells. This suggests that all the components necessary for adenyl cyclase activation are present in the CMF antisense cells and that CMF regulates the ability of an activated G protein to stimulate adenylyl cyclase.

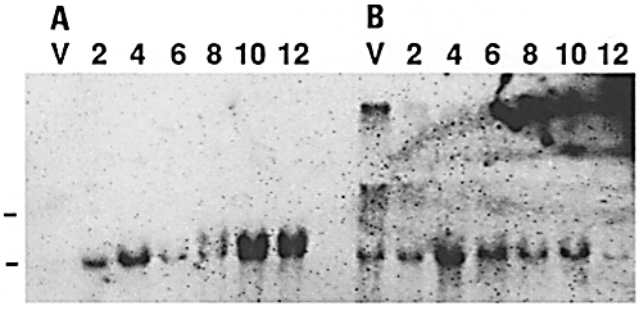

Figure 3. mRNA levels of the surface cAMP recpetor cAR1. *V* indicates vegetative cells and the number indicates the hours after starvation. *A* shows RNA from control transformant cells while *B* shows RNA from CMF antisense cells. The positions of the 4.1- and 1.9-kb ribosomal RNA bands, visualized by methylene blue staining to verify that approximately equal amounts of undegraded RNA were loaded in each lane, are indicated at the left.

Discussion

We previously found that CMF is required for aggregation. We have shown here that CMF is not required for the viability of starving cells and in fact for the first 6 h of development CMF does not have any large effect on the ability of cells to translocate randomly. During later development, CMF does affect the speed of cell translocation. The inability of CMF antisense cells to chemotax towards cAMP indicates that CMF strongly affects the ability of cAMP to regulate the direction of cell velocity during a time in which CMF has little effect on the magnitude of cell velocity. However, the production of ruffles and pseudopods is regulated by CMF. The CMF-induced increase in the frequency of pseudopod formation was seen with cells starved for the relatively short time of four hours at low cell densities, where there would be no relaying of cAMP pulses and thus little activation of cAMP pulse-induced genes. The effect of CMF on cell morphology thus would seem to be a direct effect as opposed to a secondary effect due to CMF-regulated cAMP signal transduction. CMF regulates cAMP signal transduction within a few seconds, whereas CMF alone takes an hour to affect pseudopods. The two processes thus seem to be regulated by divergent pathways.

CMF appears to be essential for several aspects of

Table VII. The Effect of CMF on [^{3}H]cAMP Binding to Membranes

Cell line	[^{3}H]cAMP binding, % of control		Fold stimulation by GTPγS
	–GTPγS	+GTPγS	
Ax-4	216	55	0.25
Ax-4 + CMF	77	29	0.38
Control transformant	100	57 ± 6	0.56 ± 0.06
Control transformant + CMF	184 ± 66	32 ± 3	0.24 ± 0.08
CMF antisense	207 ± 124	41 ± 18	0.30 ± 0.13
CMF antisense + CMF	279 ± 162	53 ± 29	0.29 ± 0.16

Untransformed Ax-4 cells, control transformant, or CMF antisense cells were starved for 5 h in the presence (*+CMF*) or absence of 1 ng/ml recombinant CMF. The binding of [^{3}H]cAMP to membranes was measured in the presence or absence of GTPγS in quadruplicate and normalized to the amount bound to control transformant cells. Values are means ± SD from one (for AX-4) or three (all others) independent experiments. In each experiment, the ratio of the average amount of [^{3}H]cAMP bound in the presence of GTPγS to the average amount bound in the absence of GTPγS was calculated, and the means ± SD of the ratios (fold stimulations) are shown. A fold stimulation of 1.00 would indicate that GTPγS has no effect on the binding of cAMP to membranes.

Table VIII. The Effect of CMF on [^{35}S] GTPγS Binding to Membranes

Cell line	[^{35}S]GTPγS binding, % of control		Fold stimulation by cAMP
	–	+cAMP	
Ax-4	92 ± 15	129 ± 6	1.36 ± 0.17
Ax-4 + CMF	108 ± 5	131 ± 15	1.21 ± 0.08
Control transformant	100	120 ± 12	1.20 ± 0.12
Control transformant + CMF	116 ± 11	151 ± 30	1.30 ± 0.24
CMF antisense	84 ± 22	111 ± 28	1.35 ± 0.17
CMF antisense + CMF	86 ± 14	122 ± 19	1.42 ± 0.04

Untransformed Ax-4 cells, control transformant, or CMF antisense cells were starved for 5 h in the presence (*+CMF*) or absence of 1 ng /ml recombinant CMF. The binding of [^{35}S]GTPγS to membranes was measured in the presence or absence of cAMP in quadruplicate, and then normalized to the amount of [^{35}S]GTPγS bound to control transformant membranes in the absence of cAMP. The means ± SD from three independent experiments are shown. For each separate experiment, the ratio of the average amount of [^{35}S]GTPγS bound in the presence of cAMP to the average amount bound in the absence of cAMP was calculated; the average and standard deviations of the three ratios is shown. A value of 1.00 would indicate that cAMP has no effect on the binding of [^{35}S]GTPγS to membranes.

Table IX. The Effect of CMF on [^{3}H]GTP Binding to Membranes

Cell line	Binding time	[^{3}H]GTP binding to membranes		Fold stimulation by cAMP
		–	+cAMP	
	s	*cpm bound*		
Control transformant	20	2104 ± 186	2572 ± 66	1.22 ± 0.07
Control transformant	60	2572 ± 37	2850 ± 66	1.11 ± 0.01
CMF antisense	20	2158 ± 24	2499 ± 96	1.12 ± 0.00
CMF antisense	60	2388 ± 37	2756 ± 15	1.15 ± 0.01
722 *Synag 7*	60	486	632	1.3

Control transformant, CMF antisense cells or 722 axenic *Synag 7* cells were starved for 5 h and membranes were prepared. [^{3}H]GTP and buffer or [^{3}H]GTP and cAMP was added and incubated for the time indicated; the amount bound was measured in triplicate. The average from one experiment (722 cells) or means ± SD from three independent experiments are shown. For each experiment, the ratio of the average amount of [^{3}H]GTP bound in the presence of cAMP to the average amount bound in the absence of cAMP was calculated; the average of the ratios ± SD is shown. A value of 1.00 would indicate that cAMP has no effect on [^{3}H]GTP binding to membranes.

cAMP-mediated signal transduction (Fig. 4). CMF regulates the stimulation of Ca^{2+} influx and both adenylyl cyclase and guanylyl cyclase by cAMP. CMF has to be present for only a few seconds to permit these cAMP-induced responses. CMF does not affect the cAMP-activation of phospholipase C; however this enzyme is not necessary for growth or development of *Dictyostelium* in the laboratory (Drayer et al., 1994) and thus it is probably not necessary that CMF regulates its activity. We found that CMF is not required for the presence of cAMP receptors. In addition, the stimulation of GTP binding to membranes induced by cAMP and the affinity modulation of cAMP binding to membranes induced by GTPγS are essentially unaffected by CMF. This suggests that the cAMP receptor and its interaction with a majority of G proteins is unaffected by CMF in vitro. CMF strongly affects the ability of GTPγS to activate adenylyl cyclase in vitro. This could be due to either CMF regulating the activity of the cytosolic protein, CRAC, which in turn regulates the activation of adenylyl cyclase by G proteins, or by CMF somehow regulating the release of activated G proteins from the cAR1 receptor.

In *frigid A* mutants, which lack Gα2 (Kumagai et al., 1989), GTPγS has a reduced ability to inhibit the binding of cAMP to membranes, and cAMP does not stimulate the binding of GTP to membranes (Kesbeke et al., 1988). Since these two interactions are unaffected by CMF, our data suggest that Gα2 is present and at least partially functional in the CMF antisense cells; this is supported by the observation that Gα2 mRNA is present in CMF antisense

Table X. The Effect of CMF on the Activity of Adenylyl Cyclase In Vitro

Cell line	Adenylyl cyclase activity, pmol/min/mg protein at 22°C		Fold stimulation by GTPγS
	–	+GTPγS	
Ax-4	2.9 ± 0.0	21.3 ± 1.0	7.3 ± 0.7
Ax-4 + 10 s of CMF	2.2 ± 1.6	23.7 ± 0.5	10.8 ± 0.8
CMF antisense	3.8 ± 0.6	4.6 ± 1.1	1.21 ± 0.3
CMF antisense + 10 s of CMF	2.8 ± 2.9	41.4 ± 9.2	14.8 ± 3.2

Untransformed Ax-4 cells or CMF antisense cells were starved for 5 h. Cells were then harvested and lysed (either immediately or after a 10-s incubation with 1 ng/ml recombinant CMF at 22°C) in the presence or absence of GTPγS and assayed for cAMP synthesis in triplicate essentially following Theibert and Devreotes (1986); see Materials and Methods for modifications. Values are means ± SD from three independent experiments (CMF antisense cells) or two experiments (Ax-4 cells). The ratio of (average adenylyl cyclase activity in the presence of GTPγS)/(average adenylyl cyclase activity in the absence of GTPγS) was calculated for each separate experiment. The means ± SD for the ratios is shown. A value of 1.00 would indicate that GTPγS does not stimulate adenylyl cyclase.

cells (Lindsey and Gomer, manuscript in preparation). Furthermore, a 10-s exposure of cells to CMF immediately before cAMP stimulation is sufficient to permit the cAMP activation of Ca^{2+} influx and guanylyl cyclase. This rapid response indicates that both the CMF signal transduction machinery and those components of the cAMP signal transduction pathway that we have found to be regulated by CMF are present in cells which have not been previously exposed to CMF. The ability of a 10-s exposure of cells to CMF to permit the GTPγS activation of adenylyl cyclase in vitro then indicates that the CRAC protein is indeed present in CMF antisense cells and that CMF thus regulates either its activity or subcellular localization.

A non-aggregating mutant, *Synag 7*, appears to lack CRAC activity (Theibert and Devreotes, 1986). *Synag 7* exhibits normal chemotaxis towards cAMP and activation of guanylyl cyclase by cAMP (Schaap et al., 1986), whereas the CMF antisense cells can do neither. Also unlike CMF antisense cells, the activation of guanylyl cyclase by cAMP in vivo (Schaap et al., 1986) or by GTPγS in vitro in *Synag 7* mutants (Janssens and Van Haastert, unpublished observation) is similar to that in wild-type cells. This suggests that CMF is not CRAC per se, and that CMF does not regulate CRAC exclusively.

CMF regulates cAMP signal transduction during development, but it is unclear whether CMF also regulates events in vegetative cells. In transformants engineered to express cAMP receptors in vegetative cells (which do not secrete CMF), cAMP will stimulate Ca^{2+} influx in the vegetative cells (Milne and Coukell, 1991; Milne and Devreotes, 1993). This might suggest that a process that we have shown to require high levels of extracellular CMF occurs when there are low levels of extracellular CMF. However, for the Ca^{2+} influx assays, the vegetative cells were collected by centrifugation, and then resuspended and collected by centrifugation twice in a buffer which would cause starvation and thus initiate CMF secretion. The cells were then shaken at a density of 10^8 cells/ml in the starvation buffer for 10 more min before being assayed (Milne and Coukell, 1991; Milne and Devreotes, 1993). We have found that the rate at which the 80-kD CMF accumulates in starvation buffer is essentially invariant for the first 10 h of starvation, at ~12 molecules/cell/min (Gomer et al., 1991; Yuen and Gomer, 1994). This would indicate that at 10^8 cells/ml, the CMF concentration would reach the 0.3 ng/ml threshold value in 2 min and the 1 ng/ml optimal concentation in 7 min, and thus the vegetative cells that were being assayed were actually starving in the presence of high levels of CMF.

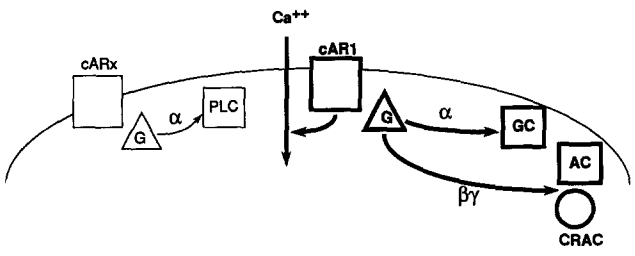

Figure 4. Model of the cAMP signal transduction system in *Dictyostelium.* CMF appears to regulate the processes shown with bold lines. *cARx*, the unknown cAMP receptor; *G*, a heterotrimeric G protein containing Gα2; *PLC*, phospholipase C; *cAR1*, cAMP receptor 1; *GC*, guanylyl cyclase; *AG*, adenylyl cyclase; *CRAC*, cytosolic regulator of adenylyl cyclase.

Our results thus indicate that CMF regulates cAMP-stimulated Ca^{2+} influx and activations of adenylyl cyclase and guanylyl cyclase, but not the cAMP-stimulated activation of phospholipase C. Ca^{2+} influx does not require the presence of Gα subunits 1, 2, 3, 4, 7, or 8 (Milne and Devreotes, 1993), or the Gβ subunit (Devreotes, 1994; Milne et al., 1995), suggesting that CMF does not solely affect these proteins, and as described above CMF does not appear to solely regulate CRAC. Mutants lacking adenylyl cyclase have normal cAMP-stimulated guanylyl cyclase activation, and vice versa (Kuwayama et al., 1993); CMF thus appears to regulate two independent cyclases. In mutants lacking either cAR1 or CMF, the cAMP-stimulated activation of phospholipase C is normal while cAMP-stimulated Ca^{2+} influx and activations of adenylyl cyclase and guanylyl cyclase are greatly reduced. This similarity suggests that CMF may regulate the ability of cAMP to activate the cAR1-G protein complex. Alternatively, CMF might separately regulate the activation of Ca^{2+} influx by cAR1, the pathway between cAR1 and adenylyl cyclase and the pathway between cAR1 and guanylyl cyclase. In either case, since CMF concentrations allowing a peak CMF response cause occupation of ~200 receptors/cell (Jain and Gomer, 1994) and a cAMP pulse causes occupation of several thousand cAMP receptors, the CMF signal transduction mechanism appears to involve some form of amplification.

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