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Published in:
The Journal of Cell Biology

DOI:
10.1083/jcb.129.5.1251

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Yuen, I. S., Jain, R., Bishop, J. D., Lindsey, D. F., Deery, W. J., van Haastert, P. J. M., & Gomer, R. H. (1995). A Density-sensing Factor Regulates Signal Transduction in Dictyostelium. The Journal of Cell Biology, 129(5), 1251-1262. https://doi.org/10.1083/jcb.129.5.1251

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

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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 Ca²⁺ 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 regulation 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 10⁵ 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 (10⁵ cells/cm²) differentiate, whereas cells at low density (2 × 10³ cells/cm²) do not. However, when the low density cells are starved in buffer in which high density cells had previously been starved (a condi-
tioned medium), the low density cells then differentiate (Grabel and Loomis, 1978; Kay, 1982; Mehdy and Firtel, 1985). Time lapse 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) 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 NH2 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 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). Ga2 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 adenyl 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 Ca2+ after cAMP activation (Bumann et al., 1984), which does not appear to be mediated by G proteins, intracellular cAMP, or cGMP (Milne and Coughell, 1991; Milne and Devreotes, 1993).

Ga2, 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). Ga2 also activates phospholipase C, resulting in a transient rise in inositol 1,4,5-trisphosphate (IP3) which peaks at ~6 s (Europe-Finner and Newell, 1987a,b; Van Haastert et al., 1989; Okaichi et al., 1992). Interestingly, the Ga2 which activates phospholipase C is regulated by a cAMP receptor that is not cAR1 (Bominara and Van Haastert, 1994). Both the cGMP and the IP3 levels return to baseline ~30 s after their peak. The activation of adenyl cyclase occurs over several minutes and is mediated by the βγ heterodimer moiety of an activated G protein (Okaichi et al., 1992). The GTPγS activation of adenyl cyclase in vitro requires membranes and a cytosolic protein designated cytoplasmic regulator of adenyl cyclase (CRAC) (Theibert and Devreotes, 1986; Van Haastert et al., 1987; Snaar-Jagalska and Van Haastert, 1988; Insall et al., 1994). A nonaggregating mutant, Synap 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 Ca2+ influx, guanylyl cyclase, and adenyl 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 KH2PO4, 10 μM CaCl2, 1 mM MgCl2, pH 6.1, with KCl). The cells were resuspended in PBM to a final density of 1 × 106 cells/ml, and shaken at 180 rpm. For the Ca2+ 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-
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 Na2HPO4, 6.94 mM KH2PO4, pH 6.5) at 1 × 10⁷ 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/PB plates. One microliter of cells at a density of 1 × 10⁶ cells/ml in PB 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 TFC-400 TV camera connected to a Sony ETV 801 timelapse videocassette recorder. Cells were starved at low density in submerged monolayer culture in PB 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 480fold). 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 × 10⁶ cells/cm². Wild-type cells were also allowed to develop in the presence or absence of gel-purified CMF at a density of 4 × 10⁶ cells/cm². At the indicated times, cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 for 2 h at 4°C. Cells were then washed twice in PB and resuspended to 1 × 10⁷ cells/ml. 45 µl of cells was added to a mixture of 5 µl of 100 mM dithiothreitol containing 20 nCi [3H]cAMP, and 425 µl of 0.9% 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 × 10⁶ 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 × 10⁶ cells/ml; cAMP binding was immediately measured as described above. Scatchard plots of cAMP binding were done with a variety of [3H]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 [3H]cAMP to membranes followed Kesbeke et al. (1988), with the exceptions that cells were starved by shaking for 5 hours at 1 × 10⁷ cells/ml in PB, and the binding reactions were done in 50 µl of (2.56 mM Na2HPO4, 5.55 mM KH2PO4, 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 [35S]GTPγS to membranes was determined following Kesbeke et al., (1990), except that cells were starved for 5 h by shaking in PB, and the reactions were done in 50 µl and contained instead of 10 mM MgCl2. The effect of cAMP on the binding of [3H]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 × 10⁶ cells/ml and starved for 5 h at a density of 1 × 10⁵ 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 viability, 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,
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 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, 1987a,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-
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.

**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

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**Table III. The Effect of CMF on 45Ca2+ Influx**

| Cell line          | −cAMP | +cAMP | Fold increase in Ca2+ influx |
|--------------------|-------|-------|-----------------------------|
| 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 45Ca2+ 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 Ca2+.
Table IV. The Effect of CMF on IP₃ Production

| Substance assayed | IP₃ -cAMP | +cAMP | Fold increase in IP₃ production at 8 s |
|------------------|----------|-------|----------------------------------|
| 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 × 10⁶ cells/ml and aerated for 10 min. For unstimulated cells, the cell suspension was then centrifuged and the amount of IP₃ 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 IP₃ 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 IP₃ in the stimulated cells to the concentration of IP₃ 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 IP₃ 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 [3H]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 [3H]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 [35S]GTPγS bound to membranes was measured. We found that in the absence of added CMF or cAMP, the binding of [35S]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 [35S]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       | 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 [3H]cAMP to Cells

| Cell line                 | % of control | cAMP binding number/cell | $K_0$ (nM) | High affinity sites |
|---------------------------|--------------|--------------------------|------------|---------------------|
| Control transformant      | 100          | 8.3 x 10^4               | 3.1        | 2.4 x 10^4          |
| Control transformant + CMF| 155 ± 15     | ND                       | ND         | ND                  |
| CMF antisense             | 140 ± 42     | 7.7 x 10^4               | 3.1        | 2.4 x 10^4          |
| CMF antisense + CMF       | 120 ± 33     | 8.3 x 10^4               | 3.1        | 2.0 x 10^4          |
| Ax-4, low density (control)| 100          | ND                       | ND         | ND                  |
| Ax-4, low density + CMF   | 96 ± 0.07    | 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. Each experiment was done 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 sulphate.

[35S]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 [3H]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 activity of CMF-antigen receptors (Table X). We previously observed that CMF regulates the ability of an activated G protein 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 adenyl cyclase. As shown in Table X, using the method of Theibert and Devreotes (1986), we found that at 22°C the adenyl cyclase activities of Ax-4 and CMF antisense cells were roughly similar, indicating that CMF does not affect the basal level of adenyl cyclase activity. Stimulation with GTPγS resulted in a substantial increase in adenyl 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 adenyl 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 adenyl cyclase.

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, whereas CMF alone takes an hour to affect pseudopods. The two processes thus seem to be regulated by different pathways.

CMF appears to be essential for several aspects of
Table VII. The Effect of CMF on [3H]cAMP Binding to Membranes

| Cell line                  | [3H]cAMP binding, % of control | Fold stimulation by GDPγS |
|----------------------------|--------------------------------|--------------------------|
|                            | -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 [3H]cAMP to membranes was measured in the presence or absence of GDPγS 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 [3H]cAMP bound in the presence of GDPγS to the average amount bound in the absence of GDPγS was calculated, and the means ± SD of the ratios (fold stimulations) are shown. A fold stimulation of 1.00 would indicate that GDPγS has no effect on the binding of [3H]cAMP to membranes.

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

| Cell line                  | [35S]GTPγS binding, % of control | Fold stimulation by cAMP |
|----------------------------|---------------------------------|--------------------------|
|                            | -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 [35S]GTPγS to membranes was measured in the presence or absence of cAMP in quadruplicate, and then normalized to the amount of [35S]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 [35S]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 [35S]GTPγS to membranes.

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

| Cell line                  | [3H]GTP binding to membranes | Fold stimulation by cAMP |
|----------------------------|------------------------------|--------------------------|
|                            | Binding time                 | cpm bound |                         |
|                            | s                                    | -          | +cAMP                   |                         |
| 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                     |

Untransformed Ax-4 cells, control transformant, or CMF antisense cells or 722 axenic Synag 7 cells were starved for 5 h and membranes were prepared. [3H]GTP and buffer or [3H]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 [3H]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 [3H]GTP binding to membranes.

cAMP-mediated signal transduction (Fig. 4). CMF regulates the stimulation of Ca2+ 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 |
|-----------|-----------------------------------------------------|--------------------------|
| Ax-4      | 2.9 ± 0.0                                           | 7.3 ± 0.7                |
| Ax-4 + 10 s of CMF | 2.2 ± 1.6                                           | 10.8 ± 0.8               |
| CMF antisense | 3.8 ± 0.6                                           | 1.21 ± 0.3               |
| CMF antisense + 10 s of CMF | 2.8 ± 2.9                                           | 14.3 ± 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.

Figure 4. Model of the cAMP signal transduction system in Dicoryteium. CMF appears to regulate the processes shown with bold lines. cARx, the unknown cAMP receptor; G, a heterotrimeric G protein containing Ga2; PLC, phospholipase C; cARI, cAMP receptor 1; GC, guanylyl cyclase; AG, adenyl cyclase; CRAC, cytosolic regulator of adenylly cyclase.

We thank R. Ammann for assistance with cell culture, J. Segall for advice on the chemotaxis assay, J. Heath for assistance with critical point drying.
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