The Cell Density Factor CMF Regulates the Chemoattractant Receptor cAR1 in *Dictyostelium*

Peter J.M. Van Haastert,* John D. Bishop,‡ and Richard H. Gomer‡§

*Department of Biochemistry, University of Groningen, 9727 AG Groningen, The Netherlands; and ‡Department of Biochemistry and Cell Biology, and §Howard Hughes Medical Institute, Rice University, Houston, Texas 77251-1892

Abstract. Starving *Dictyostelium* cells aggregate by chemotaxis to cAMP when a secreted protein called conditioned medium factor (CMF) reaches a threshold concentration. Cells expressing CMF antisense mRNA fail to aggregate and do not transduce signals from the cAMP receptor. Signal transduction and aggregation are restored by adding recombinant CMF. We show here that two other cAMP-induced events, the formation of a slow dissociating form of the cAMP receptor and the loss of ligand binding, which is the first step of ligand-induced receptor sequestration, also require CMF. Vegetative cells have very few CMF and cAMP receptors, while starved cells possess ~40,000 receptors for CMF and cAMP. Transformants overexpressing the cAMP receptor gene cAR1 show a 10-fold increase of [^{3}H]cAMP binding and a similar increase of [^{125}I]CMF binding; disruption of the cAR1 gene abolishes both cAMP and CMF binding. In wild-type cells, downregulation of cAR1 with high levels of cAMP also downregulates CMF binding, and CMF similarly downregulates cAMP and CMF binding. This suggests that the cAMP binding and CMF binding are closely linked. Binding of ~200 molecules of CMF to starved cells affects the affinity of the majority of the cAR1 cAMP receptors within 2 min, indicating that an amplifying mechanism allows one activated CMF receptor to regulate many cARs. In cells lacking the G-protein β subunit, cAMP induces a loss of cAMP binding, but not CMF binding, while CMF induces a reduction of CMF binding without affecting cAMP binding, suggesting that the linkage of the cell density–sensing CMF receptor and the chemoattractant cAMP receptor is through a G-protein.

MANY multicellular organisms secrete molecules used to sense the size or cell-type composition of specific organs or the whole organism (Fuqua et al., 1994; Magnuson et al., 1994; Clarke and Gomer, 1995). Such molecules could be centrally involved in growth regulation, wound healing, and tissue regeneration, whereas disruption of a mass-sensing mechanism could lead to uncontrolled growth. In vertebrate embryos, transplantation of a single cell to an ectopic site can cause the cell to change its fate to match that site. When a group of cells is transplanted, however, they retain their original cell type. These and other observations suggest the widespread existence of signals that allow a cell to sense the local or total density of cells of its type (Gurdon et al., 1993; Gomer, 1994; Zhang et al., 1994).

Cell density–sensing factors have been identified in *Dictyostelium*. This organism normally exists as an individual amoeba that consumes bacteria living on soil. Upon starvation, cells aggregate using relayed pulses of cAMP as the chemoattractant. The aggregated cells develop into a fruiting body. During *Dictyostelium* development, the expression of some genes is dependent on the cell density. A protein called prestarvation factor accumulates during growth in proportion to the density of cells. Prestarvation factor sensing is inhibited by the presence of bacteria, and thus, a high level of detected prestarvation factor indicates that starvation is imminent (Rathi et al., 1991). During early starvation, a protein called conditioned medium factor (CMF)[1] is secreted. When *Dictyostelium* cells are starved at low cell densities, cAMP can not induce developmental gene expression, which is restored by addition of purified CMF (Mehdy and Firtel, 1985; Gomer and Firtel, 1987; Gomer et al., 1991; Yuen et al., 1991). Starved cells only respond to cAMP when CMF reaches a threshold concentration. Cells starve asynchronously, and thus, this mechanism allows cells to simultaneously start cAMP-mediated cell aggregation after a high density of cells has starved.

Address all correspondence to Peter J.M. Van Haastert, Department of Biochemistry, University of Groningen, Nijenborgh 4, 9727 AG Groningen, The Netherlands. Tel.: (31) 503634172. Fax: (31) 503634165. e-mail: P.J.M.van.Haastert@rugch4.chem.rug.nl.

1. *Abbreviations used in this paper*: CMF, conditioned medium factor; IP_3, inositol 1,4,5-trisphosphate; PB, phosphate buffer.

© The Rockefeller University Press, 0021-9525/96/09/1543/7 $2.00

The chemoattractant cAMP is detected by highly specific surface receptors that interact with multimeric G-proteins (Van Haastert, 1984). Four genes have been identified encoding cAMP receptors (cAR1–cAR4). The deduced amino acid sequences predict proteins that span the membrane seven times (Klein et al., 1988; Saxe et al., 1993; Johnson et al., 1993; Louis et al., 1994). Binding of cAMP to cAR1 is required for the activation of several second messenger pathways, including the G-protein–independent stimulation of calcium uptake, and the G-protein–dependent stimulation of adenylyl and guanylyl cyclases (Milne and Coukell, 1991; Milne and Devreotes, 1993; Kesbeke et al., 1988; Kumagai et al., 1989; Sun et al., 1990; Pupillo et al., 1992). Stimulation of phospholipase C is probably G-protein dependent, but it does not require the presence of the major cAMP receptor cAR1 (Bominaar and Van Haastert, 1994).

We have investigated how the cell density–sensing factor CMF interacts with cAMP signal transduction and reported previously that cAMP does not activate multiple second messenger pathways in cells with reduced levels of CMF (Yuen et al., 1995). These results suggested that CMF is required for an early step in the signal transduction cascade. In the present study, we investigated the relationship between binding of CMF and cAMP to surface receptors and show that (*a*) cAMP can not activate cAR1 in the absence of CMF; (*b*) increasing or decreasing cAR1 levels increase or decrease both cAMP and CMF binding; (*c*) CMF and cAMP interact through an intermediate that allows the binding of each CMF molecule to affect hundreds of cAMP receptors; and (*d*) the interaction between the cAMP receptor and the CMF receptor requires the presence of a G-protein. The results suggest that signaling through the cell surface cAMP receptor requires the binding of two ligands to cells. Binding of CMF to its receptor does not induce responses, but it is permissive for cAMP to induce receptor-mediated second messenger responses, leading to directed cell movement and developmental gene expression.

Materials and Methods

Materials

[2,8-^{3}H]cAMP (1.85 TBq/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Recombinant CMF and [^{125}I]CMF were prepared and assayed for protein concentration and CMF activity as described in Jain and Gomer (1994).

Cells and Culture Conditions

The following cell lines were used: AX3 wild-type cells; K3 CMF antisense cells, obtained and recloned as described by Jain and Gomer (1994); and LW6 cells with a disruption of the Gβ gene, 1A3 cells expressing cAR1 in the vegetative stage from an actin promotor, and d280 and 1H1 cAR1-null cells (all kindly provided by Peter Devreotes, John Hopkins University, Baltimore, MD). Cells were grown in axenic medium supplemented with 10 μg/ml G418 (all strains except AX3). Cells were harvested in the late logarithmic phase with 10 mM phosphate buffer (PB) (KH_2PO_4/Na_2HPO_4, pH 6.5), washed twice, and starved in PB at a density of 10^7 cells per ml. To obtain CMF-depleted AX3 cells, starvation was at 10^6 cells per ml.

cAMP-binding Assay

Cells were washed three times in cold PB and resuspended in this buffer to 2×10^7 cells per ml. The binding assay in phosphate buffer contained 190 μl of the cell suspensions and 10 μl of binding mixture (400 nM [^{3}H]cAMP and 200 mM DTT), yielding a final concentration of 20 nM [^{3}H]cAMP and 10 mM DTT in PB; the incubation was for 45 s at 0°C. The binding assay in ammonium sulfate contained 190 μl of the cell suspensions, 10 μl of the same binding mixture, 1 ml 90% saturated ammonium sulphate, and 50 μg BSA, yielding final concentrations of 3.33 nM [^{3}H]cAMP, 1.67 mM DTT, and 75% saturated ammonium sulfate in PB; this incubation was for 5 min at 0°C. The binding reactions for both assays were terminated by centrifugation at 14,000 *g* for 30 s and 5 min, respectively; the supernatant was aspirated, and the radioactivity in the cell pellet was determined. Nonspecific binding was measured in the presence of 0.1 mM cAMP and was subtracted from all data; nonspecific binding was 22 ± 4 and 10 ± 1% of total binding of [^{3}H]cAMP to aggregation-competent AX3 cells for the assay in phosphate buffer and ammonium sulphate, respectively.

CMF-binding Assay

Cells were resuspended to 2×10^7 cells per ml in PB with 200 ng/ml BSA. The binding incubations contained 190 μl of the cell suspension in a total volume of 200 μl containing 2 nM [^{125}I]CMF (1.4 TBq/mmol = 30,000 cpm per assay); the binding reactions were terminated after 20 min by centrifugation of the cells through silicon oil or sucrose as described (Van Haastert et al., 1986; Jain and Gomer, 1994). The cell-associated radioactivity in the pellet was determined. Nonspecific binding was measured in the presence of 40 nM rCMF and was 61 ± 8% of total binding.

To examine binding of [^{3}H]cAMP to CMF, 0.5 ml of PBMB (20 mM KH_2PO_4, 10 μM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml BSA, pH 6.1) or 0.5 ml PBMB plus 1.5×10^{-8} M rCMF were dialyzed at 4°C in Spectrapor 12-kD cutoff membranes (Spectrum Medical Industries, Inc., Houston, TX) against 150 ml of PBMB containing 5 μCi of [^{3}H]cAMP. After 20 h, the radioactivity in the dialysis bags was determined. Binding of [^{3}H]cAMP to CMF was also determined by incubating 0.2 μCi [^{3}H]cAMP in the absence or presence of 12 μg rCMF in 100 μl of PBMB with 2 mM KCl for 1 h at either 21° or 4°C. Subsequently, the mixtures were spun through ultrafree MC 30-kD cutoff spin filters (Millipore Corp., Bedford, MA), and the retained radioactivity was determined.

Results

Cells were starved in the presence of very low concentrations of CMF by either using a strain that expresses antisense CMF mRNA or by starving wild-type cells at low cell density, which prevents the accumulation of CMF above a threshold concentration. Previously, we have demonstrated that these CMF-depleted cells do not aggregate and are not chemotactic to cAMP; these cellular responses were restored upon addition of recombinant purified CMF (Yuen et al., 1995). cAMP induces several second messenger responses in *Dictyostelium* cells (Ca^{2+} uptake; cAMP, cGMP, and IP_3 accumulations). In CMF-depleted cells, cAMP no longer induced Ca^{2+} uptake or the accumulations of cAMP and cGMP, whereas the accumulation of IP_3 was similar to that in wild-type cells. This phenotype of CMF-depleted cells is similar to that of cells with a disrupted cAR1 gene (Sun et al., 1990), and different from cells with a disrupted gene encoding the G-protein subunit Gα2 (Kesbeke et al., 1988; Kumagai et al., 1989) or Gβ (Lilly et al., 1993; Wu et al., 1995), which both show a normal Ca^{2+} uptake. Addition of CMF to CMF-depleted cells for as little as 10 s was sufficient to restore all the cAMP-induced second messenger responses (Yuen et al., 1995), indicating that the signal-transducing machinery was present but could not be activated by cAMP in CMF-depleted cells. The above observations suggest that in the cAMP signal transduction pathway, CMF may interact with the cAR1 cAMP receptor or with a protein that interacts with cAR1. The effect of CMF on the interaction between cAMP and its receptor was thus investigated in more detail.

The Receptors for CMF and cAMP Are Closely Linked

To investigate whether the ability of CMF to modulate the effects of cAMP is due to CMF binding to cAMP, we performed equilibrium dialysis and direct binding assays. Repeated assays under a variety of conditions (see Materials and Methods) invariably indicated that there was no detectable binding of cAMP to CMF with a $K_d < 3 \times 10^{-5}$ M (data not shown). This then suggested that CMF binds to something on the cell surface that, in turn, modulates cAMP binding to cAR1.

We previously found that CMF binds to specific receptors on starved cells (Jain and Gomer, 1994). To examine the interaction between the cAMP receptor and the CMF receptor, we used high concentrations of the two ligands to downregulate their receptors. High levels of cAMP will downregulate cAR1 (Klein and Juliani, 1977), and we find that such treatment also downregulated CMF binding (Table I). Similarly, treatment of cells with CMF caused a downregulation of its binding, as well as a downregulation of cAMP binding. The ability of either ligand to downregulate both receptors suggests that cAR1 and the CMF receptor are coupled.

Vegetative *Dictyostelium* cells contain low but detectable amounts of cAMP and CMF receptors. Starvation induces the accumulation of both cAMP receptors and CMF receptors, and after 5 h, there are ~40,000 cAR1 and CMF receptors (Klein et al., 1988; Jain and Gomer, 1994). In postvegetative cells starved for 30 min, cAMP binding was 10%, and CMF binding was 29%, compared with the respective binding to 5-h starved cells (Table II). Transformants expressing cAR1 from an actin promoter showed ~10-fold increased levels of both cAMP binding and CMF binding in postvegetative cells. Furthermore, starved cells with a disrupted cAR1 gene showed a strong reduction in both cAMP binding and CMF binding. Finally, as described above, when wild-type cells starved at high cell density for 5 h were exposed to 1 mM cAMP for 1 h to induce downregulation of cAR1, cAMP binding and CMF binding were both reduced. These experiments reveal that the expressions of cAR1 and the CMF receptor are tightly coregulated.

Alteration of the cAMP Receptor Requires CMF

Binding experiments have revealed the existence of different kinetic forms of the cAMP receptor (Van Haastert, 1984). These forms show different affinities and/or dissociation kinetics. At least part of this heterogeneity of cAMP binding is due to the interaction of cAR1 with other proteins. A brief exposure of cells to cAMP (up to 1 min) leads to a reduction of the affinity and dissociation rate of the cAMP receptor complex. These changes are induced in membranes from wild-type cells by GTPγS, but are absent in mutants lacking the G-protein subunits Gα2 or Gβ, suggesting that the changes of cAMP binding to cells are attributed to the activation of a G-protein (Van Haastert et al., 1986; Kesbeke et al., 1988; Wu et al., 1995). Longer incubation of cells with cAMP (up to 15 min) results in the loss of cAMP binding to cells, while the receptor protein is still present on the cell surface (Klein et al., 1977; Van Haastert et al., 1992). All these cAMP-induced alterations of cAMP binding are easily detectable in phosphate buffer. However, when cAMP binding is measured in nearly saturated ammonium sulphate, all of this variation disappears, probably because ammonium sulphate disrupts protein–protein interactions (Van Haastert, 1985; Khachatrian et al., 1987).

To examine the requirement of CMF for modulation of the cAMP receptor, we measured the different receptor forms in CMF-depleted cells. In saturated ammonium sulphate, no differences of cAMP binding to control cells and CMF-depleted cells are detected (Fig. 1). In phosphate buffer, however, binding of cAMP to CMF-depleted cells was significantly increased compared with control cells.

Table I. The Binding of [^{3}H]cAMP and [^{125}I]CMF to Cells Pretreated with cAMP or CMF

Pretreatment	[^{3}H]cAMP binding	[^{125}I]CMF binding
	% of control	*% of control*
0.1 mM cAMP	42 ± 10	24 ± 16
0.1 ng/ml CMF	67 ± 20	33 ± 14
1 ng/ml CMF	70 ± 16	38 ± 7
300 ng/ml CMF	69 ± 16	30 ± 16

AX4 cells were starved for 6 h in shaking culture at a density of 10^6 cells per ml. The indicated amounts of cAMP or rCMF were then added to the cultures, and control cells were treated with buffer. After 40 min (60 min for the pretreatment with cAMP), the cells were harvested and extensively washed. Binding of [^{3}H]cAMP in the presence of nearly saturated ammonium sulfate and binding of [^{125}I]CMF were determined in parallel as described in Materials and Methods. Data shown are the means and SD of three independent experiments, and are expressed as percentage of the binding of [^{3}H]cAMP or [^{125}I]CMF to cells pretreated with buffer.

Table II. [^{3}H]cAMP and [^{125}I]CMF Binding to Cells Expressing Different Levels of cAR1

Cells	Stage	[^{3}H]cAMP binding	[^{125}I]CMF binding
		% of control	*% of control*
AX3	Agg	100	100
AX3	Post-Veg	10 ± 5	29 ± 7
cAR1^{OE}	Post-Veg	164 ± 28	209 ± 35
car1-null	Agg	9 ± 13	18 ± 30
AX3	Agg/DR	13 ± 7	7 ± 11

Cells at the indicated stage of development were assayed in parallel for the binding of [^{125}I]CMF and [^{3}H]cAMP; 100% binding is defined as the binding of [^{125}I]CMF or [^{3}H]cAMP to aggregation-competent wild-type AX3 cells. Data shown are the means and SD of four independent experiments. *cAR1^{OE}*, transformant overexpressing cAR1 from the constitutive actin promoter; *car1-null*, transformant with an indicated cAR1 gene; *Agg*, aggregation stage (5 h starved); *Post-Veg*, postvegetative stage (30 min starved); *Agg/DR*, cells in the aggregation stage incubated for 1 h with 1 mM cAMP to induce downregulation of the cAR1 protein; cAMP was removed by extensive washing.

Table III. cAMP Binding and CMF Binding to Gβ-Null Cells Pretreated with cAMP or CMF

Cell	Pretreatment	cAMP binding	CMF binding
		% of control	*% of control*
AX4	PB	100 (def)	100 (def)
	cAMP	29 ± 5	38 ± 13
	CMF	70 ± 16	38 ± 10
Gβ	PB	100 (def)	100 (def)
	cAMP	45 ± 10	145 ± 44
	CMF	98 ± 3	44 ± 27

AX4 cells and transformants with a deletion of the G-protein β subunit were treated as described in Table I with PB, 0.1 mM cAMP, or 1 ng/ml rCMF. The data shown are the means and standard errors of the mean of four (pretreated with cAMP) or five (pretreated with CMF) independent experiments. The binding of [^{3}H]cAMP or [^{125}I]CMF to cells treated with PB is defined (*def*) as 100% for each cell line.

Addition of CMF induced a time- and dose-dependent decrease of cAMP binding (Fig. 1, *A* and *B*). In this experiment, wild-type cells were starved at low cell density. Cells without added CMF showed a slow decrease of cAMP binding with a half-maximal effect at 5.7 min. During the binding experiment, these cells are expected to secrete CMF at a rate of 12 molecules per cell per min, resulting in a CMF concentration of 0.4 ng/ml at 5.7 min (Yuen and Gomer, 1994). The dose response measured at 3 min after CMF addition (Fig. 1 *B*) revealed a half-maximal effect at ~0.2 ng/ml of added CMF; taking into account that during 3 min 0.24 ng/ml CMF is secreted, this observation implies that a half-maximal reduction of cAMP binding to cAR1 is induced by ~0.4 ng/ml CMF. This is the CMF concentration that causes half-maximal cell differentiation (Jain et al., 1992). Since CMF does not affect cAMP binding in ammonium sulfate, these results suggest that CMF does not alter the amount of cAR1 protein, but that it affects its physical or functional state. This raises the question of whether cAMP can activate cAR1 in the absence of CMF.

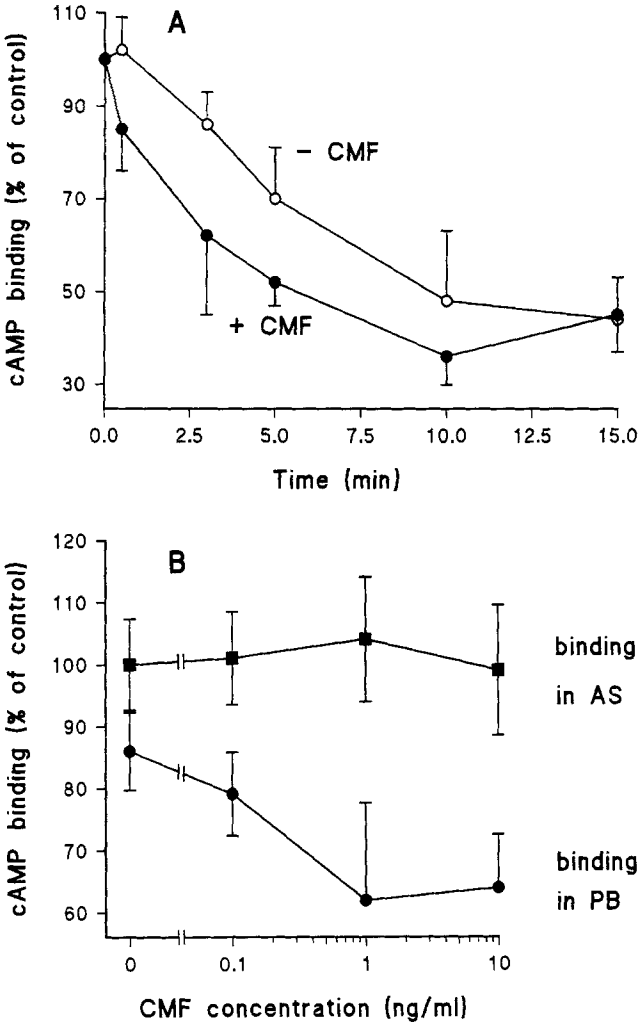

Figure 1. CMF modulates cAMP binding. *Dictyostelium* wild-type AX3 cells were starved in 10 mM PB at low cell density (10^6 cells per ml) for 5 h. Cells were collected and washed once with ice-cold PB, resuspended in PB to a density of 5×10^7 cells per ml, and used within 1 min for the experiment. (*A*) Time course. At t = 0 min, cells were transferred to room temperature and incubated in the absence (○) or presence (●) of 1 ng/ml purified recombinant CMF. At the times indicated, cAMP binding was measured in phosphate buffer. (*B*) Dose–response curve. Cells were incubated for 3 min with different concentrations of CMF, followed by assay of cAMP binding in phosphate buffer (●) or in nearly saturated ammonium sulfate (■). The data are shown as the means and SD of three independent experiments with triplicate determinations. The binding to cells immediately before the addition of CMF is set at 100%.

An initial response of cAR1 to cAMP is the conversion of some of the receptors from a high affinity form A^H to a low affinity form A^L; other receptors convert from a fast dissociating form B^F to a slowly dissociating form B^{SS}. These changes of the kinetic properties of the receptor have been related to the activation of a G-protein (Van Haastert et al., 1986). A delayed response is a loss of cAMP-binding activity due to sequestration, the first step in the process of downregulation (Van Haastert et al., 1992). The formation of the low affinity receptor form A^L by cAMP was detected in Scatchard analysis of equilibrium cAMP binding to cAR1 (Fig. 2). The data indicate that the number and K_d of the high affinity sites on control and CMF-depleted cells were essentially identical. The

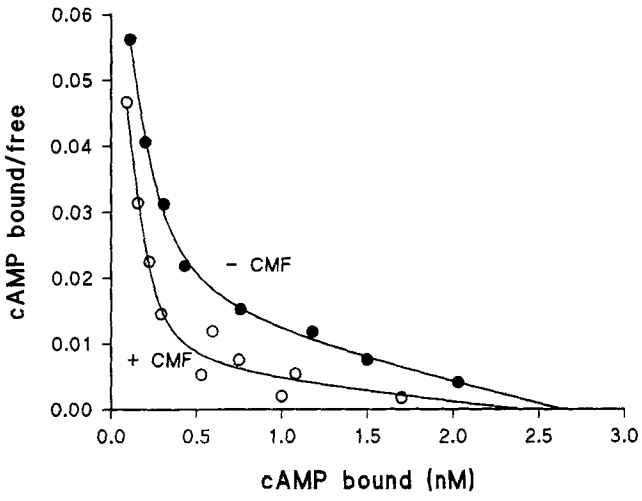

Figure 2. cAMP does not induce the formation of the receptor form A^L in the absence of CMF. CMF antisense cells were starved at a density of 10^7/ml for 5 h, washed, and resuspended in PB. Cells were incubated in the absence or presence of 1 ng/ml rCMF for 10 min. cAMP binding was measured in phosphate buffer using different concentrations of [^{3}H]cAMP. The data shown are the means of two experiments with triplicate determinations. The curves were analyzed by computer-assisted curve fitting with program FigP using a model of two independent binding sites (Van Haastert, 1994); the two-site model fits significantly better then a one-site model. The results and 95% confidence limits are: control with rCMF added (○), $B_1 = 0.223 \pm 0.021$ nM, $K_{d1} = 3.49 \pm 0.48$ nM, $B_2 = 2.17 \pm 0.48$ nM, $K_{d2} = 367 \pm 85$ nM; CMF-depleted cells (●), $B_1 = 0.220 \pm 0.012$ nM, $K_{d1} = 3.28 \pm 0.29$ nM, $B_2 = 2.40 \pm 0.19$ nM, $K_{d2} = 163 \pm 12$ nM (B is the number of binding sites; 1 nM = 12,500 sites per cell). The values for CMF-depleted cells and cells with added CMF are not significantly different, except that the value of the dissociation constant of the low affinity component ($P < 0.01$) with $K_{d2} = 163$ nM without CMF, and 367 nM with CMF.

number of the low affinity sites was also similar, but their K_d increased from 163 ± 12 to 363 ± 85 nM in the presence of CMF, indicating that CMF is required for the formation of the low affinity state A^L of cAR1. Incubation of cells with 2 nM cAMP for 1 min induces the formation of the B^{SS} state; this response is undetectable in cells lacking the G-protein Gα2 (Kesbeke et al., 1988). The fraction of cAR1 receptors converted to the B^{SS} form was 10 ± 0.9% in control cells and 2.6 ± 1.3% in CMF-depleted cells (Fig. 3). Preincubation of *Dictyostelium* cells with 0.1 mM cAMP for 15 min induced sequestration of 82.4 ± 7.8% of all receptors in control cells (Fig. 4); this response is still present in cells lacking the G-protein α2 or β subunits (Kesbeke et al., 1988; Wu et al., 1995). However, in CMF-depleted cells, cAMP-mediated sequestration of cAR1 was only 21 ± 17% (Fig. 4). Thus, CMF is required to allow cAMP to induce changes of the cAR1 protein itself.

The Interaction between CMF and cAMP Receptors

To examine the nature of the linkage between the CMF receptor and the cAMP receptor, sequestration was examined in cells with a defective G-protein. Whereas downregulation of wild-type cells with cAMP or CMF induced a reduction of both cAMP binding and CMF binding, this was not observed in cells with a deletion of the G-protein β subunit. Pretreatment of these cells with cAMP reduced cAMP binding, but it did not affect CMF binding. Conversely, pretreatment with CMF reduced CMF binding but not cAMP binding. Thus, in the absence of G-protein activation, a ligand induces a loss of its own binding, but it has no effect on the binding of the other ligand. The simplest interpretation is that cAMP and CMF bind to separate but closely linked receptors; communication between these receptors requires the activation of a G-protein.

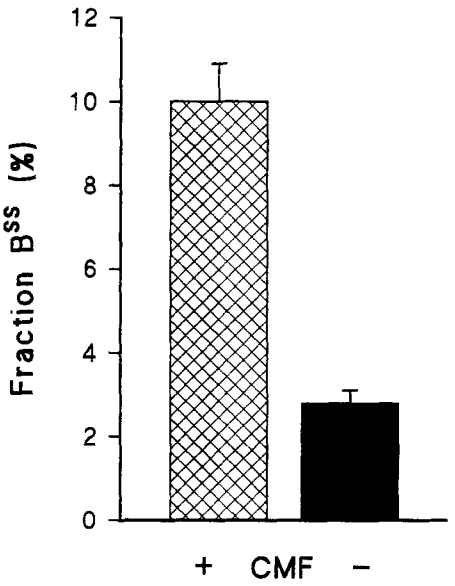

Figure 3. cAMP does not induce the formation of the receptor form B^{SS} in the absence of CMF. CMF antisense cells were starved at a density of 10^7/ml for 5 h, washed, and resuspended in PB. The binding of cAMP to the receptor form was then measured as follows: cells were incubated in the absence or presence of 1 ng/ml rCMF for 10 min. Cells were incubated for 1 min on ice with 2 nM [^{3}H]cAMP in PB; the dissociation of the bound [^{3}H]cAMP was measured after addition of 1 ml ice-cold buffer containing 1 mM cAMP, and the fraction of bound [^{3}H]cAMP released from the slowly dissociating form B^{SS} was calculated as described (Van Haastert et al., 1986). The results and 95% confidence limits are: control with CMF added (*crosshatched bar*), Fraction B^{SS} = (10.0 ± 0.9)%, k_{-1} = (2.15 ± 0.7) × 10^{-3} s^{-1}; CMF-depleted cells (*solid bar*), Fraction B^{SS} = (2.8 ± 0.3)%, k_{-1} = (3.13 ± 1.3) × 10^{-3} s^{-1} (k_{-1} is the rate constant of dissociation of B^{SS}). The difference between the rate constants of dissociation is not statistically different; the difference between the fraction of B^{SS} is significant at $P < 0.01$. The data shown are the means of two experiments with triplicate determinations.

Discussion

We have previously shown that CMF is required for cell aggregation. Cells without CMF do not show cAMP-mediated chemotaxis or the activation of several second messengers. cAMP binds to a G-protein–coupled seven-transmembrane receptor. CMF is required for both G-protein–dependent (chemotaxis, cAMP response, and cGMP response) and G-protein–independent (Ca^{2+} uptake) responses (Yuen et al., 1995). All these responses are mediated by the major cAMP receptor cAR1. CMF is not required for cAMP binding to cAR1, indicating that CMF acts downstream of the binding of cAMP to cAR1. Interestingly, the cAMP-induced increase of IP_3, which is G-protein dependent but cAR1 independent, still occurs in cells

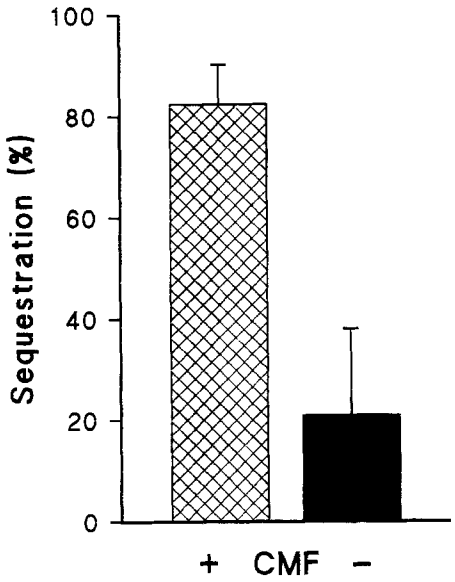

Figure 4. cAMP does not induce loss of ligand binding in the absence of CMF. CMF antisense cells were starved at a density of 10^7/ml for 5 h, washed, and resuspended in PB. Cells were incubated at room temperature with 0.1 mM cAMP for 15 min in the presence (*crosshatched bar*) or the absence (*solid bar*) of 1 ng/ml recombinant CMF, washed extensively, resuspended in buffer to 5 × 10^7 cells per ml, and assayed for cAMP binding in the presence of ammonium sulfate as described in the legend of Fig. 1 *B*. The data presented are the decrease of cAMP binding when compared with cells not preincubated with 0.1 mM cAMP. The data shown are the means of two experiments with triplicate determinations.

without CMF (Bominaar and Van Haastert, 1994; Yuen et al., 1995). Thus, the signal transduction phenotype of CMF-depleted cells somewhat resembles that of cAR1-null cells. In this study, we find that CMF is required not only for the stimulation of second messenger responses, but also for cAMP-mediated alterations of the cAR1 protein itself. In control cells, cAMP induces the alteration of the affinity and dissociation rates of cAR1, which are mediated in membranes by GTPγS and are absent in cells with a deletion of the G-protein subunits Gα2 or Gβ (Kesbeke et al., 1988; Kumagai et al., 1989; Wu et al., 1995). These combined data suggest that in cells without CMF, cAMP binding to the receptor cAR1 does not lead to the interaction and activation of the G-protein. In control cells, cAMP also induces the loss of ligand binding, which is probably the first step in a process of receptor sequestration and downregulation. Loss of ligand binding does not require the activation of a G-protein, as it is unaltered in cells lacking Gα2 or Gβ. In cells without CMF, cAMP does not induce loss of ligand binding. Since both G-protein–dependent and G-protein–independent alterations of cAR1, as well as responses, require CMF, it appears that CMF primarily regulates the activation of the cAR1 protein itself and indirectly regulates the activation of the G-protein by the activated cAR1.

We tested the hypothesis that CMF may directly bind to cAR1 and observed a close correlation between cAMP and CMF binding to cells with a 20-fold difference of cAR1 expression; this large variation in cAR1 levels was obtained by overexpression of cAR1 during growth, inactivation of the cAR1 gene by homologous recombination, or downregulation of cAR1 by prolonged exposure of wild-type cells to cAMP. Furthermore, we observed that CMF also can induce downregulation of both CMF and cAMP binding. This close correlation between cAMP and CMF binding may suggest that the cAMP receptor cAR1 and the CMF receptor are the same protein. Nevertheless, we investigated the possibility that CMF binds to another receptor protein, and that the expression of the CMF receptor is under the tight control of the expression of cAR1, both in transformants that overexpress cAR1, in cAR1-null cells, and in wild-type cells where cAR1 is downregulated by excess cAMP. We observed that CMF at a concentration of 1 ng/ml (12.5 pM) is sufficient to downregulate half of the cAMP receptors; taking a dissociation constant of 2.1 nM for the 39,000 CMF receptors (Jain and Gomer, 1994), and assuming binding equilibrium, this would imply that 200 occupied CMF receptors can downregulate 20,000 cAMP receptors. Secondly, in cells with a disruption of the Gβ subunit, cAMP induces a reduction of cAMP-binding activity but no reduction of CMF-binding activity; and vice versa, CMF induces a reduction of CMF-binding activity but has no effect on cAMP-binding activity. These observations strongly suggest that the cAMP receptor and the CMF receptor are two different proteins that are coupled via a G-protein.

In the absence of CMF, cAMP still binds to cAR1, but no cAR1-mediated responses are induced by cAMP. CMF alone has no effect on the levels of second messengers in *Dictyostelium*. Thus, CMF is an essential coactivator of the cAMP receptor. This implies that during *Dictyostelium* development, the cAMP sensory transduction machinery including cAR1, G-proteins, and effector enzymes are expressed, but cells must wait for CMF to accumulate above a threshold level to allow cAMP to become active as a chemoattractant. This provides the mechanism by which groups of cells communicate their density and collective state of starvation. CMF functions as a cell density–sensing factor by permitting cAR1 to be sensitive to cAMP. The dual control of a G-protein–coupled receptor by activating and permissive signals could be a general mechanism for cell density sensing.

We thank Nora Ghbeish for assistance in preparation of [^{125}I]CMF.

This work was supported by grants from the Netherlands Organization for Scientific Research and by R.A. Welch grant C-1247. R.H. Gomer is an assistant investigator of the Howard Hughes Medical Institute.

Received for publication 12 January 1996 and in revised form 29 April 1996.

References

Bominaar, A.A., and P.J.M. Van Haastert. 1994. Phospholipase C in *Dictyostelium*. Identification of stimulatory and inhibitory surface receptor and G-proteins. *Biochem. J.* 297:189–193.

Clarke, M., and R.H. Gomer. 1995. PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*. *Experientia (Basel).* 51:1124–1134.

Fuqua, W.C., S.C. Winans, and E.P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* 176:269–275.

Gomer, R.H. 1994. Knowing that you're among friends. *Curr. Biol.* 4:734–735.

Gomer, R.H., and R.A. Firtel. 1987. Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell-cycle phase. *Science (Wash. DC).* 237:758–762.

Gomer, R.H., I.S. Yuen, and R.A. Firtel. 1991. A secreted 80 × 10^3 M_r protein mediates sensing of cell density and the onset of development in *Dictyostelium*. *Development (Camb.).* 112:269–278.

Gurdon, J.B., P. Lemaire, and K. Kato. 1993. Community effects and related phenomena in development. *Cell.* 75:831–834.

Jain, R., and R.H. Gomer. 1994. A developmentally regulated cell surface receptor for a density sensing factor in *Dictyostelium*. *J. Biol. Chem.* 269:9128–9136.

Jain, R., I.S. Yuen, C.R. Taphouse, and R.H. Gomer. 1992. A density sensing factor controls development in *Dictyostelium*. *Genes & Dev.* 6:390–400.

Johnson, R.L., C.L. Saxe, R. Gollop, A.R. Kimmel, and P.N. Devreotes. 1993. Identification and targetted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development. *Genes & Dev.* 7:273–282.

Kesbeke, F., B.E. Snaar-Jagalska, and P.J.M. Van Haastert. 1988. Signal transduction in *Dictyostelium* fgd A mutant with a defective interaction between surface cAMP receptors and GTP-binding regulatory protein. *J. Cell Biol.* 107:521–528.

Khachatrian, L., A. Howlett, and C. Klein. 1987. Ammonium sulfate modifies adenylate cyclase and the chemotactic receptor for *Dictyostelium discoideum*. Evidence for a G protein effect. *J. Biol. Chem.* 262:8071–8076.

Klein, C., and M.H. Juliani. 1977. cAMP-induced changes in cAMP-binding sites on *D. discoideum* amebae. *Cell.* 10:329–335.

Klein, P.S., T.J. Sun, C.L. Saxe III, A.R. Kimmel, R.L. Johnson, and P.N. Devreotes. 1988. A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science (Wash. DC).* 241:1467–1472.

Kumagai, A., M. Pupillo, R. Gundersen, R. Miake-Lye, P.N. Devreotes, and R.A. Firtel. 1989. Regulation and function of Gα protein subunits in *Dictyostelium*. *Cell.* 57:265–275.

Lilly, P.J., L. Wu, D.L. Welker, and P.N. Devreotes. 1993. A G-protein β-subunit is essential for *Dictyostelium* development. *Genes & Dev.* 7:986–995.

Louis, J.M., G.T. Ginsburg, and A.R. Kimmel. 1994. The cAMP receptor cAR4 regulates axial patterning and cellular differentiation during late development of *Dictyostelium*. *Genes & Dev.* 8:2086–2096.

Magnuson, R., J. Solomon, and A.D. Grossman. 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. *Cell.* 77:207–216.

Mehdy, M.C., and R.A. Firtel. 1985. A secreted factor and cAMP jointly regulate cell-type specific gene expression. *Mol. Cell. Biol.* 5:705–713.

Milne, J.L., and M.B. Coukell. 1991. A Ca^{2+} transport system associated with the plasma membrane of *Dictyostelium discoideum* is activated by different chemoattractant receptors. *J. Cell Biol.* 112:103–110.

Milne, J.L., and P.N. Devreotes. 1993.The surface cAMP receptors cAR1, cAR2, and cAR3 promote Ca^{2+} influx in *Dictyostelium discoideum* by a Gα2-independent mechanism. *Mol. Biol. Cell.* 4:283–292.

Pupillo, M., R.H. Insall, G.S. Pitt, and P.N. Devreotes. 1992. Multiple cAMP receptors are linked to adenylyl cyclase in *Dictyostelium*. *Mol. Biol. Cell.* 3:

1229–1234.
Rathi, A., S.C Kayman, and M. Clarke. 1991. *Dev. Genet.* 12:82–87.
Saxe, C.L., G.T. Ginsburg, J.M. Louis, R. Johnson, P.N. Devreotes, and A.R. Kimmel. 1993. cAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum. Genes & Dev.* 7: 262–272.
Sun, T.J., P.J.M. Van Haastert, and P.N. Devreotes. 1990. A surface receptor controls development in *Dictyostelium discoideum*. Evidence by antisense mutagenesis. *J. Cell Biol.* 110:1559–1564.
Van Haastert, P.J.M. 1984. Guanine nucleotides modulate cell surface cAMP-binding sites in membranes from *Dictyostelium discoideum. Biochem. Biophys. Res. Commun.* 124:597–604.
Van Haastert, P.J.M. 1985. The modulation of cell surface cAMP receptors from *Dictyostelium discoideum* by ammonium sulfate. *Biochim. Biophys. Acta.* 845:254–260.
Van Haastert, P.J.M. 1994. Intracellular adenosine 3′,5′-phosphate is essential for down-regulation of adenosine 3′,5′-phosphate surface receptors in *Dictyostelium. Biochem. J.* 303:539–545.
Van Haastert, P.J.M., R.J.W. De Wit, P.M.W. Janssens, F. Kesbeke, and J. DeGoede. 1986. G-protein mediated interconversions of cell-surface cAMP receptors and their involvement in the activation and desensitization of guanylate cyclase in *Dictyostelium discoideum. J. Biol. Chem.* 261:6904–6911.
Van Haastert, P.J.M., M. Wang, A.A. Bominaar, P.N. Devreotes, and P. Schaap. 1992. cAMP-induced desensitization of surface cAMP receptors in *Dictyostelium*: different second messengers mediate phosphorylation, loss of ligand binding, degradation of receptor and reduction of receptor mRNA levels. *Mol. Biol. Cell.*, 3:603–612.
Wu, L., R. Valkema, P.J.M. Van Haastert, and P.N. Devreotes. 1995. The G protein β-subunit is essential for multiple responses to chemoattractants in *Dictyostelium. J. Cell Biol.* 129:1667–1675.
Yuen, I.S., and R.H. Gomer. 1994. Cell density sensing in *Dictyostelium* by means of the accumulation rate, diffusion coefficient and activity threshold of a protein secreted by starved cells. *J. Theor. Biol.* 167:273–284.
Yuen, I.S., C.R. Taphouse, K.A. Halfant, and R.H. Gomer. 1991. Regulation and processing of a secreted protein that mediates sensing of cell density in *Dictyostelium. Genes & Dev.* 113:1375–1385.
Yuen, I.S., R. Jain, J.D. Bishop, D.F. Lindsey, W.J. Deery, P.J.M. Van Haastert, and R.H. Gomer. 1995. A density-sensing factor regulates signal transduction in *Dictyostelium. J. Cell Biol.* 129:1251–1262.
Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J.M. Friedman. 1994. Positional cloning of the mouse *obese* gene and its human homologue. *Nature (Lond.).* 372:425–432.