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A cell-density sensing factor regulates the lifetime of a chemoattractant-induced Gα-GTP conformation

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Abstract **Starving *Dictyostelium discoideum* cells monitor the local density of other starving cells by simultaneously secreting and sensing CMF. CMF regulates signal transduction through the chemoattractant cAMP receptor, cAR1. cAR1 activates a heterotrimeric G protein by stimulating Gα2 to release GDP and bind GTP. We show here that the rate of cAMP-stimulated GTP hydrolysis in membranes from cells exposed to CMF is roughly 4 times slower than in membranes from untreated cells, even though the rate of GTP binding is the same. This hydrolysis is abolished in cells lacking Gα2. Our data thus suggest that CMF regulates cAMP signal transduction in part by prolonging the lifetime of the Gα2-GTP complex.**

Key words: Cell density; GTPase; G-protein; Conditioned media factor; (*Dictyostelium discoideum*)

1. Introduction

Dictyostelium is a unicellular amoeba which feeds on bacteria. The cells eventually overgrow their food supply, and when most of the cells in a given area starve, they aggregate using pulses of cyclic AMP as the chemoattractant. The aggregated cells develop into a fruiting body consisting of spores supported on a column of stalk cells (see [1] for review). When a starving *Dictyostelium* cell receives a pulse of cAMP, it releases a burst of cAMP to relay the signal, moves towards the source of cAMP, and activates the expression of specific classes of genes [2]. The incoming cAMP pulse is sensed by cAR1 cell surface cAMP receptors, which cause Gα2 subunits to release GDP and bind GTP [3–5]. This activation causes the Gβγ subunits, along with a cytosolic protein called CRAC, to transiently activate adenylyl cyclase while the Gα2 subunits activate guanylyl cyclase and become phosphorylated [6]. Activation of cAR1 also causes a transient influx of Ca^{2+}, which is G-protein independent (see [7–11] for review). The GTP which bound to Gα2 in response to cAMP is then hydrolysed to GDP. *Dictyostelium* membranes contain several membrane-associated GTPases [12–14]. These include a basal level of a low-affinity GTPase, and a high-affinity GTPase activity which is stimulated by a pulse of cAMP [12].

The aggregation and development of *Dictyostelium* require the presence of the extracellular molecule conditioned media factor (CMF) [15,16], which is secreted and sensed only by starving cells. CMF is an 80 kDa glycoprotein with no similarity to any known protein [17]. CMF antisense cells do not aggregate when starved, unless they are allowed to develop in the presence of exogenous CMF or recombinant CMF (rCMF) [17]. We have hypothesized that the function of CMF is to coordinate the development of large fruiting bodies by triggering aggregation only when most of the cells in an area have starved, as signaled by a high level of CMF. Without such a mechanism to sense the density of starved cells, small cohorts of cells which starved at the same time might each form a small, ineffective fruiting body.

The predicted diffusion of CMF indicates that CMF might be able to mediate density sensing in the wild [18]. CMF is secreted by starved cells at a rate of 12 molecules/cell per min, and the theoretical diffusion from a cell on a soil surface or submerged in water predicts that the concentration of CMF in the immediate vicinity of an isolated starved cell remains below 0.3 ng/ml, the half-maximal activity of CMF, by a factor of at least 10 even after 10 h of continuous secretion. Similar calculations showed that when many cells in a region starve, the extracellular CMF concentration could reach 0.3 ng/ml after 2 h of secretion. Interestingly, for aggregates of fewer than roughly 45 cells, the CMF concentration can never rise to 0.3 ng/ml, indicating that CMF could be used to sense whether there are more than 45 cells in an aggregate [19]. The solutions are not unique: many combinations of secretion rate, diffusion coefficient, and threshold sensitivity will allow density sensing. These calculations illustrate that as a general principle cells can sense their local density by simultaneously secreting and recognizing a molecule. Such a mechanism could also be used for determining the total number of cells in a tissue.

CMF regulates several aspects of cAMP signal transduction [20]. 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 restored by a 10 s exposure of the cells to CMF. Down-regulation of cAR1 with high levels of cAMP also down-regulates CMF binding [21], and CMF similarly downregulates cAMP and CMF binding, indicating a linkage between the two signal transduction systems [22]. Binding of roughly 200 molecules of CMF to starved cells affects the affinity of the majority of the 40 000 cAR1s within 2 min, indicating that the linkage involves an amplifying mechanism. In cells lacking Gβ, 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 between the CMF and cAMP signal transduction pathways is through a G protein [22]. Cells lacking CMF have normal levels of cAR1, cAMP-induced binding of GTP or GTPγS to membranes, and GTPγS modulatable cAMP binding, suggesting that the interaction of the cAMP receptor with G proteins in vitro is not measurably affected by CMF. However, the activation of adenylyl cyclase by GTPγS requires cells to

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have been exposed to CMF [20]. CMF thus appears to control aggregation by regulating cAMP signal transduction at a step after cAMP induces Gα2 to exchange GDP for GTP, but before the Gα2-GTP complex can activate downstream effectors. In this report we show that this phenomenon is due in part to the fact that CMF regulates the lifetime of the Gα2-GTP configuration.

2. Materials and methods

2.1. Cell culture

Ax4 wild-type cells and Gα2 knockout cells (a gift from Dr. Peter Devreotes, Johns Hopkins University, Baltimore, MD) were grown in shaking culture in HL5 medium as previously described [17,21]. Conditioned medium (CM) was prepared by starving Ax4 cells at 5×10^6 cells/ml in PBM (20 mM KH_2PO_4, 10 μM $CaCl_2$, 1 mM $MgCl_2$, pH 6.1 with KOH) in shaking culture for 20 h, and then clarifying the conditioned medium as described by Gomer et al. [16].

2.2. GTP binding

The binding of GTP to membranes was measured following Snaar-Jagalska and Van Haastert [23] with the following modifications. Vegetative cells were harvested by centrifugation at $500\times g$ in PBM, washed once in PBM, and starved in PBM by shaking at a density of 10^7 cells/ml. After 6 h, starved cells were collected by centrifugation as before, and washed twice with PBM. The resulting pellet was resuspended in 40 mM HEPES, 0.5 mM EDTA, 250 mM sucrose, pH 7.7, to a density of 10^8 cells/ml, and immediately lysed to prevent the accumulation of CMF. If used, recombinant CMF [21] was added to the cells at a concentration of 1 ng/ml 30 s before cell lysis. Cells were lysed by passing the cell suspension through a Cameo 25N 5 μm pore size syringe filter (MSI, Westboro, MA) at 4°C. The crude membranes were isolated by centrifugation at $17000\times g$ for 5 min at 4°C, washed once with PBM, and resuspended in PBM to the equivalent of 5×10^7 cells/ml. The binding reaction mixture was preincubated at room temperature for 3 min, and binding was then initiated by the addition of 80 μl of membranes to 20 μl of reaction mixture. The reaction was stopped after 3 min and bound [^{3}H]GTP was separated from free [^{3}H]GTP by centrifugation at $17000\times g$ for 1 min. The supernatant was removed, the pellets were centrifuged as before, the residual supernatant was removed. The membranes were resuspended in 100 μl of 1 M acetic acid, and the amount of bound [^{3}H]GTP was determined by scintillation counting.

2.3. GTPase assay

GTPase activity was determined as described by Snaar-Jagalska et al. [12], with the exception that cells were starved and crude membranes were prepared as described above for GTP binding. The membranes were washed once with 10 mM triethanolamine HCl, 0.5 mM EDTA pH 7.4 and the final pellet resuspended in 10 mM triethanolamine HCl, pH 7.4, to the equivalent of 10^8 cells/ml.

2.4. Gα2 phosphorylation

Phosphorylation of Gα2 was assessed by a shift in Gα2 mobility on SDS-PAGE as previously described [6] with the following modifications. Cells were starved at 2×10^5 cells/ml with 50 nM pulses of cAMP delivered every 6 min. After 5 h of starvation, cells were pelleted and washed once with fresh DB and quickly resuspended in DB at three cell densities: 5×10^5, 5×10^6, and 5×10^7 cells/ml. Caffeine was added to the cell suspensions to 2 mM and these were shaken for 20 min at 200 rpm. Cells were sampled either just prior to or 1 min after cAMP stimulation (1 μM cAMP plus 10 mM DDT). Samples for SDS-PAGE were made from 4×10^5 cells pelleted in a microcentrifuge at $2000\times g$ for 1 min. The supernatant was quickly aspirated and the cell pellet was resuspended in 1×SDS-PAGE sample buffer at 100°C, vortexed, and heated at 100°C for 4 min. Western blots of the whole cell protein samples were examined for a Gα2 gel mobility shift by staining with anti-Gα2 peptide antiserum.

3. Results

We previously found that CMF regulates cAMP signal transduction at a step after cAMP induces Gα2 to exchange GDP for GTP, but before Gα2-GTP and Gβγ can activate guanylyl and adenylyl cyclases respectively [20]. One possible mechanism which could account for regulation at this step would involve CMF regulating the lifetime of the Gα2-GTP configuration by regulating hydrolysis of the GTP to GDP. GTP hydrolysis in *Dictyostelium* membranes is performed by at least two enzymes, one with a high affinity for GTP and one with low affinity. Only the high-affinity GTPase is stimulated by cAMP [12]. To examine if CMF regulates the low- or high-affinity GTPases, wild-type cells were starved, washed free of CMF, lysed, and the resulting membranes assayed for the hydrolysis of [γ-^{32}P]GTP. In the absence of cAMP stimulation, the high- and low-affinity GTPase activities were slightly decreased by the presence of CMF, with p values less than 0.005 and 0.01, respectively (Table 1). Thus, CMF is able to regulate both GTPase activities in membranes when cAMP is not present. Addition of cAMP in the presence or absence of CMF had no significant effect on the low-affinity GTPase activity (data not shown). However, addition of cAMP caused a 42% increase in high-affinity GTPase activity in the absence of CMF (Fig. 1 and [12,24]), whereas in the presence of CMF, addition of cAMP caused only an 6% increase in high-affinity GTPase activity. Therefore, it appears that the presence of CMF greatly reduces cAMP-stimulated, high-affinity GTP hydrolysis activity.

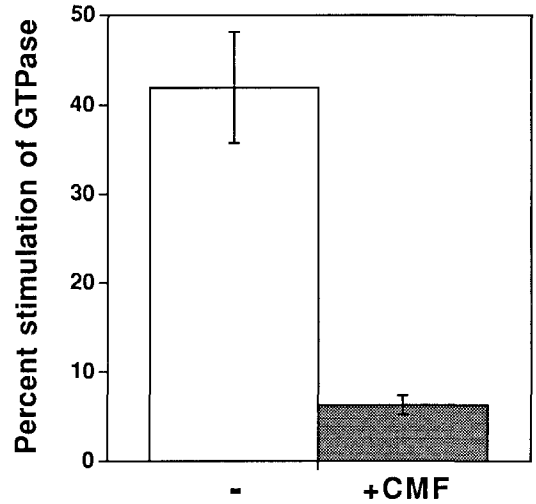

Fig. 1. Effect of CMF on cAMP-stimulated high-affinity GTPase. Ax4 cells were starved for 6 h and then divided into two aliquots. Recombinant CMF was added to one flask to 1 ng/ml (+CMF), and nothing was added to the other flask (−). 30 s later cells were lysed. The membranes were isolated, incubated in the presence or absence of 10 μM cAMP and assayed for their ability to hydrolyse [γ-^{32}P]GTP. cAMP stimulation was defined as the percentage increase in high-affinity GTPase activity after addition of cAMP. The results are the means of seven experiments with error bars representing the standard error of the mean.

CMF could be decreasing cAMP-stimulated GTP hydrolysis in membranes in two ways. It could be altering the GTPase activity of G proteins, or it could be decreasing cAMP-stimulated GTP binding to membranes. We previously found that CMF has no effect on cAMP-stimulated binding of GTP to membranes in cells starved at low cell density [20]. To examine the possibility that CMF may affect GTP binding at the cell density used in the GTPase assay, binding of [^{3}H]GTP to membranes was again measured. CMF had no significant ef-

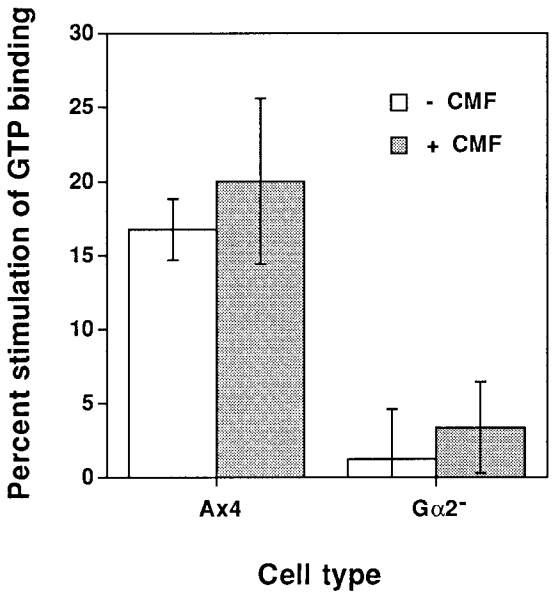

Fig. 2. Effect of CMF on cAMP-stimulated high-affinity GTP binding. Membranes isolated as described in Fig. 1 were incubated in the presence or absence of 10 μM cAMP and assayed for their ability to bind [^{3}H]GTP. cAMP stimulation was defined as the percentage increase in high-affinity GTP binding activity after addition of cAMP. The results are the means of five experiments with error bars representing the standard error of the mean.

fect on low-affinity or high-affinity GTP binding in the absence of cAMP, and addition of cAMP had no significant effect on low-affinity GTP binding in the presence or absence of CMF (data not shown). In the absence of CMF, cAMP induced a 15% increase in high-affinity GTP binding (Fig. 2 and [20,23]). Addition of CMF caused a slight but insignificant increase in cAMP-stimulated GTP binding (Fig. 2). Therefore, CMF affects the rate of cAMP-stimulated, high-affinity GTP hydrolysis without influencing cAMP-stimulated GTP binding.

It is unclear what protein is responsible for cAMP-stimulated, high-affinity GTP hydrolysis. A nitrosoguanidine-generated mutant called *fgdA* HC85 [25] contains a 2 kb deletion of the Gα2 genomic region, resulting in a loss of part of the Gα2 coding region [26]. Stimulation of GTPγS (a non-hydrolysable analogue of GTP) binding by (Sp)cAMPS (a cAMP receptor agonist) is abolished in *fgdA* HC85 cells [24]. However, the cAMP-stimulated high-affinity GTPase is 75% that of wild-type [24]. Since *fgdA* HC85 cells may express part of Gα2 and also are known to contain additional mutations [27], cAMP-stimulated GTPase and GTP binding activities were measured in membranes from cells in which homologous recombination was used to delete specifically all of Gα2 [27]. We found that in the absence of CMF, addition of cAMP to Gα2$^-$ membranes caused a slight but statistically insignificant increase in high-affinity GTP binding. In the presence of CMF, addition of cAMP caused a similar increase in high-affinity GTP binding in these membranes (Fig. 2). Thus, the vast majority of cAMP-stimulated, high-affinity GTP binding is due to Gα2 protein. To determine the role of Gα2 in cAMP-stimulated, high-affinity GTPase activity, we examined the contribution of the high-affinity GTPase activity to the total GTPase activity in Ax-4 and Gα2$^-$ membranes. In wild type membranes, approx. 80% of the total cAMP-stimulated GTPase activity is due to the high-affinity activity. However, in Gα2$^-$ membranes, none of the total GTPase activity is due to the high-affinity activity (Table 2). Therefore, both membrane-associated cAMP-stimulated high-affinity GTP binding and GTPase activity appear to be mediated by Gα2.

Extracellular cAMP causes two changes in Gα2. GDP is exchanged for GTP, and Gα2 becomes phosphorylated on Ser-113 [28]. Phosphorylation of Gα2 causes a characteristic mobility shift on SDS-PAGE, which can be detected by staining Western blots of whole cells with anti-Gα2 antibodies (Gundersen and Devreotes [6] and Fig. 3). To determine if CMF also regulates Gα2 phosphorylation, aggregation-stage cells were incubated for 20 min at different densities and then assayed for a cAMP-induced Gα2 mobility shift. At the lowest density, 5×10^5 cells/ml, the calculated CMF concentration was 0.016 ng/ml, well below the 0.3 ng/ml CMF threshold concentration [16]. At the highest concentration, 5×10^7 cells/ml, the optimal CMF concentration of 1 ng/ml would have been reached in approx. 12 min. We found that cAMP caused a shift of Gα2 in the cells incubated at all 3 densities (Fig. 3). Similar results were also seen with cells starved at 2×10^5 cells/ml (data not shown). The data from the Western blots thus indicates that the presence or absence of an activating concentration of CMF does not affect cAMP-stimulated Gα2 phosphorylation.

4. Discussion

We have confirmed that Gα2 is responsible for all of the detectable cAMP-stimulated high-affinity binding of GTP to membranes, and have shown that Gα2 is similarly responsible for all of the detectable membrane-associated cAMP-stimulated high-affinity GTPase. Using the specific activities of the radiolabelled GTPs, we calculate that in the presence or absence of CMF, a pulse of cAMP causes approx. 250 molecules of GTP to bind to a cell's membrane (and thus Gα2 proteins) over the course of 2 min, after which no more GTP binds [20 and this report]. During the same period, in the absence of CMF, the GTP which bound to membranes in response to a pulse of cAMP is hydrolyzed at a rate of roughly 80 molecules/cell per min. The combination of these binding and hydrolysis rates would therefore cause the amount of Gα2-bound GTP to remain low after a pulse of cAMP, and to approach 0 by 3 min. In the presence of CMF, we similarly calculate that the GTP which bound to membranes in response to a pulse of cAMP is hydrolyzed at a rate of 17 molecules/cell per min. This lower hydrolysis rate would cause the amount of Gα2-bound GTP to be high for several minutes, roughly matching the kinetics observed for Gα2-GTP-stimulated guanylyl cyclase in vitro [29]. In vivo,

Table 1
Effect of CMF on basal GTPase levels in membranes

	−CMF	+CMF
Low-affinity GTPase	100	81 ± 5
High-affinity GTPase	100	90 ± 4

Ax4 cells were starved for 6 h and membranes were isolated. The membranes were incubated for 30 s in the presence or absence of 1 ng/ml of recombinant CMF, and the GTPase activities were then measured in the absence of cAMP. In each experiment, the activity was normalized to the activity found in the absence of CMF. The results are the means of seven experiments and the associated standard errors of the mean.

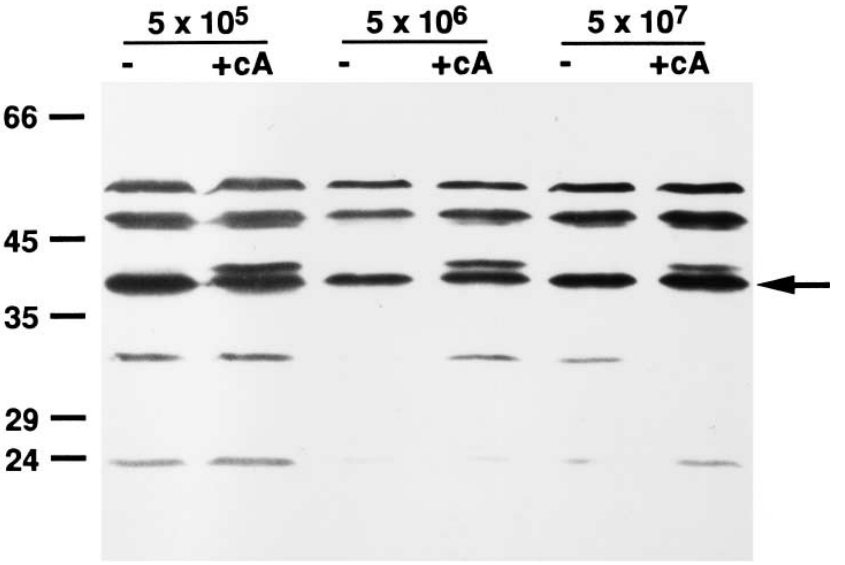

Fig. 3. cAMP-induced phosphorylation of Gα2. Starved cells were incubated at the indicated densities. Whole cells samples were taken either just prior to (−) or 1 min after stimulation with 1 μM cAMP (+cA). Gα2 is approx. 40 kDa (arrow) and the shifted band is approx. 42 kDa. The bands above 42 kDa and below 40 kDa represent variable non-specific staining [6].

cAMP-stimulated guanylyl cyclase activity is deactivated much more quickly due to a concomitant cAMP-stimulated influx of Ca^{2+} which inhibits the cyclase [29,30]. Our data thus suggest that CMF regulates cAMP signal transduction in part by regulating the lifetime of GTP-bound Gα2 in vitro. A similar decrease in Gα2 GTPase activity can be obtained by inhibiting an unknown G_i by treatment with pertussis toxin [12], raising the possibility that CMF signal transduction may involve inhibiting this unknown G_i.

There are three known methods of regulation of Gα subunit activity. One is localization (for review see [31]). The presence of CMF could allow Gα2 to localize to the membrane. In this case, addition of CMF should cause an increase in membrane associated Gα2 GTPase activity. However, we observe the opposite. Therefore, CMF cannot be regulating Gα2 activity by controlling its localization to the plasma membrane.

A second form of regulation of Gα2 could be through its phosphorylation. Gα2 is rapidly phosphorylated in response to cAMP [6]. Eliminating this phosphorylation has no effect on development at high cell densities, where cells accumulate CMF [28]. However, the function of CMF might be to dephosphorylate Gα2. Our data (Fig. 3) indicate that CMF has no observable effect on either the basal or the cAMP-stimulated phosphorylation of Gα2. Since cAMP-stimulated ^{32}P labelling of Gα2 has always been associated with a shift in gel mobility, and phosphorylation appears to occur on a single serine [28], it seems unlikely that CMF is regulating a phosphorylation or dephosphorylation of Gα2 that does not cause a shift in gel mobility. In conjunction with our data, this indicates that CMF does not regulate cAMP signal transduction via phosphorylation of Gα2.

Another possibility is that CMF controls Gα2 activity by regulating its association with GTPase activating or inhibiting proteins. Such proteins are known to regulate the GTPase activity of small GTP binding proteins such as Ras [32]. There are proteins called Regulators of G protein Signaling (RGS) which appear to regulate the activity of Gα subunits in yeast, worms and mammals [33]. Although genetic analysis has placed RGS proteins upstream of the G protein they regulate [34,35], it is not known if they activate the GTPase activity of Gα subunits. We have thus found a potentially general way for one signal transduction pathway to regulate a second G protein-mediated signal transduction pathway by regulating the lifetime of the Gα-GTP state.

Table 2
Contribution of the high-affinity GTPase activity to the total GTPase activity in membranes

	−CMF	+CMF
Ax4	81 ± 2%	78 ± 2%
Gα2⁻	0 ± 7%	0 ± 13%

Ax4 and Gα2⁻ cells were starved for 6 h, and membranes were isolated. The membranes were incubated in the presence or absence of 1 ng/ml of recombinant CMF, and the GTPase activities were measured in the presence of cAMP. In each experiment, the high-affinity and total GTPase activities were measured and the ratio of high-affinity to total GTPase activity was calculated as a percentage. Thus, if all of the GTPase activity was due to the high-affinity activity, the percentage would be 100%. The results are the means of 3 experiments and the associated standard errors of the mean.

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