

Signal Transduction in *Dictyostelium fgd A* Mutants with a Defective Interaction between Surface cAMP Receptors and a GTP-binding Regulatory Protein

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Abstract. Transmembrane signal transduction was investigated in four *Dictyostelium discoideum* mutants that belong to the *fgd A* complementation group. The results show the following. (a) Cell surface cAMP receptors are present in *fgd A* mutants, but cAMP does not induce any of the intracellular responses, including the activation of adenylate or guanylate cyclase and chemotaxis. (b) cAMP induces down-regulation and the covalent modification (presumably phosphorylation) of the cAMP receptor. (c) The inhibitory effects of GTP γ S and GDP β S on cAMP binding are reduced;

the stimulatory effect of cAMP on GTP γ S binding is lost in *fgd A* mutants. (d) Basal high-affinity GTPase activity is reduced 40% and the stimulatory effect of cAMP is decreased from 40% in wild type to 30% in *fgd A*. (e) GTP-mediated stimulation and inhibition of adenylate cyclase is normal in mutant membranes. The results suggest a defective interaction between cell surface cAMP receptors and a specific G-protein in *fgd A* mutants. This interaction appears to be essential for nearly all signal transduction pathways in *Dictyostelium discoideum*.

THE cellular slime mold *Dictyostelium discoideum* is a suitable organism to study signal transduction. Exogenous cAMP induces several responses, which lead to cell aggregation and finally to the formation of a fruiting body with spore and stalk cells (11). Stimulation of aggregative cells with cAMP induces the activation of guanylate and adenylate cyclase, leading to a rise of intracellular guanosine 3':5'-monophosphate (cGMP)¹ and cAMP. The increase in cGMP is transient, reaching a maximum ~ 10 s after stimulation. Whereas cGMP remains intracellular and is probably involved in the chemotactic reaction, the cAMP produced is secreted and the signal is thus relayed (for reviews see references 2, 5, 6).

Extracellular cAMP is detected by specific cell surface receptors, which have been subdivided in two classes, A- and B-sites, that are probably coupled to the activation of adenylate and guanylate cyclase, respectively (7, 17). Binding of cAMP to both subclasses is complex and reveals in each class different forms that interconvert after stimulation of cells with cAMP (19, 20). The A-sites are fast dissociating ($t_{1/2} = 2$ s) and may exist in two states (A^H and A^L) with high and low affinity, while the B-sites are slow dissociating and may exist in at least two states (B^S and B^{SS}) which release bound cAMP with $t_{1/2} = 15$ and 150 s, respectively.

1. *Abbreviations used in this paper:* ATP γ S, adenosine 5'-0-(3-thio)triphosphate; cGMP, guanosine 3':5'-monophosphate; dcAMP, 2'-deoxyadenosine 3':5'-monophosphate; GDP β S, guanosine 5'-0-(2-thio)diphosphate; GTP γ S, guanosine 5'-0-(3-thio)triphosphate; PB, phosphate buffer; (Sp)cAMP β S, adenosine 3':5'-monophosphorothioate, Sp-isomer.

Several observations point to a role of G-proteins in transmembrane signal transduction in *D. discoideum*. Guanine nucleotides alter the heterogeneity of cAMP binding to isolated membranes, suggesting the interaction of G-protein(s) with cAMP receptors (16, 20). [³H]GTP or [³⁵S]GTP γ S-binding to *D. discoideum* membranes and its potentiation by cAMP also point to a functional coupling between cell surface receptors and G-proteins (3, 13). Furthermore, depending on the assay conditions, GTP stimulates or inhibits adenylate cyclase activity in vitro, which supports the idea of the involvement of G-proteins (14, 21).

The small haploid genome of *D. discoideum* makes this eukaryotic organism an excellent object to study signal transduction in chemosensory mutants. Potentially, this may provide tools to elucidate the intricacies of signal transduction pathways that are not easily obtained with other means. Mutant studies may also lead to the identification of components whose participation in signal transduction is presently unknown. We have started to analyze signal transduction in mutants which possess cAMP receptors, but do not react to cAMP with the normal set of responses.

Amebas of the so-called "frigid" mutants are nearly completely unresponsive to cAMP signals (1). 11 frigid mutants were isolated; genetic evidence indicates that they belong to five complementation groups (*fgd A*–*fgd E*). Biochemical data allowed the distinction of two subtypes. One type is unable to respond to cAMP signals because development is blocked very early after starvation and so the cells do not make cell surface cAMP receptors (*fgd B*, *D*, and *E*). The

other type (*fgd A* and *C*) enters the developmental pathway to some extent and does make significant levels of surface cAMP receptors. Some of these mutants show a weak chemotactic response to cAMP, but in none could differentiation be accelerated by the addition of cAMP pulses (1). These characteristics make the *fgd A* and *C* mutants very suitable for use in investigation of the cAMP signal-response coupling process. In this paper we describe the biochemical characterization of mutants from the *fgd A* group. We tried to determine why these cells are unable to respond to cAMP signals and where the defect in the signal transduction pathway is localized. The results show a dramatic defect in the interaction between cAMP receptors and a putative G-protein, probably caused by a defect at one of the G-proteins.

Materials and Methods

Materials

[2,8-³H]cAMP and the cGMP radioimmunoassay kit were obtained from Amersham International (Buckinghamshire, England). [³⁵S]GTPγS and [³²P]GTP were from New England Nuclear (Dreieich, Federal Republic of Germany [FRG]); GTPγS, GDPβS, GTP, cAMP, (Sp)cAMPs, dcAMP, adenosine 5'-(2,3-imido)triphosphate, ATP, ATPγS, creatine phosphate, and creatine kinase were purchased from Boehringer Mannheim GmbH, Mannheim, FRG; dithiothreitol (DTT) and BSA were from Sigma Chemical Co. (St. Louis, MO); silicon oil AR 200 and AR 20 were obtained from Wacker Chemie GmbH (Munich, FRG).

Strains and Culture Conditions

The strains of the *fgd A* group, HC 33, HC 85, HC 112, and HC 213, and the parental strains, HC 6 and HC 91, were kindly provided by Dr. M. B. Coukell (York University, Toronto, Ontario, Canada). The parental strains are derivatives of the wild-type NC₄, HC 6 being the parental strain of HC 33 and HC 85, and HC 91 the parental strain of HC 112 and HC 213 (1).

Cells were grown in association with *Escherichia coli* 281 on a buffered glucose/peptone medium (20). Cells were harvested in the late log phase with 10 mM sodium/potassium phosphate buffer (PB), pH 6.5, washed, and starved either on nonnutrient agar (1.5% agar in PB) at a density of 2×10^6 cells/cm² or in a shaking suspension in PB at a density of 10^7 cells/ml. After 5 h, cells were collected by centrifugation, washed twice, and resuspended in PB.

Crude membranes were prepared by cell lysis (21). If not otherwise indicated, the membranes were washed once and finally resuspended in PB.

Chemotaxis Assay

Chemotaxis to cAMP was measured using the small population test on hydrophobic agar (10). Briefly, vegetative amoebas were harvested from growth plates, washed twice, and resuspended in PB at a density of 5×10^6 cells/ml. Subsequently, small droplets of the cell suspension were deposited on the agar surface. After 30 min or 7 h at 22°C, test solutions containing 10 nM–10 μM cAMP were placed close to the populations of amoebas. At 5–10 min intervals the distribution of the cells within the small population was observed.

cGMP Response

Cells were starved on nonnutrient agar and resuspended at a density of 6.25×10^7 cells/ml (wild-type cells) or 10^8 cells/ml (mutant cells). Cells were stimulated with cAMP at a final concentration of 5×10^{-7} M and then lysed at the times indicated by adding 3.5% (vol/vol) perchloric acid. Lysates were neutralized with 50% saturated KHCO₃ and centrifuged at 8,000 g for 2 min. The cGMP concentration in the supernatant was measured using the radioimmunoassay.

cAMP Relay Response

Cells were starved on nonnutrient agar and resuspended as described above. They were then stimulated with dcAMP (final concentration 10^{-5} M) in the presence of 5 mM DTT and lysed at the times indicated by adding 3.5%

perchloric acid. Lysates were neutralized as described above and the cAMP content was measured by using the isotope dilution assay as described in reference 15.

cAMP Binding Assay

Cells were starved in suspension, resuspended at a density of 10^8 cells/ml, and used for Scatchard analysis and dissociation kinetics. cAMP binding was measured at 0°C in a total vol of 100 μl containing different concentrations of [³H]cAMP, 5 mM DTT, and 80 μl cell suspension. At the times indicated, bound and free [³H]cAMP was separated by centrifuging the cells through 200 μl silicon oil (AR 200/AR 20 = 11:4) in a swing-out rotor at 10,000 g for 15 s; after centrifugation the tubes were frozen and the bottoms, containing the cell pellet, were cut. Radioactivity was determined by liquid scintillation counting.

Membranes were used for analyzing the interaction of guanine nucleotides with cAMP binding. The final concentration of the membrane suspension in PB was equivalent to 10^8 cells/ml. cAMP binding was measured in a volume of 100 μl, containing 5 nM [³H]cAMP, 5 mM DTT, different concentrations of GTPγS or GDPβS, and 80 μl membranes. The incubation time was 5 min at 0°C. Samples were centrifuged for 3 min at 10,000 g, the supernatant was aspirated, and the pellet was dissolved in 80 μl of 1 M acetic acid; 1.2 ml scintillation liquid was added and radioactivity was determined. Nonspecific binding was measured by including 0.1 mM cAMP in the incubation mixture and subtracted from all data.

Down Regulation and Receptor Modification

After 4.5 h of starvation, the cell suspension was divided into two portions. One portion was treated with 1 μM cAMP and 10 mM DTT during 15 min; the other portion was taken as a control. Then cells were collected, washed twice in ice cold PB, resuspended in PB, and used for binding studies (see above) or for examination of receptor modification by Western blot analysis (16).

GTPγS Binding Assay

Cells were starved in suspension for 4.5 h and collected by centrifugation; membranes were prepared, resuspended in PB, and adjusted to a density equivalent to 2×10^8 cells/ml. Binding of [³⁵S]GTPγS to membranes has been described extensively elsewhere (13). Briefly, the incubation mixture (100 μl) contained 0.1 nM [³⁵S]GTPγS, PB, 10 mM MgCl₂, 10 mM DTT, and 80 μl membranes. The incubation time was 30 min at 0°C. Samples were centrifuged at 10,000 g for 3 min, the supernatant was aspirated, the pellet was dissolved in 80 μl 1 M acetic acid, and radioactivity was determined. Nonspecific binding was measured by including 0.1 mM GTP in the incubation mixture and subtracted from all data.

GTPase Assay

After 4.5 h of starvation in suspension, cells were collected and used for membrane preparation. Crude membranes were washed and resuspended in 10 mM triethanolamine-HCl, pH 7.4, at a density equivalent to 10^8 cells/ml, and GTPase activity was determined (13a). The reaction mixture was preincubated at 25°C for 5 min and contained [³²P]GTP (3.7 kBq), 2 mM MgCl₂, 0.1 mM EGTA, 0.2 mM adenosine 5'-(2,3-imido)triphosphate, 0.1 mM ATPγS, 10 mM DTT, 5 mM creatine phosphate (Tris-salt), 0.4 mg/ml creatine kinase, and 2 mg/ml BSA (purified) in 50 mM triethanolamine-HCl, pH 7.4, in a total volume of 100 μl. The reaction was initiated by the addition of 30 μl of membranes to 70 μl of the reaction mixture and conducted for 3 min. The reaction was stopped by the addition of 0.5 ml PB (50 mM), pH 2.0, containing 5% (wt/vol) activated charcoal. The reaction tubes were centrifuged for 5 min at 10,000 g at 4°C and radioactivity of the supernatant was determined using Cerenkov radiation.

Adenylate Cyclase Assay

Cells were starved in suspension. After 4.5 h cells were collected by centrifugation, lysed, and crude membranes were resuspended in 40 mM HEPES/NaOH, 5 mM EDTA, 250 mM sucrose, pH 7.7, to a volume equivalent to 2×10^8 cells/ml. Enzyme activity was measured as described (21). Briefly, 20 μl of isolated membranes were incubated with buffer (40 mM HEPES/NaOH, 3 mM EDTA, 250 mM sucrose, pH 7.7), 6 mM MgCl₂, 0.5 mM ATP, 10 mM DTT in a total volume of 40 μl. The incubation time was 40 min at 0°C. The reaction was terminated by the addition of 10 μl

of 0.1 M EDTA. Enzyme activities were destroyed by boiling the samples for 2 min. Produced cAMP was determined by isotope dilution assay (15).

Stimulation by 100 μ M GTP γ S was measured in membranes in the presence of 15% of their own supernatant and inhibition of adenylate cyclase activity by GTP γ S was measured after preincubation of the membranes with Mg/ATP γ S (11).

Results

We first determined the capability of *fgd A* mutants to respond to exogenous cAMP signals; then the presence and heterogeneity of cAMP binding to cells was analyzed; and finally the interaction between surface cAMP receptors and G-proteins and between G-proteins and adenylate cyclase was investigated. During this study we found no differences in developmental, physiological, and biochemical properties between the two parental strains and the wild-type NC₄.

cAMP-induced Responses

Extracellular cAMP induces the accumulation of cAMP and cGMP and a chemotactic response. The responses of wild-type and *fgd A* mutant cells are summarized in Table I and some typical experiments are presented in Figs. 1 and 2. The cGMP response at different time points after stimulation with cAMP is shown for one wild-type strain and two of the *fgd A* mutants in Fig. 1 A. No response could be detected in HC 85, HC 33, or HC 213. Mutant HC 112 gave a small response which was maximal at 20 s after stimulation. However, this rise in concentration was not very reproducible and the maximal levels were not always significantly different from basal levels.

The agonist dcAMP was used to measure the receptor-stimulated cAMP accumulation. The results were similar to the cGMP response; i.e., a barely detectable response in HC 112 and no response at all in the other mutants of the *fgd A* group (Fig. 1 B).

The four mutant strains and their parents were also tested for chemotactic activity to cAMP. Cells were used either in the vegetative stage or after 7 h of starvation at 22°C, the time point at which the wild-type cells are aggregation competent. Vegetative cells of the wild type showed a positive chemotactic response within 30 min after cAMP (10^{-7} or 10^{-6} M) was

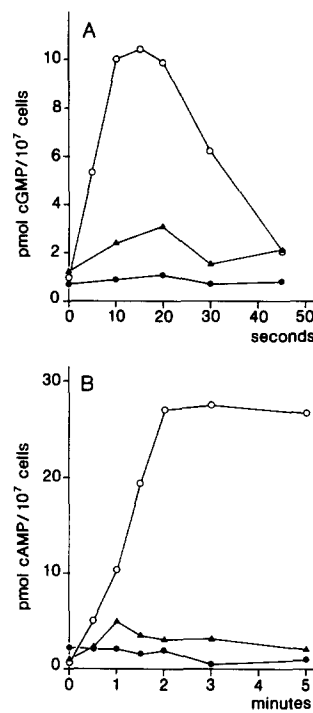


Figure 1. Kinetics of the cGMP and cAMP response in wild-type and *fgd A* mutant cells. Cells starved for 5 h on nonnutrient plates were stimulated with 0.5 μ M cAMP (A) or 10 μ M dcAMP with 10 mM DTT (B) at 0 s. At the times indicated, cells were lysed and the cAMP or cGMP content was determined. Symbols in A and B represent wild-type NC₄ (○) and mutant strains HC 85 (●) and HC 112 (▲). The results shown are the means of duplicate determinations of a typical experiment that was reproduced three times.

placed on the agar surface. Only mutant HC 112 showed a small but significant response at the same concentrations.

After 7 h of starvation, a chemotactic response was observed in HC 112 cells, at 100-fold higher cAMP concentrations than in wild-type cells; also the time dependence of this response in HC 112 differed from that in wild-type cells. HC 33, HC 85, and HC 213 didn't show chemoresponsiveness at any cAMP concentrations tested (Fig. 2). These results clearly demonstrate that, in the *fgd A* mutants, cAMP is unable to elicit cellular responses as normally found in wild-type cells of *D. discoideum*.

cAMP Binding

Previous studies showed that *fgd A* cells have low levels of cAMP binding sites, however the production of these sites is

Table I. cAMP-induced Responses, cAMP Binding, and Down-Regulation of cAMP Binding Sites of Different Strains of *D. discoideum*

Strain	Class	cGMP response*	cAMP response*	Chemotactic response*	cAMP binding [‡] (% of wild type)	Binding after down-regulation [§] (% of control)
NC 4	<i>agg</i> +	+	+	+	100	11
HC 6	<i>agg</i> +	+	+	+	100	ND
HC 91	<i>agg</i> +	+	+	+	100	ND
HC 33	<i>fgd A</i>	—	—	—	25 ± 17	4
HC 85	<i>fgd A</i>	—	—	—	28 ± 3	ND
HC 112	<i>fgd A</i>	±	±	±	65 ± 23	5
HC 213	<i>fgd A</i>	—	—	—	35 ± 15	0

* Responses were measured as described in Materials and Methods. ±, small response (see Figs. 1 and 2).

‡ Binding was measured at a final concentration of 5 nM [³H]cAMP, 100% being 1,000 cpm per incubation.

§ 100% is the binding before down-regulation.

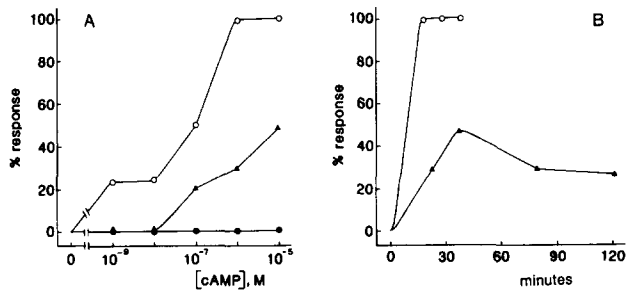


Figure 2. Chemotaxis in wild-type and mutant cells. The chemotactic response as a function of cAMP concentration (A) or as a function of time (B) is presented. The time dependence was measured at a cAMP concentration of 10^{-6} M. Symbols in A and B represent wild-type NC4 (○), *fgd A* mutants HC 85 (●), and HC 112 (▲). A positive chemotactic response was defined when at least twice as many cells were pressed against the proximal edge to the cAMP droplet than against the opposite edge. 100% is achieved when all populations, tested at the same cAMP concentration, show a positive response.

not stimulated by treatment with cAMP pulses (1). We measured cAMP binding at a final concentration of 5 nM [³H]cAMP. The percentage of binding activity on mutant cells with respect to wild-type cells varied from 25 to 65%, depending on the mutant used. Strain HC 112 showed considerably more binding activity than the other mutants of the *fgd A* group (Table I). cAMP binding was investigated in more detail to determine the presence of several binding types on the cell surface of the *fgd A* mutants.

Equilibrium Kinetics. For Scatchard analysis, cells were incubated with different concentrations of [³H]cAMP and binding was measured at equilibrium (Fig. 3). As found previously (19), the Scatchard plot of cAMP binding to wild-type cells is nonlinear, indicating two types of binding sites with high and low affinity. The number of binding sites in HC 85 was 50% less, but the curve was also nonlinear suggesting the presence of high and low affinity sites in HC 85. The other *fgd A* strains revealed the same tendency, a nonlinear Scatchard plot with slightly different apparent affinities of the two binding components (data not shown).

Nonequilibrium Kinetics. In Fig. 4, the dissociation of the cAMP-receptor complex after equilibration with 4 nM [³H]cAMP is shown. In wild-type HC 91 cells, the dissociation was multiphasic and cAMP was released by at least three components with different off-rates: 61% with $t_{1/2} = 1.8$ s; 29% with $t_{1/2} = 23$ s (B^S -sites); and 10% with $t_{1/2} = 283$ s (B^{SS} -sites). The dissociation of cAMP in the *fgd A*

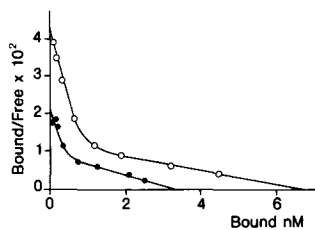


Figure 3. Equilibrium cAMP binding to wild-type and mutant cells. The binding of different concentrations of [³H]cAMP (2,000–1,000 nM) to cells starved for 5 h in PB was measured. Equilibrium binding was reached after an incubation period of 75 s at 0°C; at

that moment cells were centrifuged through silicon oil. The results are presented as a Scatchard plot and are the means of two independent experiments with duplicate (○, wild-type NC4) or triplicate (●, mutant HC 85) determinations.

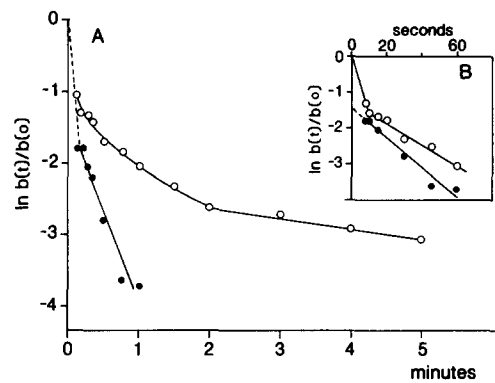


Figure 4. Dissociation kinetics of bound [³H]cAMP from wild-type and mutant cells. Cells were preincubated in a total volume of 100 μ l with 4 nM [³H]cAMP for 75 s to reach binding equilibrium. Then, at 0 s in the figure, 1 ml buffer with 0.1 mM cAMP was added and 1 ml of the mixture was centrifuged through the silicon oil at the times indicated. $b(t)$, the specific binding at t min after the onset of dissociation; $b(0)$, the specific binding before the cAMP chase. The strains are wild-type HC 91 (○) and mutant HC 213 (●). (B) The binding to the very slowly dissociating component B^{SS} in wild-type cells was calculated from the linear part of the curve in A and was subtracted from the observed binding during the first minute of dissociation. Residual binding is plotted together with the original data of the mutant cells. The results shown are the means of triplicate determinations from an experiment reproduced once.

strain HC 213 is only biphasic with a fast ($t_{1/2} = 1.7$ s) and a slow ($t_{1/2} = 17$ s) dissociating component. In strain HC 213 all bound [³H]cAMP is released after 1 min of dissociation, whereas 10% of the radioactivity is still bound to wild-type cells. The lack of B^{SS} -sites was also observed in mutant HC 85 where the two dissociation components showed a $t_{1/2} = 1$ s and 8 s, respectively.

Down Regulation and Modification of the cAMP Receptor

We examined whether cAMP induced down-regulation of cAMP binding sites. In wild type, only 10% of cAMP binding is left after treating the cells with 1 μ M cAMP during 15 min. Table I shows that in the mutants the remaining cAMP binding after down-regulation is even less than in the wild-type strain.

cAMP also induces the modification of the receptor which caused a shift of its apparent molecular mass from 40 to 43 kD. This modification is probably caused by phosphorylation of the receptor at multiple sites (8, 9). It can be detected with the antireceptor antiserum on Western blot transfers of size-fractionated membrane proteins (9). Fig. 5 shows the shift in electrophoretic mobility of the receptor from the R form (M_r 40,000) to the D form (M_r 43,000) in response to the presence of 1 μ M cAMP during 15 min in a wild-type and a *fgd A* strain. This receptor shift is observed in all four mutants of the *fgd A* group (data not shown). The difference between wild type and *fgd A* in staining of the other bands was not reproducible.

These results indicate that the size of the surface receptor protein of mutant and wild-type cells are identical and that

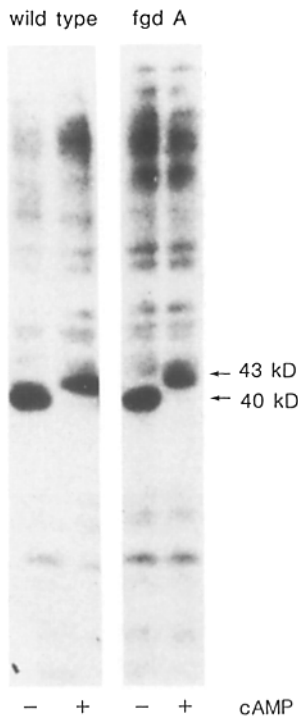


Figure 5. Western Blot analysis of the cAMP receptor from wild-type and mutant cells. Wild-type NC4 and mutant HC 112 were incubated for 15 min in the absence (-) or presence (+) of 0.1 μ M cAMP for 15 min and membranes were isolated. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained with the antireceptor antiserum.

These results indicate that the size of the surface receptor protein of mutant and wild-type cells are identical and that the receptor protein reacts in the same way to high exogenous cAMP concentrations with respect to down-regulation and covalent modification. Nonetheless, *fgd A* mutants do not respond to cAMP with an activation of adenylate or guanylate cyclase. These responses are probably mediated by signal transducing G-proteins. Therefore, the interactions between receptors, G-proteins, and adenylate cyclase were investigated.

Inhibition of cAMP Binding to Membranes by Guanine Nucleotides

Guanine nucleotides affect the different cAMP-binding types in membranes of *D. discoideum* by reducing the affinity of the A-sites and accelerating the dissociation rate of the B-sites (16, 20). At binding equilibrium, this results in a 50–70% inhibition of cAMP binding in wild-type cells. Inhibition of equilibrium binding of 5 nM [3 H]cAMP to membranes by GTP γ S and GDP β S is presented in Table II. In the wild-type strains binding of cAMP was reduced to 35% in the presence of GTP γ S and to ~55% in the presence of GDP β S. In all mutants tested, the effect of GTP γ S and GDP β S was less pronounced than in wild-type cells. This defect is stronger in HC 85 than in other mutants of the *fgd A* group.

Dose-response curves of the inhibition of cAMP binding by GTP γ S are shown in Fig. 6. Half-maximal effects were obtained at similar GTP γ S concentrations in wild-type HC 6 and mutants HC 112 and HC 85 (respectively at 3, 3.5, and 1.5 μ M). In contrast, maximal inhibition was significantly lower in the *fgd A* strains.

These results suggest defects in the interaction between the cAMP receptor and a GTP-binding protein in the *fgd A* mutants and that these defects are more or less pronounced in the different strains.

It was not possible to determine, specifically, the effects of

Table II. Inhibition of cAMP Binding to Membranes by 30 μ M GTP γ S and 30 μ M GDP β S

Strain	Class	% of control	
		+ GTP γ S	+ GDP β S
NC 4	<i>agg +</i>	36 \pm 15	63 \pm 14
HC 6	<i>agg +</i>	32 \pm 13	53 \pm 9
HC 91	<i>agg +</i>	32 \pm 9	48 \pm 11
HC 33	<i>fgd A</i>	51 \pm 13*	75 \pm 7*
HC 85	<i>fgd A</i>	92 \pm 20*	94 \pm 3*
HC 112	<i>fgd A</i>	52 \pm 8*	66 \pm 13
HC 213	<i>fgd A</i>	64 \pm 5*	70 \pm 18*

Equilibrium binding was measured at a final concentration of 5 nM [3 H]-cAMP. Data presented as relative to control binding; the control is cAMP bound to the same strain in the absence of guanine nucleotides. Values are means \pm SDs of triplicate determinations from three independent experiments. For statistical analysis, data of wild-type strains were pooled.

* Significant differences (according to *t* test) as compared to the wild-type cells, $P < 0.05$.

guanine nucleotides on the slowly dissociating B-sites in the mutant strains. Firstly, control equilibrium binding to mutant cells is only 30% of that in wild-type cells. Secondly, at 10 s after the onset of dissociation most of the bound [3 H]cAMP was released from the *fgd* membranes and not enough binding activity was left to measure the differences caused by GTP γ S or GDP β S.

GTP γ S Binding and Modulation of Binding by (Sp)cAMPS

The interactions between surface receptors and G-proteins has also been detected by receptor-agonist stimulation of GTP or GTP γ S binding to membranes. GTP γ S-binding activity to *D. discoideum* membranes is heterogeneous and composed of high affinity ($K_d = 0.22 \mu$ M) and low affinity ($K_d = 6.3 \mu$ M) components (13). In the presence of micromolar concentrations of surface-receptor agonist (Sp)cAMPS, GTP γ S binding was enhanced as the result of an increase in affinity and the number of high-affinity binding sites. Results of binding activity in mutant membranes and the effects of (Sp)cAMPS are presented in Table III. Control binding in mutant membranes reached the same values as in wild-type

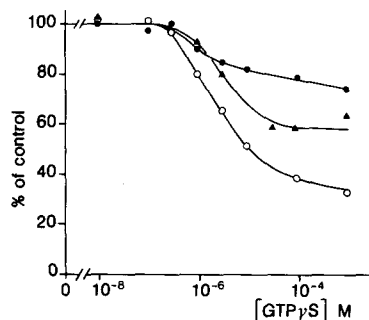


Figure 6. Dose-response curve of the inhibition by GTP γ S of cAMP binding to membranes of wild-type and mutant cells. Membranes from wild-type strain HC 6 (○) and mutant strains HC 85 (●) and HC 112 (▲) were incubated with 5 nM [3 H]cAMP in the presence of different concentrations of GTP γ S for 5 min at 0°C; and the binding of [3 H]cAMP was determined. The results shown are the means of triplicate determinations from a single experiment.

strains. In contrast, stimulation of GTP γ S binding by (Sp)cAMPS is very small (HC 85 and HC 213) or essentially absent (HC 33). Statistical analysis of the data (according to the *t* test) revealed that the increase of GTP γ S binding by (Sp)cAMPS in HC 85 is not significantly different from control binding ($P > 0.1$), while in wild-type strains the percentage of stimulation of GTP γ S binding by (Sp)cAMPS is significantly higher than in mutant strains ($P < 0.001$).

These observations also point to a disturbance in the pathway from cell surface cAMP receptor to a G-protein.

GTPase Activity and Activation by cAMP

G-proteins have GTPase activity that can be stimulated by receptor agonists. GTP hydrolysis in *D. discoideum* membranes is caused by at least two enzymes with high ($K_m = 6.5 \mu\text{M}$) and low ($K_m > 1 \text{ mM}$) affinity. The high affinity GTPase is stimulated by cAMP, with half-maximal effects at a cAMP concentration of $3 \mu\text{M}$. Treatment of wild-type cells with pertussis toxin decreased the cAMP-induced stimulation of GTPase activity (13a).

In Table IV, results are shown of GTPase activity in membranes of *fgd A* mutant HC 85 and its parental strain HC 6. GTPase activity was measured at $0.01 \mu\text{M}$ GTP; at this concentration mainly the high-affinity enzyme is detected and stimulation by cAMP is optimal. Basal activity in mutant HC 85 is significantly lower than in wild-type membranes, the difference being $\sim 40\%$. Activation of the high-affinity GTPase was measured in the presence of $3 \mu\text{M}$ cAMP. The enhancement of activity was significantly lower in the mutant strain. In two other *fgd A* strains, HC 33 and HC 213, similar results were found (data not shown).

The effect of pertussis toxin was examined in *fgd A* mutant HC 85 and the parental strain HC 6 (Table IV). In wild-type as well as in mutant membranes cAMP stimulation of the high-affinity GTPase was significantly reduced as a result of pertussis toxin treatment in vivo.

Regulation of Adenylate Cyclase Activity by GTP γ S

Recently, assays have been developed for the stimulation and inhibition of *D. discoideum* adenylate cyclase by GTP γ S (21). Both the stimulation and inhibition of adenylate cyclase

activity is specific for guanosine triphosphates and antagonized by guanosine diphosphates.

The regulation of adenylate cyclase by GTP γ S in a *fgd A* mutant and a parent strain is shown in Table V. The results indicate that stimulation as well as inhibition of adenylate cyclase by GTP γ S was not significantly different in mutant and wild-type membranes.

Discussion

Transmembrane signal transduction was investigated in *D. discoideum* mutants of the *fgd A* complementation group that were isolated by Coukell et al. (1). These investigators showed that *fgd A* mutants possess cell surface cAMP receptors, but are unable to respond to cAMP with a chemotactic reaction or the induction of EDTA-resistant contact sites. These observations were the basis of the present work, which aimed at a biochemical and functional characterization of chemosensory mutants. Since the cAMP chemosensory system of *D. discoideum* is developmentally regulated, cAMP chemosensory mutants may be blocked early in development. Therefore, mutants of interest to us must bear at least the surface cAMP receptor. These mutants are expected to have a defect in the transduction pathway distal to the receptor. An alternative strategy was recently proposed by Segall et al. (12), who isolated folate chemotaxis mutants that are also defective for cAMP chemotaxis; the folate chemotaxis system does not require the development of *D. discoideum* cells.

The *fgd A* mutants show the following characteristics: (a) cell surface cAMP receptors were present, high and low affinity forms were detected, but the B^{SS}-form was absent; (b) cAMP induced the down-regulation of the receptors, as well as their covalent modification; (c) cAMP did not induce any of the (intra)cellular responses, such as the activation of adenylate cyclase, guanylate cyclase, and chemotaxis; (d) in membranes, the inhibition of cAMP binding by GTP γ S was reduced to different extents depending on the mutant used; (e) stimulation of GTP γ S binding to membranes by (Sp)-cAMPs was lost in all *fgd A* mutants; (f) basal high-affinity GTPase activity was reduced as well as the cAMP-mediated stimulation of this enzyme, and pertussis toxin treatment of cells resulted in a decrease of the cAMP-induced activation of GTPase in both wild-type and mutant membranes; (g) GTP γ S-mediated stimulation and inhibition of adenylate cyclase was not defective in *fgd A* mutant cells; and (h) the biochemical and physiological phenotypes are qualitatively identical in the four *fgd A* mutants, but show quantitative differences with HC 112 being least defective followed at some distance by HC 33 and HC 213, with HC 85 being the most defective mutant.

These results suggest that transmembrane signal transduction in *fgd A* mutants is severely defective in vivo, and that the defect is probably located somewhere between the surface cAMP receptor and a specific G-protein. Transmembrane signal transduction is essentially the transduction of a signal from the surface receptor to effector enzymes through an unknown number of intermediate transducer proteins. The transducing components are bifunctional; i.e., G-proteins interact with at least the surface receptors and effector enzymes. Therefore, defect in signal transduction can be due either to the absence of one of the signal transducing compo-

Table III. Effect of (Sp)cAMPS on GTP γ S Binding to Membranes

Strain	Class	GTP γ S binding		Stimulation by (Sp)cAMPS
		control	+ (Sp)cAMPS	
		%	%	%
HC 6	<i>agg</i> +	100 \pm 2.8	* 127 \pm 2.7	27 \pm 4.5
HC 91	<i>agg</i> +	100 \pm 2.2	* 127 \pm 4.5	27 \pm 4.2
HC 33	<i>fgd A</i>	103 \pm 5.9	* 86 \pm 5.8	-16 \pm 6.5 [†]
HC 85	<i>fgd A</i>	100 \pm 4.9	NS 104 \pm 2.9	4 \pm 5.9 [†]
HC 213	<i>fgd A</i>	102 \pm 4.0	* 110 \pm 5.6	8 \pm 6.9 [†]

Experiments were performed as described in Materials and Methods. (Sp)cAMPS was present at a concentration of $10 \mu\text{M}$. Data shown are means \pm SDs of triplicate determinations from two independent experiments, 100% being $\sim 10,000$ cpm per incubation.

* Significant difference (according to *t* test) $P < 0.02$.

[†] Significant differences (% of stimulation by (Sp)cAMPS compared to wild-type strains) $P < 0.01$.

Table IV. GTPase Activity in Wild-type and *fgd A* Membranes and cAMP-induced Stimulation and Influence of Pertussis Toxin

Strain	Class	Activity (pmol Pi hydrolyzed · min ⁻¹ · mg protein ⁻¹)		Stimulation of activity by cAMP	
		Control	PT	% Control*	% PT†
HC 6	<i>agg</i> +	1.99 ± 0.36	2.85 ± 0.34	39.1 ± 2.75	17.2 ± 2.25
HC 85	<i>fgd A</i>	*1.14 ± 0.24	1.19 ± 0.14	29.3 ± 3.53	21.7 ± 3.04

Cells were starved for 4.5 h in the absence or presence of 0.1 µg/ml pertussis toxin, washed, and used for membrane preparation. GTP hydrolysis by high affinity GTPase was determined in the absence and presence of 3 µM cAMP at a GTP concentration of 10 nM.

The results are means of three independent experiments with triplicate determinations.

PT, Pertussis toxin-treated cells.

* Significant differences from wild-type (tested according to *t* test) *P* < 0.05.

† Significant differences from control (according to *t* test) *P* < 0.05.

nents or to a mutation so that the component has lost only one of its functions (e.g., G-proteins may still interact with the receptor, but not with the effector enzyme). It is thus difficult to identify the component that causes a defective interaction between two proteins, unless one of the proteins is absent. The inability of cAMP to induce responses in *fgd A* mutants is not due to the absence of the receptor protein. Two cAMP-induced responses which modify the cAMP receptor, down-regulation and its covalent modification, are perfectly normal in *fgd A* mutants. Furthermore, the target proteins through which adenylate cyclase is regulated by GTPγS *in vitro*, which may be equivalent to G_s and G_i in other systems, are functionally not altered in *fgd A* mutants. The receptor-stimulated GTPase activity in *fgd A* mutants indicates the functional coupling between receptor and at least one G-protein, possibly G_i, since receptor-stimulated GTPase activity is strongly inhibited by pertussis toxin in wild type and *fgd A*.

We anticipate that defects in three proteins could lead to the observed biochemical phenotype. Firstly, the cAMP receptor may be defective in such a way that it still binds cAMP and responds to cAMP with its modification and down-regulation. Furthermore, the defective receptor may still activate one or more G-proteins, but a specific G-protein is no longer activated. This hypothesis probably requires a mutation in the structural gene of the receptor, because the function of the protein is altered in a very specific way. Secondly, *D. discoideum* cells may contain multiple G-proteins; a specific G-protein is missing or nonfunctional in *fgd A*. Mutations can be both in the structural or regulatory gene. Thirdly, an unknown component that is essential for the receptor-mediated activation of a G-protein is nonfunctional. The present

data give no formal clue to discriminate between these possibilities. However, four independent *fgd A* mutants were isolated that belong to the same complementation group. Mutants with nonsense mutations in the receptor gene would have been detected as a *fgd* mutant, assuming that the cAMP receptor is not essential for growth. Assuming then that the mutation in *fgd A* is in the structural gene of the receptor, the probability that none of the four *fgd A* mutants shows a defect in the molecular mass of the receptor protein is very small. Therefore we consider it as unlikely that the mutation in *fgd A* is in the structural gene of the receptor.

Detection of putative G-protein α-subunits with the G_α-common antiserum A569 reveals at least two proteins with apparent molecular masses of 52 and 40 kD in *D. discoideum* cells. Interestingly, *fgd A* mutant HC 213 shows a strongly reduced staining of the 40-kD band, suggesting that the defect in *fgd A* mutants could indeed be located at the level of a G-protein (Snaar-Jagalska, B. E., F. Kesbeke, M. Pupillo, and P. J. M. Van Haastert; manuscript submitted for publication). Consistent with this hypothesis is the observation that one of the receptor forms, B_{SS}, is not present in *fgd A* mutant cells. Previous data suggest that this form of the receptor can only arise through the activation of a G-protein, and that its formation is indicative for the transduction of the cAMP signal towards the activation of guanylate cyclase (18, 20). The activation of guanylate cyclase has been proposed to proceed through the receptor and G-protein-stimulated production of inositol-(1, 4, 5)-trisphosphate (4). It is possible that the primary defect in the *fgd A* mutants is located in the G-protein that mediates the receptor stimulation of the phosphatidylinositol cycle. This would imply that this transduction pathway is a master regulator of cAMP-induced processes in *D. discoideum* and is also essential for chemotaxis and the stimulation of adenylate cyclase.

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Table V. Regulation of Adenylate Cyclase by GTP

Conditions	Activity (pmol · min ⁻¹ · mg protein ⁻¹)	
	Wild-type NC4	Mutant HC 213
Basal*	0.49 ± 0.04	0.40 ± 0.02
+ GTPγS	0.89 ± 0.11	0.79 ± 0.06
Ratio ± GTPγS	1.82 ± 0.25	1.98 ± 0.17
After Mg/ATPγS	0.35 ± 0.04	0.42 ± 0.04
+ GTPγS	0.25 ± 0.03	0.29 ± 0.02
Ratio ± GTPγS	0.71 ± 0.10	0.69 ± 0.09

Data from a typical experiment with quadruplicate determinations; a duplicate experiment yields similar results.

* Activity measured in the presence of 15% of the supernatant.

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