Chemotaxis to cyclic AMP and folic acid is mediated by different G proteins in *Dictyostelium discoideum*

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Summary

Mutant Frigid A (*fgd*A) of *Dictyostelium discoideum* is defective in a functional Gα2 subunit of a G protein and is characterized by a complete blockade of the cyclic AMP-mediated sensory transduction steps, including cyclic AMP relay, chemotaxis and the cyclic GMP response. Folic acid-mediated transmembrane signal transduction was investigated in this mutant; the results show that: (1) cell surface folic acid receptors are present in *fgd*A mutants. (2) Folic acid induces intracellular responses, including activation of guanylate cyclase and chemotaxis. (3) The inhibitory effect of GTP on folic acid binding to membranes is present. (4) GTPγS binding and high-affinity GTPase are stimulated by folic acid. These data strongly suggest that folic acid receptors are coupled to guanylate cyclase and chemotaxis *via* a Gα protein that is different from Gα2. The results imply that surface receptors for cyclic AMP and folic acid are coupled to different G proteins.

Key words: chemotaxis, cyclic AMP, folic acid, *D. discoideum*, G protein.

Introduction

During the life cycle of the cellular slime mold *Dictyostelium discoideum*, two different extracellular signals play an important role. In the vegetative stage the amoebae feed on bacteria, which secrete folic acid (FA). Amoebae in this growth phase react to FA by a chemotactic response (Pan *et al.* 1972, 1975) and FA induces an increase in the cyclic GMP content (Wurster *et al.* 1977; Mato *et al.* 1977; De Wit and Bulgakov, 1986*a*). After starvation the cells become less responsive to FA and gain responsiveness to cyclic AMP. The starved cells start to release cyclic AMP in periodic pulses, which leads to cell aggregation and finally the formation of a multicellular structure with spores and stalk cells (Loomis, 1982). In aggregation-competent cells cyclic AMP elicits several responses including a chemotactic response and the activation of guanylate and adenylate cyclase, by which intracellular cyclic GMP and cyclic AMP are produced. cyclic GMP is probably involved in the chemotactic response, while cyclic AMP is secreted and attracts other cells (see reviews, Gerisch, 1987; Janssens and Van Haastert, 1987).

Extracellular cyclic AMP and FA are detected by specific cell surface receptors (Van Haastert and De Wit, 1984; De Wit and Van Haastert, 1985); the sequence of the cyclic AMP receptor is known and has been described (Klein *et al.* 1988). After binding of the ligand to the receptor the extracellular signal is transduced, probably *via* one or more G proteins. The binding of FA and cyclic AMP to cell surface receptors is heterogeneous, showing different kinetic forms. A and B-sites have been described for both ligands, and it has been proposed that the B-sites mediate chemotaxis to FA and cyclic AMP (De Wit *et al.* 1985; Van Haastert, 1985; Kesbeke and Van Haastert, 1985). Guanine nucleotides alter the heterogeneity of cyclic AMP and FA binding to membranes (Van Haastert *et al.* 1986; De Wit and Bulgakov, 1986*b*), while GTPγS binding and high-affinity GTPase activity in membranes are potentiated by cyclic AMP (Snaar-Jagalska *et al.* 1988*a*,*b*).

FA and cyclic AMP both induce chemotaxis by a transduction mechanism involving separate surface receptors and the activation of one or more G proteins. It is likely that the transduction pathways of FA and cyclic AMP meet each other at a step close to the receptor. This could imply that both receptors transduce signals through common G proteins that activate different effector enzymes (adenylate cyclase and guanylate cyclase, phospholipase C). Alternatively, each receptor may interact with a specific G protein, which may activate the same effector enzyme.

A study on cyclic AMP-mediated signal transduction in mutant *fgd*A of *D. discoideum* that lacks a functional Gα2 subunit (Kumagai *et al.* 1989), revealed that all cyclic AMP sensory transduction is blocked (Kesbeke *et al.* 1988). In the present study we show that the FA sensory transduction is essentially normal in this mutant. These results imply that the cyclic AMP and the FA receptors interact with distinct G proteins.

Materials and methods

Chemicals

The cyclic GMP radioimmunoassay, [7,9,3′,5′-^{3}H]folic acid (FA, 0.55 TBq mmol^{-1}) and [7,3′,5′-^{3}H]methotrexate (MTX, 0.50 TBq mmol^{-1}) were obtained from Amersham International (Buckinghamshire, UK). N^{10}-methyl[7,3′,5′-^{3}H]folic acid (MFA) was prepared by alkaline hydrolysis from [7,3′,5′-^{3}H]MTX. [^{35}S]GTPγS and [γ-^{32}P]GTP were from New England Nuclear (Dreieich, FRG). 8-Azaguanine was purchased from Fluka A.G. (Buchs SG, Switzerland). 2-Deaminofolic acid (DAFA) was prepared by enzymatic degradation of FA (BDH Biochemicals, Poole, UK) as described (Van Haastert *et al.* 1982). GTPγS (adenosine 5′-(2,3-imido)triphosphate), ATPγS, creatine phosphate and creatine kinase were obtained from Boehringer Mannheim GmbH (Mannheim, FRG) and bovine serum albumin was from Sigma Co. (St Louis, MO).

Strains and culture conditions

The wild-type *Dictyostelium discoideum* strains are NC4, HC6 and HC91; the latter two are the parents of the different *fgd*A strains (Coukell *et al.* 1983). The strains of the *fgd*A group, HC33, HC85 and HC213, and the parental strains were kindly provided by Dr M. B. Coukell (York University, Toronto, Ontario, Canada).

Cells were grown in association with *Escherichia coli* 281 on a solid medium, containing 3.3 g peptone, 3.3 g glucose 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4.2H_2O$ and 15 g agar per liter. Cells were harvested with cold 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB), before clearing of the bacterial lawns, and starved at 22°C either on non-nutrient agar (1.5 % agar in PB) at a density of 2×10^6 cells cm^{-2} or in a shaking suspension in PB at a density of 10^7 cells ml^{-1}. After starvation cells were collected by centrifugation, washed twice and resuspended in the appropriate buffer.

To prepare membranes cells were resuspended to 2×10^8 cells ml^{-1} in buffer A (40 mM Hepes/NaOH, 5 mM EDTA, 250 mM sucrose, pH 7.7). Cells were lysed at 0°C by pressing them through a Nuclepore filter with 3 μm pores (Das and Henderson, 1983). The homogenate was centrifuged at 10 000 ***g*** for 5 min, and the pellet washed once in buffer A. The final pellet was resuspended in PB.

Cyclic GMP response

After starvation on non-nutrient agar cells were resuspended in PB at a density of 10^8 cells ml^{-1} and aerated for at least 10 min. Cells were stimulated with folic acid in the presence of the deaminase inhibitor 8-azaguanine (final concentration 0.33 mM) and at the times indicated the reaction was terminated by addition of 3.5 % (v/v) perchloric acid. Lysates were neutralized with 50 % saturated $KHCO_3$ and centrifuged at 8000 ***g*** for 2 min. The cyclic GMP concentration in the supernatant was determined by radioimmunoassay.

Folate binding assay

Cells were starved in suspension, collected by centrifugation, resuspended in PB and used for membrane preparation. The final concentration of the membrane suspension in PB was equivalent to 1.5×10^8 cells ml^{-1}. Folate binding was measured at 0°C in a total volume of 150 μl, containing 120 μl membrane suspension, 5 nM [^{3}H]FA and 0.33 mM 8-azaguanine (final concentrations). The mixture for MFA-binding contained 120 μl membrane suspension, 2 nM [^{3}H]MFA, 0.33 mM 8-azaguanine and 3.33 μM DAFA. GTPγS (if present) was preincubated with the membrane suspensison for 1 min. The binding reaction was started by the addition of membrane suspension and after 5 min incubation 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; 1.5 ml scintillation liquid was added and radioactivity was determined. Nonspecific binding was measured in the presence of 0.1 mM FA and subtracted from all data.

GTPγS binding assay

After collecting the cells, they were used directly to prepare membranes, which were resuspended in PB to a density equivalent to 2×10^8 cells ml^{-1}. Binding of [^{35}S]GTPγS to membranes was performed as described (Snaar-Jagalska *et al.* 1988*a*). Briefly, 100 μl of reaction mixture contained 0.1 nM [^{35}S]GTPγS, 3 mM $MgCl_2$, 10 mM PB, 0.33 mM 8-azaguanine and 50 μl membranes. After 30 min incubation at 0°C, samples were centrifuged at 10 000 ***g***, the supernatant was aspirated and the pellet dissolved in 80 μl 1 M acetic acid; 1.5 ml scintillation liquid was added and radioactivity was determined. Nonspecific binding was determined in the presence of 0.1 mM GTP and subtracted from all data.

GTPase assay

For this assay, membranes were washed in 10 mM triethanolamine–HCl, pH 7.4, containing 0.5 mM EDTA and the final pellet was resuspended in the same buffer to the equivalent of 1×10^8 cells ml^{-1}. GTPase activity of the membranes was determined in a reaction mixture containing [γ-^{32}P]GTP (3.7 kBq), 2 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM adenosine 5′-(2,3-imido)triphosphate, 0.1 mM ATPγS, 10 mM dithiothreitol (DTT), 5 mM creatine phosphate, 0.4 mg ml^{-1} creatine kinase, 0.33 mM 8-azaguanine and 2 mg ml^{-1} bovine serum albumin (purified) in 50 mM triethanolamine–HCl, pH 7.4, in a total volume of 100 μl (Snaar-Jagalska *et al.* 1988*b*). The reaction was started by the addition of 30 μl of membranes to the reaction mixture. After a 3 min incubation at 25°C, the reaction was terminated by the addition of 0.5 ml sodium phosphate buffer (50 mM), pH 8, containing 5 % activated charcoal. Samples were centrifuged for 5 min at 10 000 ***g*** at 4°C and the radioactivity of the supernatant was measured using Čerenkov radiation.

Results

*Dictyostelium fgd*A mutants are unable to respond to exogenous cyclic AMP signals; these strains show no chemotactic response to cyclic AMP and no activation of adenylate or guanylate cyclase is measurable *in vivo* upon stimulation with cyclic AMP. These defects are related to the absence of a functional G*α*2 protein (Kesbeke *et al.* 1988; Kumagai *et al.* 1989). However, the different *fgd*A cell lines do respond chemotactically to folate (Coukell *et al.* 1983), suggesting that FA-mediated chemotaxis is not transduced by a G protein, or that it is transduced by a G protein other than G*α*2. Therefore we measured the interaction of FA receptors and putative G proteins in mutant *fgd*A. During these investigations no differences were found between wild-type strain NC4 and the parental wild-type strains HC6 or HC91.

FA-induced cyclic GMP response

During the first hours of starvation extracellular FA induces the accumulation of cyclic GMP. In Fig. 1 the kinetics of the response in wild-type and two *fgd*A strains are shown. There is a fast and transient response in all *fgd*A strains tested, and the kinetics of the response are similar to that in parental strain HC6 with maximal cyclic GMP levels at about 10 s after stimulation and a return to basal levels after 30 s.

FA binding to membranes and modulation of binding by GTPγS

To study the coupling between folate receptors and a possible G protein in the *fgd*A mutants and their parents, we measured folate binding to membranes in the absence or presence of GTPγS (Table 1). Multiple FA receptors may exist (De Wit and Van Haastert, 1985). Using FA as a ligand, all binding forms are detected. In wild-type GTPγS induces a 43 % inhibition of [^{3}H]FA binding; in the *fgd*A

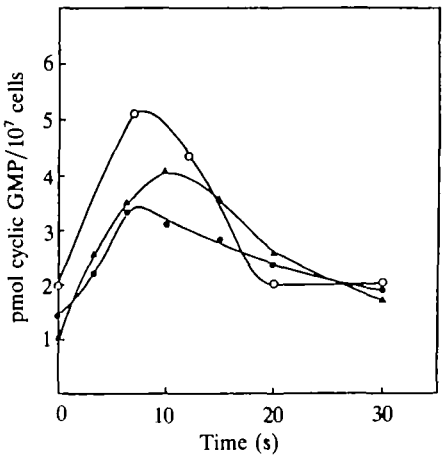

Fig. 1. FA-induced accumulation of cyclic GMP levels. Cells, starved for 2 h on non-nutrient agar, were stimulated with 1 μM FA at $t=0$ s. At the times indicated, cells were lysed and the cyclic GMP content was determined. Symbols represent: wild-type (○), *fgd*A mutants HC85 (●) and HC213 (▲). The results shown are the means of duplicate determinations of a single experiment. Two duplicate experiments gave similar results.

Table 1. *Effect of 100 μM GTPγS on folate binding to membranes*

Strain	Class	Binding in the presence of GTPγS: % of control FA-binding	% of control MFA-binding
Wild-type	*agg*+	57±6	70±12
HC33	*fgd*A	51±9	69±6
HC85	*fgd*A	57±23	68±8
HC213	*fgd*A	51±7	72±12

Cells were starved for 2 h in shaking suspension and used for membrane preparations. Equilibrium binding was performed as described in Materials and methods. Data are presented as % of control binding; the control is the binding of FA or MFA to membranes from the same strain in the absence of GTPγS. Values are means±S.D. of triplicate determinations from two or more independent experiments.

strains this inhibition is somewhat higher, but differences between wild-type and mutant strains are not significant ($P>0.05$, tested according to Student's t-test). When [^{3}H]MFA is used as radioligand in the presence of DAFA, only B-sites are detected that are probably involved in chemotaxis. Also in these measurements GTPγS induced a similar inhibition of binding in wild-type and *fgd*A strains.

The results of these binding studies point to an unaltered interaction between cell surface folate binding sites and a putative G protein in mutant *fgd*A.

Regulation of GTPase activity by folates

Recently we have shown that GTP hydrolysis in *D. discoideum* membranes from aggregatiom-competent cells is caused by at least two enzymes with high (K_M=6.5 μM) and low (K_M>1 mM) affinity. The high-affinity GTPase is stimulated by cyclic AMP (Snaar-Jagalska *et al.* 1988*b*). In *fgd*A mutants the stimulatory effect of cyclic AMP was decreased, while basal high-affinity GTPase activity was reduced 40 % (Kesbeke *et al.* 1988).

Table 2 presents GTPase activity in membranes isolated from vegetative cells of *fgd*A mutant HC85 and its parental strain HC6. GTPase activity was measured at 0.01 μM GTP; at this concentration mainly the high-affinity enzyme is detected. Activation of the high-affinity GTPase was measured in the presence of 3 μM FA or its degradation-resistant analog, MTX (De Wit *et al.* 1985). In wild-type and mutant membranes FA and MTX induced similar stimulation of the high-affinity GTPase of about 30 % in both wild-type and mutant membranes.

Table 2. *FA-induced stimulation of high-affinity GTPase activity in wild-type and a* fgdA *mutant*

Strain	Class	GTPase activity (pmol P_i min^{-1} mg^{-1})	% GTPase stimulation by: FA	MTX
HC6	*agg*+	1.25±0.11	33±5	27±4
HC85	*fgd*A	1.21±0 12	26±3	31±9

GTP hydrolysis by high-affinity GTPase was determined in the absence or presence of 3 μM FA or MTX at a GTP concentration of 10 nM. GTP hydrolysis by low-affinity GTPase was determined in the presence of 100 μM GTP. High affinity is defined as the difference between total GTPase and low-affinity GTPase. Means±S.D. of three experiments are presented. Differences between % stimulation were not significant (according to t-test, $P>0.05$).

Table 3. *Effect of FA on GTPγS binding to membranes, isolated from wild-type cells and* fgdA *mutants*

Strain	Class	GTPγS binding (% of wild-type)	% Stimulation by: FA	MTX
HC6	*agg*+	100	*29±6	*19±3
HC85	*fgd*A	93±8	*30±4	*21±4

Equilibrium binding of GTPγS was measured in the absence or presence of 3 μM FA and MTX. Means±S.D. of three experiments are presented, 100 % being 7000 cts min^{-1} bound per incubation.
* % Stimulation is significant, according to t-test, $P<0.05$.

GTPγS binding and modulation of binding by folates

Previously it has been observed that FA enhances [^{3}H]GTP equilibrium binding and causes an increased dissociation of the bound GTP from the membranes (De Wit and Snaar-Jagalska, 1985). In the present study we investigated [^{35}S]GTPγS binding on membranes isolated from vegetative cells of wild-type and *fgd*A mutant HC85 (Table 3). Equilibrium binding of GTPγS on membranes of both cell lines is enhanced about 30 % in the presence of FA and about 20 % in the presence of MTX. Statistical analysis of these data (according to Student's t-test) revealed that the increases in GTPγS binding by FA and MTX are significant for both HC85 and wild-type cells ($P<0.05$), and that there are no significant differences between HC85 and wild-type cells ($P>0.05$).

Discussion

Chemotaxis plays an important role during the life cycle of a slime mold cell, because it is involved in food seeking during the single-cell phase, and during the social phase in the formation of the multicellular structure. During development from the vegetative to the aggregation-competent stage the cells loose their sensitivity for folates but acquire sensitivity for cyclic AMP. Both compounds induce signal transduction involving cell surface receptors and G proteins (Janssens and Van Haastert, 1987). Stimulation of *D.*

discoideum with the appropriate chemoattractant induces a fast cyclic GMP response, which is involved in chemotaxis (Wurster *et al.* 1977; Ross and Newell, 1981; De Wit *et al.* 1987). The cyclic GMP responses to saturated FA and cyclic AMP stimuli are not additive, which suggests that the transduction pathways of these two stimuli start at separate cell surface receptors but meet each other at or before guanylate cyclase. Cyclic AMP and FA induce both adaptation of guanylate cyclase and chemotaxis, but cells adapted to cyclic AMP are still responsive to FA and *vice versa* (Van Haastert, 1983). This suggests that the transduction pathways from cyclic AMP and FA surface receptors toward guanylate cyclase and chemotaxis meet each other between the adaptation step and the effector enzyme, perhaps at the G protein level.

In the present study we address the question: do FA and cyclic AMP receptors transduce signals to guanylate cyclase through a common G protein, or does each receptor interact with a specific G protein, which may activate guanylate cyclase? The sequence of two G-protein α-subunit cDNAs (Gα1 and Gα2) from *D. discoideum* has been reported (Pupillo *et al.* 1989). In *fgd*A mutants, isolated by Coukell *et al.* (1983), Gα1 is normally expressed, while Gα2 is absent (Kumagai *et al.* 1989); this mutation caused complete blockade of all cyclic AMP sensory transduction, suggesting that the cyclic AMP receptor operates *via* Gα2 (Kesbeke *et al.* 1988). Coukell demonstrated that FA-mediated chemotaxis was normal in this mutant, suggesting that FA-induced chemotaxis is not transduced by a G protein, or by a G protein other than Gα2. Therefore we measured the interaction of the FA receptor and putative G proteins in *fgd*A mutants.

FA-induced signal transduction in *fgd*A mutants shows the following characteristics: (1) cell surface FA receptors are present; (2) FA does induce a cyclic GMP response and chemotaxis; (3) in membranes isolated from the vegetative mutant cells GTPγS does inhibit FA-binding; (4) FA and MTX do stimulate GTPase activity and GTPγS binding on membranes. These results lead to the conclusion that in *fgd*A mutant cells signal transduction *via* the folic acid receptor is normal. Since in the same mutants the interaction between cell surface cyclic AMP receptor and G proteins is defective, this implies that cyclic AMP and FA receptors must interact with distinct G proteins. Cyclic AMP receptors probably interact with Gα2, while FA receptors operate *via* a different Gα. Gα1 is expressed when FA receptors are present. However, several observations suggest that FA receptors are not coupled to Gα1. Recently cell lines have been constructed in which the expression of the Gα1 protein was reduced by more than 95 %, using antisense mRNA inactivation. In these transformants FA induces normal chemotaxis, activation of guanylate cyclase and stimulation of GTP binding (Firtel, personal communication). These results suggest that neither Gα1 nor Gα2 is associated with folic acid receptors. The present observations imply that the G protein that interacts with FA receptors has not yet been identified. Furthermore, *Dictyostelium* does not contain a common chemotaxis G protein, since chemotaxis to FA and cyclic AMP appears to be mediated by distinct G proteins.

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