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

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**Summary**

Mutant Frigid A (*fgdA*) of *Dictyostelium discoideum* is defective in a functional Ga2 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 *fgdA* 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) GTPyS 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 Ga protein that is different from Ga2. 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, 1986a). 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, 1986a), while GTPyS binding and high-affinity GTPase activity in membranes are potentiated by cyclic AMP (Snaar-Jagalska et al. 1988a, 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 *fgdA* of *D. discoideum* that lacks a functional Go2 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, [7,3',5'-3H]folic acid (FA, 0.557TBq mmol⁻¹) and [7,3',5'-3H]methtrexate (MTX, 0.60TBq mmol⁻¹) were obtained from Amersham International (Buckinghamshire, UK). N⁵'-methyl[7,3',5'-3H]folic acid (MFA) was prepared by enzymatic degradation of FA (BDH Biochemicals, Poole, O.S.) and [7,3',5'-3H]methotrexate from Amersham International (Buckinghamshire, UK). Ar 10-methyl[7,3',5'-3H]folic acid (MFA) was obtained from Boehringer Mannheim GmbH (Mannheim, 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. 1989). GTPyS (adenosine 5'-[2-(3-imido)triphosphate]), ATPyS, 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 fgdA strains (Coukell et al. 1983). The strains of the fgdA group, HC33, HC65 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 yeast extract, 0.2 g Na₂HPO₄, 1.5 g Na₂HPO₄, 2H₂O 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 (15% agar in PB) at a density of 2x10⁸ cells ml⁻¹ or in a shaking suspension in PB at a density of 10⁷ 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 2x10⁸ 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 10⁶ 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 potassium acetate 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 1.5x10⁶ cells ml⁻¹. Folate binding was measured at 0°C in a total volume of 150 μl, containing 120 μl membrane suspension, 5 mM [³H]FA and 0.33 mM 8-azaguanine (final concentrations). The mixture for MFA-binding contained 120 μl membrane suspension, 2 nM [³H]MFA, 0.33 mM 8-azaguanine and 3.33 μM DAFA. GTPγS (if present) was preincubated with the membrane suspension for 1 min. The binding reaction was started by the addition of membrane suspension and after 5 min incubation by alkaline hydrolysis from PB at a density of 10⁷ 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 2x10⁸ 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.

**GTPγS binding assay**

After collecting the cells, they were used directly to prepare membranes, which were resuspended in PB to a density equivalent to 2x10⁶ cells ml⁻¹. Binding of [³H]GTPγS to membranes was performed as described (Snaar-Jagalska et al. 1988a). Briefly, 100 μl of reaction mixture containing 0.1 mM [³H]GTPγS, 3 mM MgCl₂, 10 mM PB, 0.33 mM 8-azaguanine and 50 μl membranes. After 90 min incubation at 0°C, samples were centrifuged at 10 000 g, the supernatant was aspirated and the pellet dissolved in 80 μl 1 mM 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.

**GTmaze 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 1x10⁶ cells ml⁻¹. GTmaze activity of the membranes was determined in a reaction mixture containing (γ⁻³²P]GTP (3.7 kBq), 2 mM MgCl₂, 0.1 mM EDTA, 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. 1988b). 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 fgdA 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 Ga2 protein (Kesbeke et al. 1988; Kumagai et al. 1989). However, the different fgdA 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 Ga2. Therefore we measured the interaction of FA receptors and putative G proteins in mutant fgdA. 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 fgdA strains are shown. There is a fast and transient response in all fgdA strains tested, and the kinetics of the response are similar to that in parental strain NC4 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 fgdA 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 [³H]IFA binding; in the fgdA...
Table 1. Effect of 100 µM GTPyS on folate binding to membranes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Class</th>
<th>% of control FA-binding</th>
<th>% of control MPA-binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>agg+</td>
<td>57±6</td>
<td>70±12</td>
</tr>
<tr>
<td>HC33</td>
<td>fgdA</td>
<td>51±9</td>
<td>69±6</td>
</tr>
<tr>
<td>HC85</td>
<td>fgdA</td>
<td>57±23</td>
<td>68±8</td>
</tr>
<tr>
<td>HC213</td>
<td>fgdA</td>
<td>51±7</td>
<td>72±12</td>
</tr>
</tbody>
</table>


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 GTPyS. Values are means ± S.D. of triplicate determinations from two or more independent experiments.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Class</th>
<th>GTPase activity (pmol Pi·min⁻¹·mg⁻¹)</th>
<th>% GTPase stimulation by</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC8</td>
<td>agg+</td>
<td>1.25±0.11</td>
<td>33±5</td>
</tr>
<tr>
<td>HC85</td>
<td>fgdA</td>
<td>1.21±0.12</td>
<td>26±3</td>
</tr>
</tbody>
</table>

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. The results shown are means ± S.D. of three experiments. Differences between % stimulation were not significant (according to t-test, P>0.05).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Class</th>
<th>GTPyS binding (% of wild-type)</th>
<th>% Stimulation by</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC6</td>
<td>agg+</td>
<td>100</td>
<td>29±4</td>
</tr>
<tr>
<td>HC85</td>
<td>fgdA</td>
<td>33±6</td>
<td>29±4</td>
</tr>
</tbody>
</table>

Equilibrium binding of GTPyS was measured in the absence or presence of 3 µM FA and MTX. Values are means ± S.D. of three experiments presented. 100% being 7000 cts min⁻¹ bound per incubation. *% Stimulation is significant, according to t-test, 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 lose 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 membranes from aggregation-competent cells is caused by at least two enzymes with high (Km=6.5 µM) and low (Km>1 mM) affinity. The high-affinity GTPase is stimulated by cyclic AMP (Snaar-Jagalska et al. 1988b). In fgdA 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 fgdA mutant HC85 and its parent 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.

FA-induced accumulation of cyclic GMP levels. Symbols represent: wild-type (○), fgdA mutants HC85 (●) and HC213 (▲). The results shown are the means of duplicate determinations of a single experiment. Two duplicate experiments gave similar results.

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. At the times indicated, cells were lysed and the cyclic GMP content was determined. Symbols represent: wild-type (○), fgdA mutants HC85 (●) and HC213 (▲). The results shown are the means of duplicate determinations of a single experiment. Two duplicate experiments gave similar results.
discoideum with the appropriate chemosensitizer 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 different loci. The cyclic GMP responses to saturated FA and cyclic AMP are still responsive to FA and cyclic AMP, respectively. The presence of G protein suggests that the G protein is involved in cyclic AMP receptor desensitization (Van Haastert, 1983). This suggests that the transduction pathways from cyclic AMP and FA surface receptors to 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 subunit cDNAs (Ga-1 and Ga2) from Dictyostelium discoideum has been reported (Pupillo et al. 1989). In fgdA mutants, isolated by Coukell et al. (1983), Ga1 is normally expressed, while Ga2 is absent (Kumagai et al. 1989); this mutation caused a complete blockade of all cyclic AMP sensory transduction, suggesting that the cyclic AMP receptor operates via Ga2 (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 Ga2. Therefore we measured the interaction of the FA receptor and putative G proteins in fgdA mutants.

FA-induced signal transduction in fgdA 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 not promote :FA-binding; (4) FA and MTX do stimulate GTPase activity and GTPγS binding on membranes. These results lead to the conclusion that in fgdA 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 Ga2, while FA receptors operate via a different Ga. Ga1 is expressed in some FA receptors. However, several observations suggest that FA receptors are not coupled to Ga1. Recently cell lines have been constructed in which the expression of the Ga1 protein was reduced by more than 95%, leading to antisense mRNA inactivation. In these transformants FA induces normal chemotaxis, activation of guanylate cyclase and stimulation of cGMP production (Firtel, personal communication). These results suggest that neither Ga1 nor Ga2 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 chemotaxic G protein, since chemotaxis to FA and cyclic AMP appears to be mediated by distinct G proteins.

References


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