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Abberant chemotaxis and differentiation in *Dictyostelium* mutant *fgd*C with a defective regulation of receptor-stimulated phosphoinositidase C

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Summary

Dictyostelium **cells use extracellular cyclic AMP both as a chemoattractant and as a morphogen inducing cell-type-specific gene expression. Cyclic AMP binds to surface receptors, activates one or more G-proteins, and stimulates adenylate cyclase, guanylate cyclase and phosphoinositidase C. Mutant *fgd*C showed aberrant chemotaxis, and was devoid of cyclic AMP-induced gene expression and differentiation. Both the receptor- and G-protein-mediated stimulation of adenylate cyclase and guanylate cyclase were unaltered in mutant *fgd*C as compared to wild-type cells. In wild-type cells phosphoinositidase C was activated about twofold by the cyclic AMP receptor. In mutant *fgd*C cells, however, the enzyme was inhibited by about 60%. These results suggest that phosphoinositidase C is regulated by a receptor-operated activation/inhibition switch that is defective in mutant *fgd*C. We conclude that activation of phosphoinositidase C is essential for *Dictyostelium* development.**

Key words: phosphoinositidase C, *Dictyostelium*, chemotaxis, differentiation.

Introduction

Dictyostelium is used as a model organism in which to study the regulation of cell locomotion, chemotaxis and gene expression by extracellular signal molecules (Janssens and Van Haastert, 1987). In aggregation-competent cells these functions are controlled by extracellular cyclic AMP, which binds to surface receptors that have a predicted structure with seven transmembrane spanning domains like many G-protein coupled receptors (Klein *et al.* 1988). Activation of the receptor leads to the G-protein-mediated stimulation of adenylate cyclase, guanylate cyclase and phosphoinositidase C (Europe-Finner *et al.* 1989; Janssens *et al.* 1989; Mato *et al.* 1977; Schaffer, 1975; Theibert and Devreotes, 1986; Van Haastert, 1989; Van Haastert *et al.* 1989; Van Haastert *et al.* 1987). These enzymes produce the second messengers cyclic AMP, cyclic GMP, Ins(1,4,5)P_3 and diacylglycerol, respectively.

In eukaryotes the mechanism by which extracellular signals induce chemotaxis and gene expression, and the role of the different second messengers in these processes, are still largely unknown. *Dictyostelium* mutants and transformants are potentially useful in elucidating these sensory transduction pathways. Transformed cells with reduced cyclic AMP receptor expression show aberrant chemotaxis and differentiation (Klein *et al.* 1988; Sun *et al.* 1990). Experiments with mutants *stm*F and *synag*7 demonstrate that activation of guanylate cyclase is important for chemotaxis but possibly not for differentiation, whereas activation of adenylate cyclase is not directly involved in either of these responses (Ross and Newell, 1981; Schaap *et al.* 1986; Snaar-Jagalska and Van Haastert, 1988). *Frigid* mutants are insensitive to extracellular cyclic AMP for the induction of contact sites (Coukell *et al.* 1983). Mutant *fgd*A is defective in the gene coding for the G-protein α-subunit Gα2 and is defective in all known second-messenger functions, chemotaxis and cyclic AMP-induced gene expression (Coukell *et al.* 1983; Kesbeke *et al.* 1988; Kumagai *et al.* 1989; Snaar-Jagalska *et al.* 1988*a*,*b*). Here we describe mutant *fgd*C, which is specifically defective in the regulation of phosphoinositidase C activity.

Materials and methods

Materials

Cyclic AMP and GTP[S] were from Boehringer-Mannheim, dithiothreitol and EGTA were obtained from Sigma. [2,8-^{3}H]cyclic AMP (40 Ci mmol^{-1}), [8-^{3}H]cyclic GMP (25 Ci mmol^{-1}) and [^{3}H]Ins(1,4,5)P_3 (60 Ci mmol^{-1}) were from Amersham, Buckinghamshire. The Ins(1,4,5)P_3 binding protein was isolated from bovine adrenal glands as described (Baukal *et al.* 1985).

Culture conditions and chemotaxis

Dictyostelium cells (wild-type strains NC4 and XP-55 and mutant *fgd*C strain HC317) were grown in association with *Escherichia coli* as described (Kesbeke *et al.* 1988). Cells were harvested in 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (PB). Since no significant differences could be observed between XP-55 and NC-4 the data presented are those of the more generally used wild-type strain

NC-4. Chemotaxis towards different cyclic AMP concentrations was determined using the small-population assay (Konijn, 1970)

Cyclic AMP-binding to surface receptors

Cells were starved in PB at a density of 10^7 cells ml^{-1} for 5 h, washed and resuspended in PB at a density of 10^8 cells ml^{-1}. Cyclic AMP binding by cells was measured in incubations containing different concentrations of [^{3}H]cyclic AMP, 10 mM dithiothreitol and 8×10^7 cells. After 5 min at 0°C cells were centrifuged through silicon oil, and cell-associated radioactivity was determined (Kesbeke *et al.* 1988). Non-specific binding was determined in the presence of 10^{-4} M unlabeled cyclic AMP. Cyclic AMP binding towards membranes was measured at 5 nM [^{3}H]cyclic AMP in the presence or absense of 30 μM GTP[S] as described (Kesbeke *et al.* 1988).

Second messenger responses in vivo

Cells were starved for 5 h in PB by shaking at a density of 10^7 cells ml^{-1}. Cells were collected, resuspended to a density of 10^8 cells ml^{-1} in PB and stimulated. For cyclic AMP response cells were stimulated with 5 μM 2'deoxy-cyclic AMP and 5 mM dithiothreitol; the stimulus for cyclic GMP and Ins(1,4,5)P_3 responses was 0.1 μM cyclic AMP. At the times indicated in the figure legends cells were lysed by the addition of perchloric acid. The levels of cyclic AMP, cyclic GMP or Ins(1,4,5)P_3 were determined in the neutralysed lysates using specific isotope dilution assays (Kesbeke *et al.* 1988; Van Haastert, 1989).

Regulation of phosphoinositidase C by cyclic AMP in vivo

Wild-type (strain NC4) and mutant *fgd*C cells (strain HC317) were starved for 5 h, washed and resuspended in 40 mM Hepes/NaOH, pH 6.5, 5.9 mM EGTA (PIC-buffer). Cells were stimulated at t_0 with 0.1 μM cyclic AMP, and lysed at the times indicated in the figure legends by rapid elution through a Nuclepore polycarbonate filter (pore size 3 μm). Phosphoinositidase C activity was measured immediately in a reaction mixture of 100 μl containing 6.9 mM $CaCl_2$ and 90 μl of lysate. The reaction was terminated after 20 s by the addition of 100 μl perchloric acid (3.5 %, v/v), basal Ins(1,4,5)P_3 levels were determined in samples to which perchloric acid was added prior to addition of $CaCl_2$. Ins(1,4,5)P_3 levels were measured in the neutralized lysate by isotope-dilution assay (Van Haastert, 1989). The data presented are the Ins(1,4,5)P_3 levels found in the presence of $CaCl_2$ minus the basal level, hence they represent the amount produced during the incubation. A more-detailed description of the assay and the characteristics of PIC will be published elsewhere (A. A. Bominaar and P. J. M. Van Haastert, manuscript in preparation).

Regulation of effector enzymes by GTP[S] in cell-free systems

Wild-type NC4 and *fgd*C cells were starved for 5 h, washed and resuspended with the indicated buffer. Cells were lysed by rapid filtration through a Nuclepore polycarbonate filter (pore size 3 μm). Adenylate cyclase: lysis was in 40 mM Hepes, 0.5 mM EDTA, 250 mM sucrose, pH 7.5 (AC-buffer). The lysate was incubated for 5 min at 0°C in the absence or presence of 100 μM GTP[S] (guanosine 5'-*O*-(3-thiotriphosphate). Adenylate cyclase activity was then determined at 20°C in a reaction containing lysate, AC-buffer, 2 mM $MgCl_2$, 0.5 mM ATP and 10 mM dithiothreitol; the cyclic AMP produced was determind by isotope dilution assay as described (Theibert and Devreotes, 1986; Van Haastert *et al.* 1987). Guanylate cyclase: lysis was in 20 mM Hepes, 1.5 mM $MgCl_2$, 0.5 mM EGTA, pH 7.0 (GC-buffer). Guanylate cyclase activity was determined immediately in a reaction containing lysate, GC-buffer, 5 mM dithiothreitol and 0.3 mM GTP in the presence or absence of 25 μM GTP[S]; the cyclic GMP produced was determined by radioimmunoassay (Janssens *et al.* 1989). Phosphoinositidase C: lysis was in 40 mM Hepes, 5.9 mM EGTA, pH 6.5 (PIC-buffer), in the presence or absence of 10 μM GTP[S]. Phosphoinositidase C activity was determined within 1 min after lysis in a reaction containing lysate PIC-buffer and 6.9 mM $CaCl_2$; the Ins(1,4,5)P_3 produced was determined by isotope dilution assay (Van Haastert, 1989).

Expression of aggregation-associated genes

Vegetative wild-type NC4 and mutant *fgd*C cells were freed from bacteria, resuspended to 10^7 cells ml^{-1} in PB and shaken at 150 revs min^{-1} and 21°C. Cells were treated with either 30 nM cyclic AMP pulses delivered at 6 min intervals, a cyclic AMP influx of 5 nM min^{-1}, pulses of 100 μM cyclic AMP added at 60 min intervals or without further additions. At the time periods indicated in the figure legends total RNA was isolated, size fractionated on formaldehyde-containing agarose gels, transferred to nylon membranes and hybridized with a ^{32}P-labeled cDNA complementary to the contact site A gene.

Expression of prespore and prestalk genes

Vegetative wild-type NC4 and mutant *fgd*C cells were freed from bacteria, shaken in PB at 10^7 cells ml^{-1} and treated during 6 h with 30 nM cyclic AMP for 6 min. Cells were washed once with PB, resuspended to the same density and shaken for an additional 8 h period with either 100 μM cyclic AMP (60 min), 30 nM cyclic AMP (6 min), or without further additions. At the time periods indicated in the figure legends total RNA was isolated and hybridized with ^{32}P-labeled cDNAs, complementary to the prestalk gene CP2 or the prespore gene D19.

Results

Cyclic AMP surface receptors

The binding of cyclic AMP to *Dictyostelium* wild-type and mutant cells was measured at different cyclic AMP concentrations, and is presented as a Scatchard plot (Fig. 1). Wild-type cells possess about 54 000 binding sites per cell, which may exist in two affinity states with K_{d1}=45 nM and K_{d2}=450 nM, respectively. Mutant *fgd*C has a lower level of cyclic AMP binding (about 20 000 sites/cell). The two affinity states have similar K_d values to those of wild-type cells (K_{d1}=28 nM and K_{d2}=400 nM).

Cyclic AMP-induced second messenger responses

Wild-type and mutant cells were stimulated with cyclic AMP or 2'-deoxy-cyclic AMP and the levels of accumulation of cyclic AMP, cyclic GMP and Ins(1,4,5)P_3 were measured. The 2'-deoxy-cyclic AMP-induced level of accumulation of cyclic AMP in mutant *fgd*C was similar in respect of kinetics and magnitude to that in wild-type cells (Fig. 2A). Cyclic AMP also induced the accumulation of

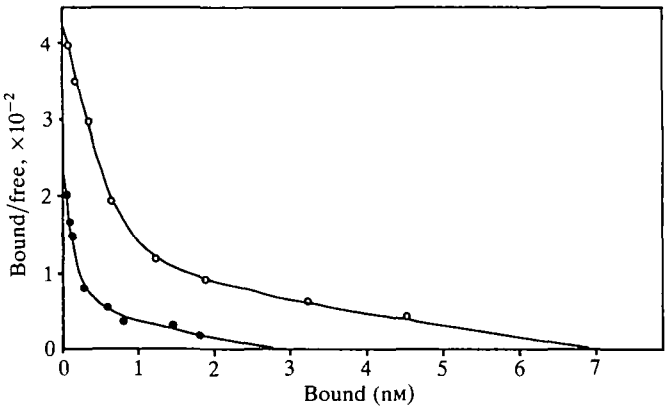

Fig. 1. Cyclic AMP-binding to surface receptors. The binding of different concentrations of [^{3}H]cyclic AMP to NC4 wild-type (○) or *fgd*C mutant (●) cells was determined. The data are presented as a Scatchard plot.

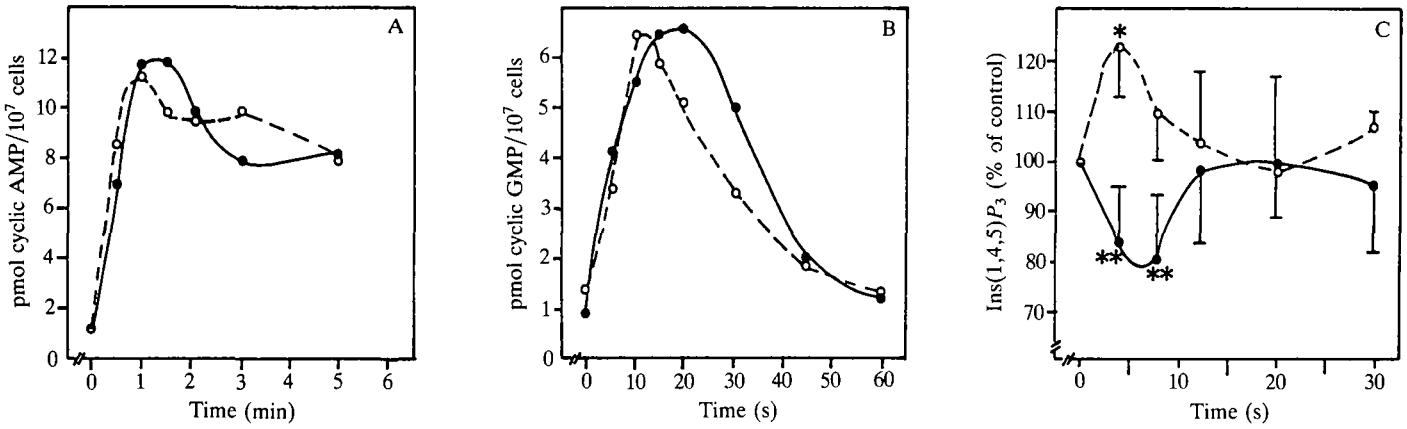

Fig. 2. Cyclic AMP-induced responses. Cells of wild-type strain NC4 (○) and mutant *fgd*C strain (●) were starved for 5 h, washed and stimulated with cyclic AMP. The accumulation of cyclic AMP (A), cyclic GMP (B) or Ins(1,4,5)P_3 (C) was determined by specific isotope-dilution assays. * Significantly above control with $P<0.05$; ** significantly below control with $P<0.05$. The results are the mean (±S.E.M. for C) of three independent experiments in triplicate.

cyclic GMP although at a slightly lower rate than in wild-type cells (Fig. 2B). Cyclic AMP stimulation of wild-type cells resulted in a 22±11 % ($n=3$, $P<0.05$) increase in the Ins(1,4,5)P_3 concentration at 4 s after stimulation. In contrast to the receptor-stimulated formation of Ins(1,4,5)P_3 in wild-type cells, cyclic AMP induced a 20±12 % ($n=3$, $P<0.05$) decrease in Ins(1,4,5)P_3 levels in mutant *fgd*C (Fig. 1C). The difference between wild-type and HC-317, at $t=4$ s, is significant, with $P<0.02$. Although these responses are significant they are relatively small, therefore phosphoinositidase C activity was determined more directly, the results of which are presented below.

Receptor–G-protein–effector interactions

The interaction between surface receptors and GTP-binding proteins is most easily studied as the GTP[S]-mediated decrease in cyclic AMP binding to membranes. The results presented in Table 1 indicate that GTP[S] induced the same inhibition of cyclic AMP binding in membranes derived from wild-type cells and mutant cells.

Interactions between G-proteins and effector enzymes can be studied by the activation of these effector enzymes by GTP[S]. Adenylate cyclase, guanylate cyclase and phosphoinositidase C can be activated by GTP[S] in lysates from wild-type cells (Janssens *et al.* 1989; Theibert and Devreotes, 1986; Van Haastert *et al.* 1987; A. A. Bominaar and P. J. M. Van Haastert, manuscript in preparation). Although basal levels of most effector enzyme activities are lower in *fgd*C cells compared to wild-type cells the relative activation of these enzymes by GTP[S] in lysates of *fgd*C was not significantly different from that in lysates from wild-type cells (Table 1). Thus a normal interaction between G-proteins and effector enzymes exists.

Assays for cyclic AMP stimulation of phosphoinositidase C in cell-free systems are not available for *Dictyostelium*. Therefore, the regulation of phosphoinositidase C by the cyclic AMP receptor was investigated *in vivo*. Cells were stimulated with cyclic AMP, rapidly lysed and phosphoinositidase C activity was determined (Fig. 3). In wild-type cells cyclic AMP induced a twofold activation of phosphoinositidase C activity when cells were lysed 20 s after stimulation. Stimulation of mutant *fgd*C cells with cyclic AMP did not result in the activation of phosphoinositidase C; in contrast, basal phosphoinositidase C activity was inhibited by 60 % (Fig. 3). Since under these assay conditions Ins(1,4,5)P_3 phosphatases are not active (data not shown), the decrease found must reflect a decrease in the enzyme activity.

Summarizing transmembrane signal transduction in *fgd*C: the results show normal cyclic AMP- and GTP[S]-mediated activation of adenylate and guanylate cyclase. Regulation of phosphoinositidase C by GTP[S] is also unaltered, but cyclic AMP inhibits the enzyme in *fgd*C, whereas it stimulates the enzyme in wild-type cells. Thus the defect in *fgd*C appears to be localized between the receptor and the G-protein.

Chemotaxis

Under physiological conditions mutant *fgd*C cells do not aggregate. However, chemotaxis was not absent, but about 100-fold higher cyclic AMP concentrations were required as compared to wild-type cells (Fig. 4). This low chemotactic efficiency towards cyclic AMP could explain

Table 1. *Receptor–G-protein–effector interactions*

	Wild-type NC4			Mutant *fgd*C		
	Basal	+GTP[S]	%	Basal	+GTP[S]	%
Cyclic AMP-binding (pmol/10^7 cells)	39.5±1 5	14.0±1.2	34±4	21.8±2.0	7.3±1.0	35±5
Adenylate cyclase (pmol min^{-1} mg^{-1} protein)	24.3±3.0	248±57	1022±267	21±4.1	179±35	854±236
Guanylate cyclase (pmol min^{-1} mg^{-1} protein)	56±10	188±13	336±64	39±12	112±20	287±102
Phosphoinositidase C (pmol min^{-1} mg^{-1} protein)	36.6±15	72.6±23.1	198±103	36.3±5.2	63.7±25	176±74

Wild-type strain NC4 and mutant *fgd*C cells were lysed and the regulation of the cyclic AMP receptor or the effector enzymes by GTP[S] was measured. Data are expressed as mean±S.D.

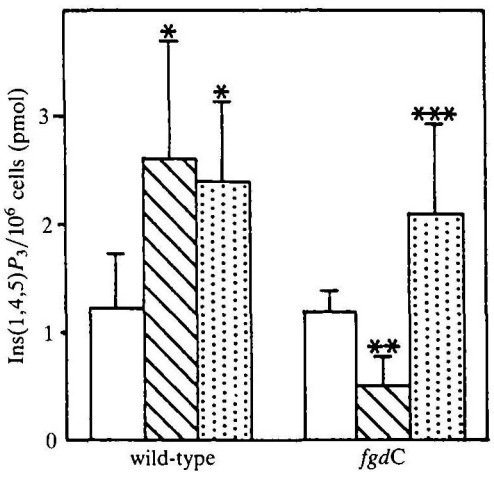

Fig. 3. Regulation of phosphoinositidase C. Cells of wild-type strain NC4 and mutant *fgdC* strain HC317 were starved for 5 h, washed and resuspended in PIC-buffer. Cells were stimulated with 0.1 μM cyclic AMP (hatched bars) and lysed after 20 s, or lysed in the presence of 10 μM GTP[S] (dotted bars). Phosphoinositidase C activity was measured. Data are presented as the mean±S.D. of 2 independent experiments in quadruplicate. * Significantly larger than control (open bars) with $P<0.02$; ** significantly lower than control with $P<0.001$; *** significantly larger than control with $P<0.05$.

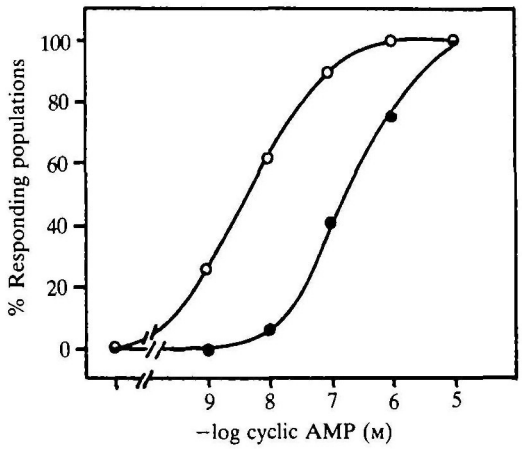

Fig. 4. Chemotaxis. The chemotactic activity of wild-type (○) and *fgdC* mutant (●) cells towards different cyclic AMP concentrations was determined with the small-population assay.

why this mutant is aggregation-deficient (Coukell *et al.* 1983).

Gene-expression

Cyclic AMP regulates the expression of several classes of genes at different stages of development (Mann and Firtel, 1989). Pulses of nanomolar cyclic AMP concentrations accelerate expression of aggregation-associated genes, such as those of cyclic AMP receptors and adhesive contact

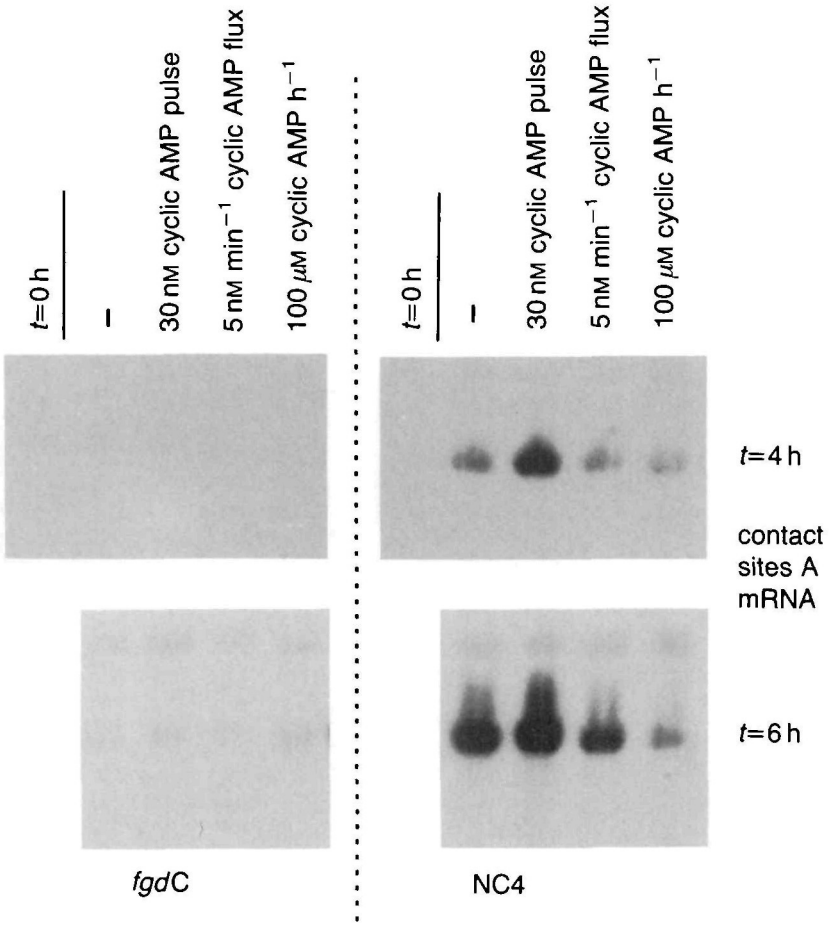

Fig. 5. Cyclic AMP-regulated expression of aggregation-associated genes. Vegetative wild-type NC4 and mutant *fgdC* cells were stimulated with either 30 nM cyclic AMP pulses delivered at 6 min intervals, a cyclic AMP influx of 5 nM min^{-1}, pulses of 100 μM cyclic AMP added at 60 min intervals or without further additions. At the indicated time periods total RNA was isolated and probed with a ^{32}P-labeled cDNA complementary to the contact site A gene.

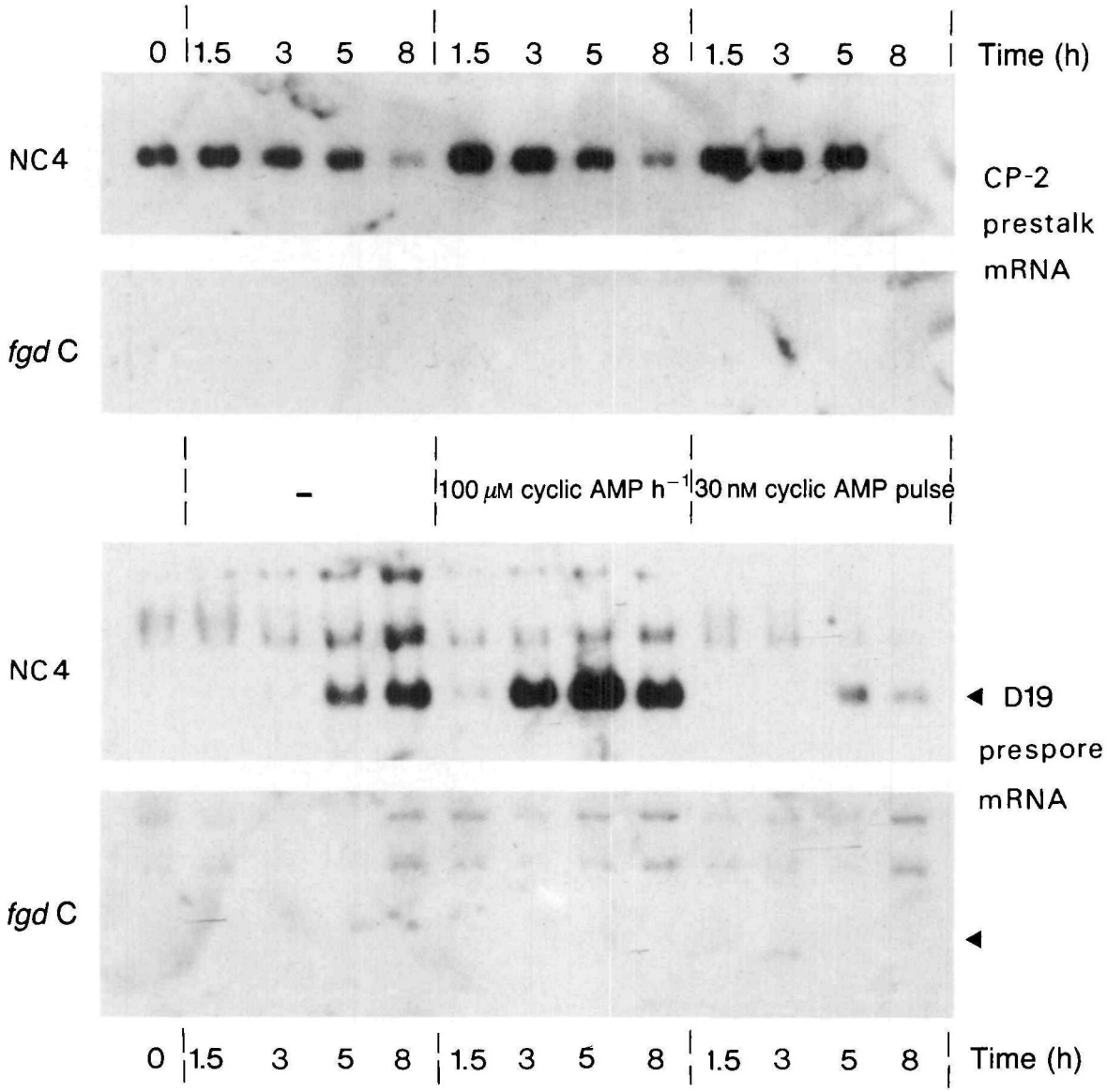

Fig. 6. Cyclic AMP-regulated expression of prespore and prestalk genes. Vegetative wild-type NC4 and mutant *fgd*C cells were treated during 6 h with pulses of 30 nM cyclic AMP for 6 min. Cells were washed and shaken for an additional 8 h period with either 100 μM cyclic AMP (60 min) or 30 nM cyclic AMP (6 min), or without further additions. At the indicated time periods total RNA was isolated and probed with ^{32}P-labeled cDNAs, complementary to the prestalk gene CP2 or the prespore gene D19.

sites A (Gerisch *et al.* 1975). Constant stimulation of aggregation-competent cells with micromolar cyclic AMP concentrations induces the expression of prespore genes, and both nanomolar pulses and micromolar stimuli induce the expression of a subclass of prestalk genes.

The induction of the different classes of cyclic AMP-regulated genes was investigated in mutant *fgd*C and in wild-type strain NC4. Fig. 5 shows that in NC4 nanomolar cyclic AMP pulses strongly enhanced contact site A gene expression, while a nanomolar cyclic AMP influx or stimulation with micromolar cyclic AMP concentrations inhibited expression of this gene. In the *fgd*C mutant, a low basal expression of contact site A gene was evident, but its expression was not regulated by cyclic AMP (Fig. 5).

In NC4 cells, which were sensitized during 6 h with nanomolar cyclic AMP pulses, expression of the prestalk gene CP2 was nearly maximal and increased only slightly by further treatment with pulses or micromolar cyclic AMP concentrations. In mutant *fgd*C neither pulses nor micromolar cyclic AMP stimuli could induce expression of this gene (Fig. 6). Micromolar cyclic AMP stimuli effectively induced expression of the D19 prespore gene in wild-type cells that were made competent by a 6 h pretreatment with cyclic AMP pulses. No expression of the D19 prespore gene was observed in the *fgd*C mutant (Fig. 6).

Discussion

Transduction of extracellular cyclic AMP signals in *Dictyostelium* is mediated by surface receptors, G-proteins and effector enzymes. The final cellular responses induced by extracellular cyclic AMP are chemotaxis and cell differentiation. The signal transduction pathways probably involve activation of adenylate cyclase, guanylate

cyclase and phosphoinositidase C, and the formation of the second messengers cyclic AMP, cyclic GMP, Ins(1,4,5)P_3, Ca^{2+} and diacylglycerol. These second messengers may transduce the signal further towards the final cellular responses.

Mutants with a defect in specific parts of the signal transduction pathways are very useful in unravelling the role of each of these pathways in the different responses. Mutant *fgd*C, which was selected for the lack of contact sites A (Coukell *et al.* 1983), a cell surface protein expressed early in development and involved in the aggregation process, shows the following characteristics: (i) surface cyclic AMP receptors are present, although at a reduced level. (ii) The receptor–G-protein interaction is essentially normal. (iii) G-protein–effector interactions are essentially normal for adenylate cyclase, guanylate cyclase and phosphoinositidase C. (iv) Cyclic AMP stimulates adenylate cyclase and guanylate cyclase as in wild-type. (v) Cyclic AMP inhibits phosphoinositidase C *versus* stimulation in wild-type. (vi) Mutant *fgd*C has low chemotactic activity towards cyclic AMP. (vii) Cyclic AMP does not induce expression of genes associated with the formation of cell aggregates, prespore cells and prestalk cells.

The biochemical defect of mutant *fgd*C is not exactly known. Genetic and biochemical evidence indicates that mutant *fgd*C is not defective in either the receptor or Gα2: cAR1, the gene for Gα2 and the *fgd*C locus are on different complementation groups (Coukell *et al.* 1983; Klein *et al.* 1988). Furthermore, mutants with a defect in Gα2 or cAR1 are defective in all sensory transduction processes (Kesbeke *et al.* 1988; Klein *et al.* 1988), whereas cyclic AMP activates adenylate and guanylate cyclase in mutant *fgd*C. Basal Ins(1,4,5)P_3 levels and both basal and GTP[S]-stimulated phosphoinositidase C activity are identical in *fgd*C and wild-type cells, excluding a defect in phosphoinositidase C. The only biochemical defect observed in mutant *fgd*C is a receptor-mediated inhibition of phosphoinositidase C, compared to stimulation of enzymes activity in wild-type cells. Apparently, sensory transduction from receptor to phosphoinositidase C contains an activation/inhibition switch between the receptor and the G-protein, which is malfunctioning in mutant *fgd*C.

The present results with mutant *fgd*C imply that activation of guanylate cyclase can occur in the absense of stimulation of phosphoinositidase C. This conclusion is in conflict with the hypothesis that guanylate cyclase is stimulated by the sequential pathway consisting of surface receptor, phosphoinositidase C, Ins(1,4,5)P_3 and cytosolic Ca^{2+} (Europe-Finner and Newell, 1985). The present observations also imply that activation of phosphoinositidase C is a not required for the activation of adenylate cyclase as we suggested previously (Snaar-Jagalska *et al.* 1988*b*).

The defect in signal transduction in mutant *fgd*C is associated with the absence of cyclic AMP-induced prespore and prestalk gene expression, and aberrant differentiation and chemotaxis. These results suggest that the activation of phosphoinositidase C is an early response required for cyclic AMP-induced differentiation in *Dictyostelium*. This view is supported by the observations that lithium inhibits prespore gene expression, possibly by inhibiting the activation of phosphoinositidase C (Peters *et al.* 1989), and that certain combinations of Ins(1,4,5)P_3 and diacylglycerol induce prespore gene expression in permeabilized *Dictyostelium* cells (Ginsburg and Kimmel, 1989). However, we cannot exclude the possibility that the blockade of all early differentiation represents the primary defect of mutant *fgd*C, and that the aberrant regulation of phosphoinositidase C is the consequence of this developmental defect.

Nevertheless, mutant *fgd*C is the first *Dictyostelium* mutant that is specifically defective in the phosphoinositidase C pathway with no discernible defects in the adenylate and guanylate cyclase pathways. Mutant *synag*7 is specifically defective in the activation of adenylate cyclase, whereas mutant *stm*F shows specifically alteration of the cyclic GMP response. The combined use of these mutants should be very useful in studying the role of each second messenger in complex regulatory processes.

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