Non-Chemotactic *Dictyostelium discoideum* Mutants with Altered cGMP Signal Transduction

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Abstract. Folic acid and cAMP are chemoattractants in *Dictyostelium discoideum*, which bind to different surface receptors. The signal is transduced from the receptors via different G proteins into a common pathway which includes guanylyl cyclase and acto-myosin. To investigate this common pathway, ten mutants which do not react chemotactically to both cAMP and folic acid were isolated with a simple new chemotactic assay. Genetic analysis shows that one of these mutants (KI-10) was dominant; the other nine mutants were recessive, and comprise nine complementation groups.

In wild-type cells, the chemoattractants activate adenylyl cyclase, phospholipase C, and guanylyl cyclase in a transient manner. In mutant cells the formation of cAMP and IP_3 were generally normal, whereas the cGMP response was altered in most of the ten mutants. Particularly, mutant KI-8 has strongly reduced basal guanylyl cyclase activity; the enzyme is present in mutant KI-10, but can not be activated by cAMP or folic acid. The cGMP response of five other mutants is altered in either magnitude, dose dependency, or kinetics.

These observations suggest that the second messenger cGMP plays a key role in chemotaxis in *Dictyostelium.*

THE cellular slime mold *Dictyostelium discoideum* provides an accessible eukaryotic model system for the study of transmembrane signaling by hormone-like messengers (4). Amoebae of this microorganism react chemotactically to two types of extracellular molecules. Because folic acid is secreted from bacteria, chemotaxis to folic acid is important for the vegetative cells to capture their food source bacteria (35, 36). After removal of bacteria, cells stop multiplying and become aggregation competent (5). In this process, cells change their chemotactic taste from folic acid to cyclic adenosine 3′,5′-monophosphate (cAMP)[1] (52) by degrading cell surface folic acid receptors and, in turn, producing cAMP receptors (29, 42, 56). Starving cells secrete cAMP periodically in response to cAMP. Cells react chemotactically towards the source of cAMP which is secreted from central collecting points (9, 17).

Binding of cAMP to its surface receptors activates several enzymes, via guanine nucleotide–binding proteins (G-proteins) (20). Adenylyl cyclase is one of these enzymes, which catalyzes the production of cAMP from ATP (23, 37). This enzyme is important for the cAMP relay system to transmit chemotactic activity to neighboring cells. The enzyme is not involved in the regulation of the motor proteins, because mutant cells which do not have adenylyl cyclase still respond chemotactically to extracellular cAMP (37). Phospholipase C is another enzyme activated by the receptor, which catalyzes the production of D-*myo*-inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol from PIP_2 (14, 15, 45, 47). IP_3 is known as a universal second messenger which opens a calcium gate on internal non-mitochondrial calcium stores (13).

Guanylyl cyclase is the third enzyme activated by the receptor, and produces cyclic guanosine 3′,5′-monophosphate (cGMP) from GTP. Accumulation of cGMP is proposed to be essential for chemotaxis, based on several lines of evidence. First, the chemoattractants folic acid and cAMP both induce a cGMP response in vegetative and starved cells, respectively (30). Second, other compounds that specifically attract *Dictyostelium lacteum*, *Dictyostelium minutum*, and *Polysphondylium violaceum* induce a cGMP response in sensitive cells (10, 31, 57). Third, mutant *stm* (Streamer) F (38) has a prolonged cGMP response (39) due to a defect in cGMP specific phosphodiesterase (6, 50), and at the same time shows a prolonged chemotactic movement (38).

The interaction of motor proteins, actin and myosin, is thought to be regulated by calcium and cGMP. Addition of millimolar concentrations of calcium chloride to saponin-permeabilized cells evokes actin polymerization (12). This

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1. *Abbreviations used in this paper:* cAMP, cyclic adenosine 3′,5′-monophosphate; dcAMP, cyclic 2′-deoxyadenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; GTPγS, guanosine 5′-[γ-thio]triphosphate; 8-Br-cGMP, cyclic 8-bromoguanosine 3′,5′-monophosphate; IP_3, D-*myo*-inositol 1,4,5-trisphosphate; *fgd* A, Frigid A; *fgd* C, Frigid C; *stm* F, Streamer F.

suggests that free calcium ions in the cytosol enhance the polymerization of actin filaments. In *stm* F mutant cells, accumulation of myosin in the cytoskeleton induced by cAMP stimuli is prolonged and phosphorylation is delayed (26, 27). This suggests that cGMP may regulate the phosphorylation of conventional myosin (2) and the disassembly of myosin filaments. This dual regulation of the motor proteins, actin and myosin, by calcium and cGMP is proposed to lead the chemotactic movement.

Binding of folic acid to its cell surface receptors activates similar signal transduction pathways as extracellular cAMP, including cGMP formation (30, 43, 55) and rapid cytoskeletal actin polymerization (32). The relation between the pathways via folic acid and cAMP receptors is not completely clear yet, although it has been shown that different receptors and different G proteins are used to detect and transduce folic acid and cAMP (22). Furthermore, it has been observed that the cGMP response of cells that are sensitive to both folic acid and cAMP is not additive (43), suggesting that the different pathways from folic acid and cAMP receptors merge into one pathway at or before guanylyl cyclase.

One approach to understand chemotactic signal transduction is to isolate mutants that are defective in various points of the chemotactic pathway. Several mutants defective in chemotaxis to cAMP or folic acid have been reported (1, 28, 40). The mutated component of the signal transduction pathway has been characterized for only a few mutants: cGMP phosphodiesterase in *stm* F (6, 50) and a G-protein α-subunit in *fgd* (Frigid) A (25). The number of characterized mutants seems to be insufficient to clarify the complete chemotactic pathway. Furthermore, most mutants are defective for chemotaxis to either cAMP or folic acid, but not to both. Therefore, we tried to isolate new chemotactic mutants which are defective in the common chemotactic pathway.

Here we report on the isolation of ten mutants that show no chemotaxis to either folic acid or cAMP, using a simple assay to measure chemotaxis. The genetic and biochemical analyses of these non-chemotactic mutants show that most mutants are defective in the regulation of the second messengers, notably cGMP.

Materials and Methods

Materials

Nitrosoguanidine and folic acid were obtained from NAKARAI Chemical. Cycloheximide, thiabendazole, streptomycin, were from Sigma Chem. Co. (St. Louis, MO). cAMP, cGMP, and cyclic 2'-deoxyadenosine 3',5'-monophosphate (dcAMP) were from Boehringer Mannheim Corp., Germany). Polycarbonate filters were from Nucleopore (Costar, Badhoevedorp, The Netherlands).

Strains and Culture Condition

KI mutants, isolated as described below, and the parental strain XP55 (33) were grown on 1/3 SM plate (0.3% glucose, 0.3% bactopeptone, 1.5% agar, and 40 mM KH_2PO_4/Na_2HPO_4, pH 6.0) with *Klebsiella aerogenes*. Cells were harvested in the late logarithmic phase with 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (PB). Bacteria were removed by repeated centrifugations at 300 *g* for 3 min. Then cells were starved for 1 h (for vegetative cells) and 5 h (for starved cells) by shaking in PB at a density of 10^7 cells/ml at 21°C.

Isolation of KI Mutants

Vegetative XP55 cells were shaken at a density of 10^7 cells/ml for 30 min at 21°C in PB containing 500 μg/ml nitrosoguanidine. Subsequently, they were washed three times with PB, shaken for 1 d in PB, and inoculated on 5 LP plates (0.5% lactose, 0.5% peptone, and 1.5% agar) with *E. coli* B/r at 25 cells per 9-cm plate (18). Several days later, aggregateless clones were isolated, and chemotactic activities were measured with the agar cutting method as described below.

Chemotaxis Assay

Two methods were used in this study to assay chemotaxis. The first one is named the agar cutting assay which we developed from the idea of Bonner's cellophane square test (5). This chemotaxis assay was used for the rapid isolation of non-chemotactic mutants from aggregateless mutants. The small population assay was also performed on the isolated non-chemotactic mutants to measure chemotactic activities in a very sensitive and semi-quantitative way (24).

The agar cutting assay: each aggregateless mutant was inoculated as a spot in the center of a 5 LP agar plate on a lawn of *E. coli* B/r. After a few days of incubation at 21°C, a 4-cm colony was formed, which contained both vegetative cells in the outer regions and starved cells in center regions (see Fig. 1). An agar block of 0.5-cm width and 2-cm length was excised and put up-side-down on the surface of a 1% purified agar plate containing 100 μM folic acid or 1 μM cAMP. Due to secreted folic acid deaminase (36) from vegetative cells and cAMP phosphodiesterase (29) from starved cells, folic acid, and cAMP are degraded under the agar block thus giving rise to a gradient of folic acid and cAMP. This positive gradient of the concentration from the agar block leads to chemotactic movement outwards from the agar block. After 1 h of incubation, the dispersion of the mutants was compared with that of the parental wild type (see Fig. 2). Only very poorly dispersing mutants were picked up as potential non-chemotactic mutants (KI mutants 1-10).

Subsequently, these chemotactic mutants were analyzed with the small population assay for their chemotactic activities toward different dilutions of folic acid, cAMP, pterin, yeast extract, human urine, and bacteria (24). The concentration of chemoattractant was determined that induced a positive response in ~50% of the populations.

Preparation of Chemoattractant Sources

Yeast extract and human urine for the small population assay were prepared as follows: an equal volume of ethanol was added to human urine and a solution of yeast extract (Becton Dickinson Microbiology Systems, Cockeysville, MD; 0.4 g/ml in distilled water). Samples were incubated for 1.5 h at 6°C, followed by centrifugation at 1,000 *g* for 15 min. The supernatants were dried at 60°C and resuspended in 12 ml distilled water (yeast extract) or 7 ml distilled water (human urine). Bacterial secretion products were isolated by suspending one loop of *E. coli* bacteria in 0.3 ml distilled water. After incubation for 1.5 h at 37°C, the sample was centrifugated at 16,000 *g* for 5 min and the supernatant was used for the small population assay.

Parasexual Genetic Analysis

Standard techniques were used to construct diploids by fusion of tester strain X2 with XP55 or KI mutants (33). X2 carries a growth temperature sensitivity recessive *tsg*$^-$ mutation on linkage group III, and XP55 and KI mutants carry a growth sensitivity recessive *bsg*$^-$ mutation on linkage group III which prevents growth on *Bacillus subtilis* 36.1 (streptomycin resistant strain). Diploidization was performed by shaking two strains in Bonner's salt solution (10 mM NaCl, 10 mM KCl and 3 mM $CaCl_2$) for 20 h. Diploids were selected by growth at 27°C with *Bacillus subtilis* 36.1 on 1/3 SM plates containing 50 μg/ml streptomycin. The phenotype of the diploids was observed.

Segregation of chromosome to form haploids was induced by growing the diploids on 1/3 SM plates containing 2 μg/ml thiabendazole (54). As XP55 and KI mutants have a cycloheximide resistant recessive marker on linkage group I, the segregants were isolated on 1/3 SM plates containing 500 μg/ml cycloheximide to prevent growth of the diploids. Subsequently, the chemotactic activities of the segregants were examined by the agar cutting method.

For the complementation test, non-chemotactic segregants containing the growth temperature sensitive marker from X2 were isolated. These cells were fused to all original KI mutants and the phenotype of the diploid was inspected.

Determination of cAMP, IP_3 and cGMP

After being washed three times, vegetative or starved cells were resuspended at a density of 10^8 cells/ml in PB and the suspension was aerated

for 10 min at 21°C. Subsequently, the cell suspensions were stimulated with folic acid, cAMP, or dcAMP at the concentrations indicated in the figures and legends. The reactions were terminated at the indicated time by adding an equal volume of 3.5% perchloric acid. After neutralization of the cell lysates with half volume of 50% $KHCO_3$, the contents of cAMP, IP_3, and cGMP were measured by the appropriate isotope dilution assays (44, 45, 46).

In Vitro Guanylyl Cyclase Assay

Guanylyl cyclase activity was measured as described previously with a few modifications (19, 21). Briefly, cells were starved for 5 h, washed, and resuspended at a density of 10^8 cells/ml in lysis buffer (40 mM Hepes/NaOH, 6 mM $MgSO_4$, 5.9 mM EGTA and 0.1 mM guanosine 5'-[γ-thio]trisphosphate (GTPγS), pH 7.0). Cells were lysed by forced filtration through polycarbonate filter (pore size 3 μm), and the cell lysate was added after 30 s to an equal volume of reaction mixture (10 mM dithiothreitol, 0.6 mM GTP). The reaction was terminated at 50, 70, and 90 s after lysis by addition of an equal volume of 3.5% perchloric acid, and the cGMP content was measured as described above.

cGMP Phosphodiesterase Activity

Cells were starved for 5 h, washed three times in AC buffer without sucrose (AC buffer contains 40 mM Hepes/NaOH, pH 7.0, 250 mM sucrose and 0.5 mM EDTA), and resuspended at a density of 2×10^8 cells/ml in the AC buffer. Cells were homogenized at 0°C by forced filtration through a polycarbonate filter, the homogenate was centrifuged at 0°C for 1 h at 48,000 *g* and the supernatant was used for the assay. cGMP specific phosphodiesterase activity was measured with a modified method previously described (51). The incubation was started at 21°C by adding 100 μl of the enzyme preparation to an equal volume of cGPDE mixture (AC buffer containing 6.0 mM $MgCl_2$, 5 mM dithiothreitol, 40 μM cAMP and 10 nM [8-^{3}H] cGMP with or without 20 nM 8-Br cGMP). After 15 min, the reaction was terminated by boiling for 2 min. The mixture was cooled down to 21°C and incubated for 30 min with 100 μl of alkaline phosphatase (10 μg/ml). To remove the remaining [8-^{3}H] cGMP, 300 μl of anion-exchanger AG-1-X_2 (50% slurry in water) was added. After shaking for 1 min, the samples were centrifuged at 16,000 *g* for 1 min and the radioactivity of 300 μl of the supernatant was measured.

Results

Isolation of the Non-Chemotactic Mutants

A new assay for chemotaxis was developed to screen the chemotactic activity of a large number of mutant strains. This agar cutting assay is described in Fig. 1. From a *Dictyostelium*/bacteria growth plate, a block of agar was cut at the edge of the *Dictyostelium* growth front. This block contains bacteria, growing *Dictyostelium* cells and starving *Dictyostelium* cells. The agar block was put upside down on an agar plate containing 10^{-4} M folic acid or 10^{-6} M cAMP. Positive chemotactic outwards dispersion in the parental strain was observed on folic acid containing agar around the border area between bacteria and cells (Fig. 2). The same dispersion was seen around the starved area on cAMP containing agar. Using this procedure, mutants with reduced locomotion are not likely to be isolated, because they make only very small colonies that are not large enough for the agar cutting assay. This assay might be very useful to isolate chemotactic mutants from a large collection, because the method is simple, fast, and requires minimal manipulation of cells. The aim of this study was to isolate chemotactic mutants that do not respond to either cAMP or folic acid. Since chemotaxis to cAMP is essential for cell aggregation, aggregateless mutants were first isolated, and subsequently characterized for chemotactic activity.

To obtain aggregateless clones, cells were mutagenized

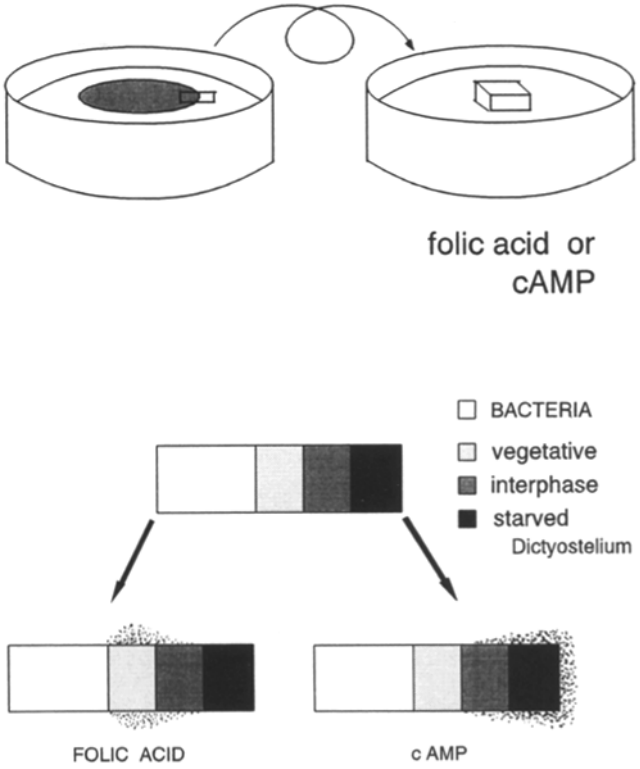

Figure 1. A schematic drawing of the agar cutting chemotactic assay method. Each tested strain was inoculated on an agar plate with *E. coli* B/r. After incubation at 21°C until the cells made a large colony, a 0.5-cm wide and 2-cm long agar block was cut off from the edge which contained starved and vegetative cells. The agar block was placed on folic acid or cAMP containing agar and the dispersion of the amoebae from the agar block was observed. The square represents an agar block which contains the bacteria and the cells in the vegetative and starved stage. The upper square represents the agar block just after cutting and the lower squares after 1 h of incubation on agar containing 10^{-4} M folic acid (*left*) or 10^{-6} M cAMP (*right*). A small dot outside of the block represents a cell.

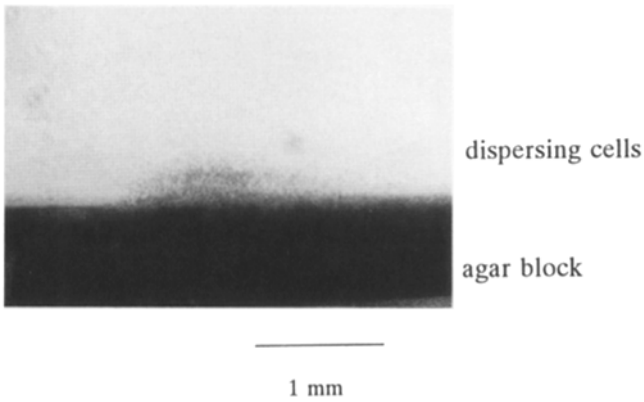

Figure 2. A photograph of chemotaxis of XP55 cells on agar containing folic acid. The agar block was placed on folic acid containing agar as described in Fig. 1. The photograph was made after 1 h of incubation. Bar, 1 mm.

with nitrosoguanidine at 5–10% survival rate. From ~10,000 clones, ~400 clones were isolated as putative aggregateless mutants, but only 243 clones had a stable aggregateless phenotype. Then these 243 aggregateless mutants were analyzed for their chemotactic activities to folic acid and cAMP using the agar cutting method. This gave 23 mutants without chemotaxis to folic acid and 51 mutants without chemotaxis to cAMP. Among these chemotactic mutants, 10 mutants (KI 1-10 mutants) were isolated as potential non-chemotactic mutants because they did not respond to either folic acid or cAMP. These ten mutants are the subject of this study.

It is possible that these aggregateless mutants develop more slowly so that it takes a longer time to acquire chemotactic sensitivity to cAMP. Therefore, a much longer agar block, which also contained cells starved for a longer period, was cut off and chemotaxis to cAMP was measured. No positive chemotaxis was seen in any KI mutant.

Detailed Chemotaxis of KI Mutants

With the agar cutting method, a positive chemotactic response will not be observed when cells lack folic acid or cAMP degrading enzymes, because this assay depends on the gradient of folic acid or cAMP that is formed by these enzyme activities. Therefore, the chemotactic abilities of the mutants were examined with the small population assay using various concentrations of folic acid and cAMP. This assay does not depend on degrading enzyme activities. All KI mutants showed reduced chemotactic activities to folic acid and cAMP. KI-1 has essentially no chemotaxis to folic acid and 1,000-fold reduced activity to cAMP, compared to wild type. On the other hand, KI-3 has weak but clear positive chemotaxis to folic acid (10-fold reduced) and cAMP (1,000-fold reduced). All other mutants showed no chemotaxis at any concentration of either folic acid or cAMP, indicating that the chemotaxis sensitivity is at least 1,000-fold reduced (Table I). These results indicate that the agar cutting assay worked well as a rapid screening method of non-chemotactic mutants.

Dictyostelium cells not only react chemotactically to folic acid and cAMP, but also to other identified compounds such as pterin, and possibly to unidentified compounds secreted from bacteria or present complex mixtures such as yeast extract and human urine. Especially, these broad-spectrum sources of chemoattractants may allow chemotactic null mutants to be detected (Table I). KI-2, 4, 5, 6, 7, 8, and 9 were shown to be perfectly chemotactic null mutants, since these cells did not respond to any chemotactic source. KI-3 shows weak chemotactic activity to yeast extract, bacteria, and urine, confirming the weak chemotaxis of this mutant to folic acid, cAMP, and pterin. Interestingly, mutant KI-10 has clear chemotaxis to yeast extract (10-fold reduced, if compared to wild type), human urine (10-fold reduced), and bacteria (100-fold reduced) but no chemotaxis to folic acid, cAMP, and pterin. This observation indicates that there exists another chemoattractant besides folic acid, cAMP, and pterin.

Parasexual Genetic Analyses

First, diploid strains were made from KI mutants and the tester strain X2. All the diploids from KI mutants showed a nearly wild-type phenotype, except KI-10. The diploid from KI-10 did not aggregate and did not show any chemotaxis to cAMP or folic acid, indicating that the mutation is dominant. The diploids from KI-3 and KI-8 were perfectly normal in their morphogenesis. The others made perfect fruiting bodies, but did not show clear streams during cell aggregation. Nevertheless, all these other diploids showed positive chemotaxis to both folic acid and cAMP using the agar cutting method, indicating that the mutations are recessive. The diploid of X2/KI-2 could not be obtained from 30 experiments.

Subsequently, haploid segregants were isolated from the diploid strains (Table II). The rationale of the genetic markers is that the cycloheximide recessive resistant marker (*cyc* A on linkage group I) and *Bacillus subtilis* growth recessive marker (*bsg* A on linkage group III) are from KI mutants,

Table I. Chemotactic Activity of KI Mutants

	Chemotaxis towards					
Strain	folic acid	cAMP	pterin	yeast extract	bacteria	urine
XP55	+++	++++	++	+++	+++	+++
KI-1	–	+	–	–	–	–
KI-2	–	–	–	–	–	–
KI-3	++	+	+	+	+	+
KI-4	–	–	–	–	–	–
KI-5	–	–	–	–	–	–
KI-6	–	–	–	–	–	–
KI-7	–	–	–	–	–	–
KI-8	–	–	–	–	–	–
KI-9	–	–	–	–	–	–
KI-10	–	–	–	++	++	+

Chemotactic activities to different concentrations of the chemoattractants were measured with the small population assay. Symbols: –, no chemotaxis. +, positive chemotaxis only at concentrated test solution (10^{-4}M folic acid, 10^{-6}M cAMP, 10^{-4}M pterin). ++, positive chemotaxis at 10-fold diluted test solution. +++, at 100-fold. ++++ at 1,000-fold diluted.

Table II. Genetic Analysis and Linkage Group Assignment

	Phenotype of segregants					
Strain	che^+ts^+	che^+ts^-	che^-ts^+	che^-ts^-	axenic	linkage group
KI-1	0	0	28	18	–	I
KI-2	ND	ND	ND	ND	ND	ND
KI-3	2	15	10	3	+	not I,II,III
KI-4	0	0	28	25	–	I
KI-5	8	7	8	9	–	not I,III
KI-6	26	15	17	2	–	not I,III
KI-7	4	2	26	4	+	not I,II,III
KI-8	11	30	13	6	+	not I,II,III
KI-9	0	0	31	21	–	I
KI-10	0	0	18	22	+	I

KI mutants (che^-ts^+) were fused to the temperature sensitive tester strain X2 (che^+ts^-). KI mutants possess the recessive cyclohexamide recessive gene on linkage group I. Segregants were isolated on cyclohexamide-containing plates, and thus all possess linkage group I from KI mutants. Phenotypes of ~50 segregants were inspected (che^-, defective chemotaxis; ts^-, temperature sensitive for growth; axenic, growth in axenic medium). This information was used to deduce the location of the mutation on a specific linkage group. ND represents not determined.

whereas the temperature sensitive recessive marker (*tsg* A on linkage group III) and the axenic markers (*axe* A on linkage group II and *axe* B on linkage group III) are from the tester strain X2. Thus, haploid segregants were isolated in the presence of cycloheximide. Since the recessive cycloheximide resistant marker is located on linkage group I from KI mutants, all segregants possess this linkage group from KI mutants and none from the tester strain X2; the other linkage groups are expected to segregate at random from KI mutants and X2. It was observed that all segregants from KI-1, 4, 9, and 10 were aggregateless, indicating that their mutations are on linkage group I. Segregation in the other diploid strains resulted in both wild-type and mutant phenotypes, indicating that the mutations are not on linkage group I. A few of the segregants from KI-3, 7, 8, and 10 were axenic, indicating that the mutant loci of these strains are not on linkage group II and III, because the axenic phenotype needs two recessive mutations on *axe* A and *axe* B on linkage group II and III, respectively.

Ten aggregateless segregants were chosen at random for each strain and tested for chemotaxis towards folic acid and cAMP using the agar cutting method. None of the segregants showed chemotaxis towards either folic acid nor cAMP. This provides evidence that none of the KI mutant are double mutants, one mutation affecting folic acid and another affecting cAMP chemotaxis.

Segregants which were temperature sensitive for growth could be isolated from all mutants. As this *tsg* A marker is derived from the tester strain X2, this observation implies that none of the mutation loci is on linkage group III. The temperature sensitive non-chemotactic segregants were subsequently fused to the original KI mutants, and the phenotype of the diploids analyzed. The diploids from KI-10 with all other KI mutants showed the non-chemotactic phenotype (aggregateless). This is consistent with the notion that KI-10 is a dominant mutant. Two diploids, KI-2/KI-7, and KI-4/KI-9, had weak aggregation but showed good chemotactic activities towards folic acid and cAMP in the small population assay. On the other hand, all other diploids made normal fruiting bodies. This suggests that the nine recessive KI mutants all belong to different complementation groups.

cAMP Response Induced by cAMP

Extracellular cAMP induces the activation of adenylyl cyclase (cAMP relay), which is known to be important to relay the chemotactic activity from cell to cell (9). However, the analysis of mutant *synag* 7, which is defective in the activation of adenylyl cyclase, indicates that cAMP relay and chemotaxis are largely independent responses (16). The examination of the cAMP-mediated cAMP response in KI mutants supports this notion (Fig. 3). All KI mutants except KI-7 showed a clear cAMP accumulation induced by 10^{-5}M dcAMP with similar magnitude and kinetics as that of the parental strain (~3 pmol cAMP/10^7 cells as a basal level and 10–30 pmol cAMP/10^7 cells as a maximal level at 1–2 min). This indicates that these KI mutants produce cAMP receptors and can develop at least to some extent upon starvation. Binding assays for surface receptors revealed that all mutants possess cAMP receptors, although the levels were low in mutants KI-3 and 4 (data not shown). KI-7 did not make any cAMP in response to dcAMP, although this mutant had substantial cAMP-binding activity.

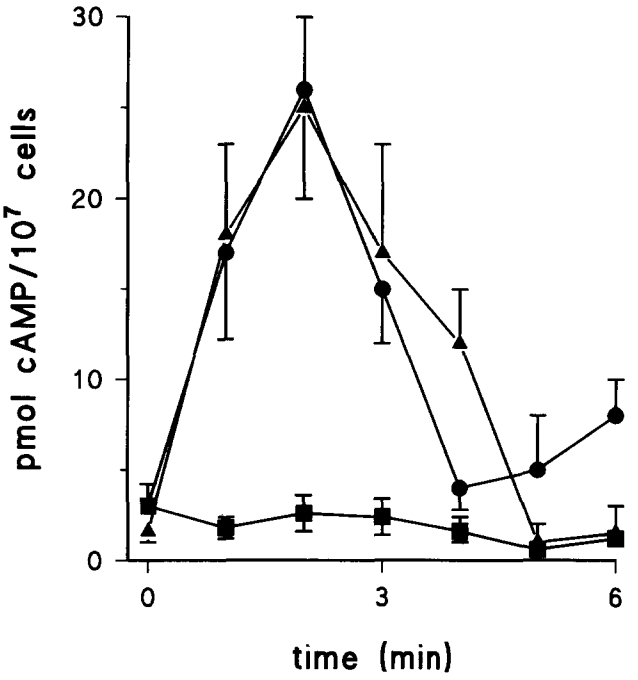

Figure 3. cAMP response. XP55 (●), KI-10 (▲), and KI-7 (■) cells were stimulated with 10^{-5} M dcAMP. Samples were quenched at the indicated time and cAMP levels were determined. The results of the other mutants are not significantly different from that in XP55 and KI-10. The results shown are the mean ± SD of two experiments with triplicate determinations.

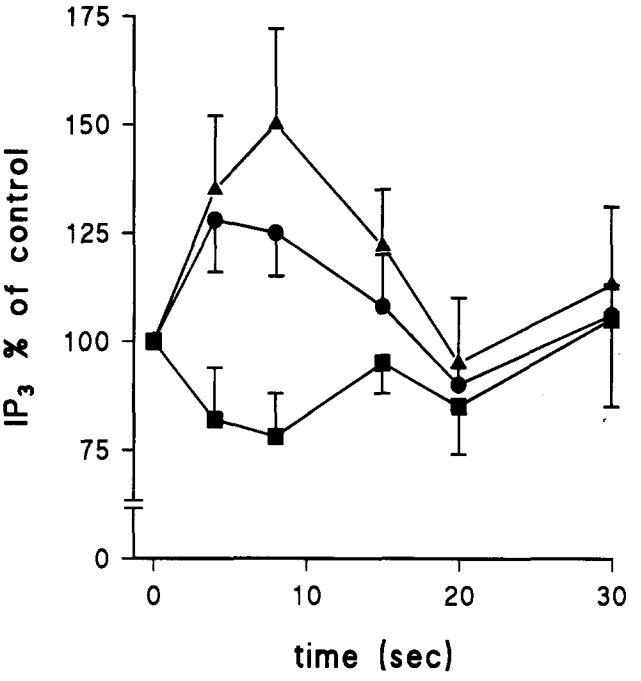

Figure 4. IP_3 response. XP55 (●), KI-10 (▲), and KI-6 (■) cells were stimulated with 10^{-7} M cAMP. The results of KI-3 and KI-7 were similar to that of KI-6. The results of the other mutants are not significantly different from that of XP55 and KI-10. The results shown are the mean ± SD of two experiments with triplicate determinations.

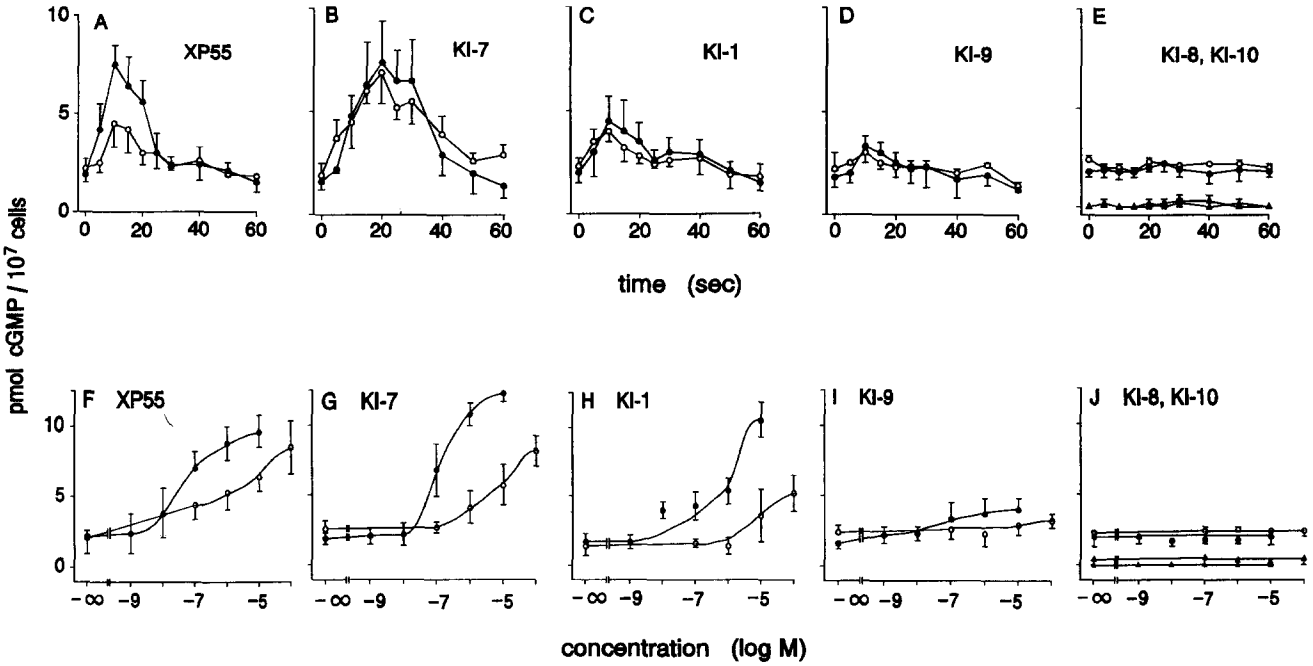

Figure 5. cGMP response. Time course of cGMP accumulation (*A–E*) of XP55 and KI mutants after stimulation with 10^{-5} M folic acid in the vegetative stage (○ or △) or 10^{-7} M cAMP in the starved stage (● or ▲). In *E*, the triangle represents KI-8 and the circle KI-10. Dose response curves of cGMP response were measured at 10 s (*F*, *H*, *I*, and *J*) and 20 s (*G*) after stimulation with folic acid (○) or cAMP (●). The results are shown as the mean ± SD of two experiments with triplicate determinations. The responses of the other mutants were KI-3 as XP55; KI-2 as KI-7; KI-4 and KI-5 as KI-1; and KI-6 as KI-9.

IP_3 Responses of KI Mutants

A transient increase of IP_3 via cAMP receptor, G protein and phospholipase C is thought to be one of the responses involved in the chemotactic movement (3, 14, 15). This is a rapid response in wild-type cells given by a small but significant increase of IP_3 on a high basal level (14, 15, 45, 47). The basal amount of IP_3 was similar between KI mutants and wild type (Fig. 4). The responses in KI-1, 2, 4, 5, 8, 9, and 10 were positive upon stimulation with cAMP. On the other hand, IP_3 levels in KI-3, 6, and 7 were reduced in response to cAMP.

cGMP Responses of KI Mutants

The rapid intracellular accumulation of cGMP, induced by the chemoattractants folic acid and cAMP, may play an important role during chemotactic movement. Indeed, two KI mutants were found to lack a cGMP response (KI-8 and KI-10; Fig. 5 *E*), two mutants had a delayed response (KI-2 and KI-7; Fig. 5 *B*), and five mutants had a strongly reduced response (KI-1, 4, 5, 6, and 9; Fig. 5, *C* and *D*). Only KI-3 showed a relatively normal cGMP response (c.f. Fig. 5 *A*).

Mutants KI-8 and KI-10 did not respond by increasing cGMP levels to stimulation with cAMP (Fig. 5 *E*). Whereas mutant KI-10 has relatively normal basal levels of 2.2 pmol/10^7 cells, mutant KI-8 appears to have no detectable cGMP (below ~0.5 pmol/10^7 cells) (Fig. 5, *E* and *J*). Even when higher concentrations of folic acid and cAMP were added, these strains did not show any stimulation of cGMP production. These results suggest that mutant KI-8 has a defect in cGMP synthesis by guanylyl cyclase, and that this enzyme is present in mutant KI-10, but cannot be activated by the receptor.

Mutants KI-2 and KI-7 showed a delayed cGMP response to both folic acid and cAMP stimuli. Their responses reached maximal levels at 20 s after stimulation and basal levels were recovered after 60 s (Fig. 5 *B*). This delayed response suggests that these KI-2 and KI-7 mutants have an abnormal activation or adaptation of guanylyl cyclase or reduced activity of cGMP specific phosphodiesterase (see below).

KI-1, 4, 5, 6, and 9 showed weak cGMP responses to stimuli that are saturating for wild-type cells (10^{-7}M cAMP and 10^{-5}M folic acid). The cGMP response was measured in these mutants using various concentrations of folic acid and cAMP. In mutant KI-1, 4, and 5, the maximal cGMP response was relatively large, but required about 40-fold (KI-1), 20-fold (KI-4), and 10-fold (KI-5) higher concentrations of folic acid and cAMP than in wild-type cells (c.f. Fig. 5 *C*). Mutant KI-6 and 9 showed very weak cGMP responses, even to very high stimulus concentrations (Fig. 5, *E–D*; *J–I*).

In Vitro Guanylyl Cyclase Activity

In a cell free system, *Dictyostelium* guanylyl cyclase activity is found in the membrane fraction, and is stimulated by GTPγS and inhibited by submicromolar concentrations of calcium ions (19, 21).

Guanylyl cyclase activity was measured with starved cells

Table III. In Vitro Guanylyl Cyclase and cGMP Phosphodiesterase Activity

Strain	Guanylyl cyclase activity			cGMP phosphodiesterase activity	
	without Ca^{2+}	with 10 nM Ca^{2+}	with 1 μM Ca^{2+}	without 8-Br-cGMP	with 8-Br-cGMP
	% of XP55 without Ca^{2+}			% of XP55 without 8-Br-cGMP	
XP55	100 ± 30	83 ± 23	0 ± 0	100 ± 16	127 ± 21
KI-1	95 ± 21	61 ± 20	0 ± 0	113 ± 13	177 ± 19
KI-2	92 ± 26	69 ± 19	2 ± 2	153 ± 16	186 ± 14
KI-3	119 ± 38	65 ± 15	7 ± 7	107 ± 8	209 ± 14
KI-4	60 ± 25	35 ± 10	2 ± 2	140 ± 8	169 ± 12
KI-5	49 ± 20	39 ± 11	1 ± 1	91 ± 9	123 ± 12
KI-6	81 ± 26	85 ± 93	2 ± 2	85 ± 7	107 ± 8
KI-7	61 ± 23	36 ± 5	2 ± 2	90 ± 11	107 ± 8
KI-8	6 ± 2	4 ± 1	2 ± 2	124 ± 22	176 ± 13
KI-9	35 ± 15	29 ± 7	2 ± 2	111 ± 14	138 ± 7
KI-10	63 ± 19	49 ± 18	0 ± 0	83 ± 19	112 ± 13

Guanylyl cyclase activity in XP55 without Ca^{2+} is 135 pmol/min.mg protein (100%), and cGMP phosphodiesterase activity in XP55 without 8-Br-cGMP is 3.2 pmol/min.mg protein (100%). The results are shown as the mean ±SD of two experiments with triplicate determinations.

in the presence of GTPγS (Table III). The activities in most KI mutants were relatively normal, when compared to XP55. In all mutants 1 μM calcium ions inhibited the enzyme by more than 90%, as in wild type. Mutant KI-10 had relatively normal guanylyl cyclase activity, although chemoattractants did not induce the cGMP response in vivo. This suggests that KI-10 has a defect on the common pathway to the activation of guanylyl cyclase in response to folic acid and cAMP, but not guanylyl cyclase itself. KI-9 and especially KI-8 have little guanylyl cyclase activity (~35% and 6% of wild type, respectively). Although the activity in KI-8 is very small, it is still inhibited by calcium ions as in XP55, suggesting that the enzyme exists in KI-8 with very low activity.

cGMP Specific Phosphodiesterase Activities in KI Mutants

cGMP specific phosphodiesterase plays a major role in degrading cGMP to 5'GMP (49). This enzyme is activated by cGMP itself and more strongly by 8-Br-cGMP (51). Basal enzyme activity and 8-Br-cGMP-stimulated activity were measured in KI mutants (Table III). In all KI mutants, this enzyme activity was essentially normal. This indicates that low production of cGMP in KI-8 and KI-10 is not due to increased activity of this phosphodiesterase, and the delayed cGMP response in KI-2 and KI-7 is not due to reduced activity of this enzyme.

Discussion

In this paper, we report on the isolation of non-chemotactic mutants using a simple chemotactic assay system, and their genetic and biochemical characterization. *Dictyostelium discoideum* cells are sensitive to different chemoattractants including folic acid and cAMP. It is known that these chemoattractants are detected by different surface receptors and G proteins. Previous isolation procedures for chemotactic mutants used chemotaxis to either folic acid (40) or cAMP (7) as the selection method. Most mutants isolated were defective in the detection of folic acid, or the development of the cAMP-signal transduction system upon starvation. Thus, these mutants showed chemotactic defects to either folic acid or cAMP, but not to both chemoattractants. The idea for the present isolation of non-chemotactic mutants is based on previous observations that folic acid and cAMP chemotactic signal transduction pathways merge at or before guanylyl cyclase (43). Based on this assumption, mutants were isolated that are insensitive to both folic acid and cAMP.

In ~10,000 mutagenized clones, 243 clones were aggregateless of which 51 clones were defective in chemotaxis to cAMP and 23 clones defective in that to folic acid. Ten clones were defective in both folic acid and cAMP chemotaxis, although detailed analysis revealed that one mutant (KI-3) showed weak chemotaxis to all chemoattractants. We were concerned about the possibility that the nine non-chemotactic mutants could have independent mutations in the separate cAMP and folic acid signal transduction pathways. Calculations and experimental observations suggest that such double mutants have not been isolated. The probability of finding a cAMP non-chemotactic mutant is 51/10,000. We assume that the folic acid chemotaxis pathway has the same sensitivity to mutagenesis (which is probably overestimated, because cells do not have to develop in order to acquire sensitivity to folic acid). Then, the probability to find a double mutant is $(51/10{,}000)^2$. With 10,000 clones inspected, this would yield 0.26 mutants. The experiments show that nine of the KI mutants do not react chemotactically to pterin, which is detected by a receptor different from those of folic acid and cAMP. Furthermore, analysis of the chemotactic activity of 10 independent segregants from each diploid always produced impaired chemotaxis to both folic acid and cAMP. These combined data strongly suggest that the chemotactic defects of each mutant is due to a single mutation in the part of the signal transduction pathway that is shared by different chemoattractants.

Genetic analysis of the non-chemotactic mutants revealed that KI-10 is dominant to the wild type. The diploid derivative of KI-10 showed the same phenotypes as KI-10, and had no cGMP response and no chemotaxis to folic acid and cAMP. This strain should be very useful for further investigation on the role of cGMP, because other recessive mutations will have no effect in the diploid. Complementation analyses on the remaining nine recessive KI mutants re-

vealed nine complementation groups. This large number of complementation groups indicates that mutagenesis of the genes leading to non-chemotactic mutants have not been saturated. We have been unable to isolate a diploid strain of KI-2 with X2. However, there were no problems in obtaining diploids of KI-2 and the *tsg* derivatives of all other KI mutants, which allowed us to assign KI-2 as a recessive mutation forming a separate complementation group.

The chemotactic activity of the KI mutants was assayed again with the small population assay, which is more sensitive than the agar cutting assay and does not depend on secreted enzymes that degrade the chemoattractant. As a result, KI-1 and KI-3 were found to have reduced but still significant chemotaxis to folic acid and/or cAMP. Using the small population assay, we also examined chemotaxis to other identified (pterin) or possibly unidentified (yeast extract, urine, and bacteria) chemoattractants. It is reported that pterin binds to a receptor that is different from those for folic acid and cAMP (42, 48, 57). All mutants except KI-1, 3, and 10 were found to have no chemotaxis to any chemoattractant, i.e., they are chemotaxis null mutants. This suggests that these mutants are defective in the common chemotactic signal transduction pathways that is shared by all chemoattractants. The interesting observation was made that the dominant mutant KI-10 shows no chemotaxis to folic acid, cAMP, and pterin, but has reduced but definitely positive chemotaxis to yeast extract, urine, and bacteria. This implies that there must exist another chemoattractant whose detection and transduction are not affected by the dominant mutation of KI-10. Mutant KI-10 can be used for the identification of this chemoattractant.

Besides chemotaxis, several second messenger responses were investigated in the KI mutants. The cAMP-mediated activation of adenylyl cyclase (cAMP relay) was normal in all KI mutants, except in KI-7, indicating that chemotaxis is not essential for cAMP relay. Previous results revealed normal chemotaxis in mutants defective in adenylyl cyclase (*aca*$^-$) (37) or the activation of adenylyl cyclase (*synag* 7) (16). On the other hand, mutants lacking the surface cAMP receptor cAR1 or the G-protein Gα2 have diminished chemotaxis and relay (25, 41). These observations suggest that cAMP-induced chemotaxis and relay are largely independent responses and may only share the surface receptor and G-protein.

Positive or negative IP_3 responses were observed in KI mutants. This negative IP_3 response was also observed in *fgd* C mutant which has reduced chemotactic activity to cAMP (3). Recently, a cDNA encoding phospholipase C has been identified in *Dictyostelium* (11). Over-expression of phospholipase C resulted in a threefold increase of cellular IP_3 levels without any effect on chemotaxis (11). Furthermore, cells with a disruption of the phospholipase C gene show normal chemotaxis to cAMP (Drayer, A. L., J. Van der Kaay, G. W. Mayr, and P. J. M. Van Haastert, manuscript in preparation). This means that the IP_3 response is not crucial for chemotaxis. We conclude that the defects of IP_3 production are not the cause for the chemotactic defects of KI mutants.

cGMP is thought to play a role in chemotaxis, due to the analyses of *stm* F mutant, which lacks a cGMP specific phosphodiesterase activity. This mutant shows prolonged cGMP production in response to cAMP stimuli, and at the same time prolonged chemotaxis and longer streams during aggregation (38, 39). Many KI mutants are defective in the folic acid and cAMP induced production of cGMP. KI-8 and KI-10 showed no cGMP response. Specifically, basal cGMP levels are below the detection limit in KI-8 and guanylyl cyclase is reduced ~16-fold. The small amount of enzyme activity present is still stimulated by GTPγS and inhibited by calcium ions, suggesting that KI-8 may have strongly reduced levels of otherwise normal guanylyl cyclase activity. Mutant KI-10 has no receptor-stimulated cGMP formation, but basal cGMP levels in vivo and guanylyl cyclase activity in vitro are essentially identical to those of wild-type cells, suggesting that KI-10 is defective before guanylyl cyclase in the chemotactic pathway.

cGMP responses of KI-2 and KI-7 are similar to each other with a cGMP peak at 20 s after stimulation with folic acid or cAMP, and a recovery of basal levels after 60 s; in wild-type cells the peak and basal levels are obtained after 10 and 30 s, respectively. This delayed cGMP response could be explained by several hypotheses. First, cGMP phosphodiesterase activities may be very low in these mutants. However, the measured cGMP specific phosphodiesterase activities in all KI mutants were found to be normal, including KI-2 and KI-7. In relation to this hypothesis, mutant *stm* F, which lacks cGMP-specific phosphodiesterase, still shows positive chemotaxis to folic acid and cAMP. A second hypothesis for a delayed cGMP response is a prolonged activation and/or defective adaptation of guanylyl cyclase in these mutants.

In some KI mutants, KI-1, 4, 5, 6, and 9, the cGMP responses to folic acid and cAMP were reduced. In KI-1, 4 and 5, the dose response curves of cGMP productions are shifted to at least 10-fold higher cAMP or folic acid concentrations. In KI-6 and KI-9 the maximal amount of cGMP was only 20–30% as that in wild-type cells. This could be the reason for non-chemotaxis of these strains, if the production of cGMP is not sufficient for the activation of chemotaxis.

One of the functions of cGMP in chemotaxis is thought to be the regulation of actin-myosin interaction. Myosin heavy chain II is polymerized transiently by folic acid and cAMP stimulation (26). Furthermore, phosphorylation of myosin II occurs also transiently after stimulation with cAMP (2, 27). Recently, it was reported that cAMP stimulated phosphorylation of myosin II plays a crucial role for its polymerization (58). In *stm* F mutant, both myosin II polymerization and phosphorylation were prolonged like the cGMP response in this mutant (26, 27). This suggests that the phosphorylation and subsequent regulation of polymerization of myosin II are regulated by cGMP, possibly via cGMP dependent protein kinase (53). In this respect, KI-8 and KI-10 as well as KI-2 and KI-7 are good tools to firmly establish this function of cGMP. Previous observations have shown that myosin II null mutants have chemotaxis to cAMP (8), indicating that myosin II is not essential for chemotaxis. Therefore, the regulations of other myosins, myosin heavy chain I and myosin light chains, by cGMP also have to be examined in KI-8 as well as in wild-type cells.

It has been supposed that cGMP may also participate in the regulation of development by cAMP. Mutants KI-8 and KI-10 are useful tools to address the function of intracellular

cGMP during cAMP-induced gene expression. Furthermore, these mutants may establish the function of cAMP chemotaxis in the multicellular stage.

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