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Regulation of Guanylyl Cyclase by a cGMP-binding Protein during Chemotaxis in *Dictyostelium discoideum**

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Chemoattractants transiently activate guanylyl cyclase in *Dictyostelium discoideum* cells. Mutant analysis demonstrates that the produced cGMP plays an essential role in chemotactic signal transduction, controlling the actomyosin-dependent motive force. Guanylyl cyclase activity is associated with the particulate fraction of a cell homogenate. The addition of the cytosol stimulates guanylyl cyclase activity, whereas the cytosol plus ATP/Mg^{2+} inhibits enzyme activity. We have analyzed the regulation of guanylyl cyclase in chemotactic mutants and present evidence that a cGMP-binding protein mediates both stimulation and ATP-dependent inhibition of guanylyl cyclase.

Upon chromatography of cytosolic proteins, cGMP binding activity co-elutes with both guanylyl cyclase-stimulating and ATP-dependent-inhibiting activities. In addition, ATP-dependent inhibition of guanylyl cyclase activity is enhanced by the cGMP analogue 8-Br-cGMP, suggesting that a cGMP-binding protein regulates guanylyl cyclase activity. Mutant KI-4 has an aberrant cGMP binding activity with very low K_d and shows a very small chemoattractant-mediated cGMP response; the cytosol from this mutant does not stimulate guanylyl cyclase. In contrast to KI-4, the aberrant cGMP binding activity of mutant KI-7 has a very high K_d and chemoattractants induce a prolonged cGMP response. The cytosol of this mutant stimulates guanylyl cyclase activity, but ATP does not inhibit the enzyme. Thus, two previously isolated chemotactic mutants are defective in the activation and inhibition of guanylyl cyclase, respectively. The positive and negative regulation of guanylyl cyclase by its product cGMP may well explain how cells process the temporospatial information of chemotactic signals, which is necessary for sensing the direction of the chemoattractant.

In a number of eukaryotic amoeboid cells, chemotaxis plays an important role in coordinating cell movement (for review, see Ref. 1). This simple behavior appears to require a precise signaling system that transmits the temporospatial gradient of chemoattractant concentrations to the localized formation of pseudopodia. Only a 2% difference of chemoattractant concentration between both ends of a *Dictyostelium* cell is sufficient to elicit chemotaxis (2). To explore the molecular mechanisms of chemotaxis in *Dictyostelium*, both biochemical and genetic approaches have been used (3).

Dictyostelium grows as amoeboid unicellular cells. On starvation, cells start to aggregate by means of chemotactic movement toward cAMP. The chemoattractant cAMP is periodically secreted at ~5-min intervals by cells from central aggregating regions. Extracellular cAMP is detected by G-protein-coupled surface receptors, the binding of which leads to the transient activation of adenylyl cyclase and guanylyl cyclase (for review, see Ref. 3). The major role of adenylyl cyclase in chemotaxis is the propagation of the extracellular cAMP signal through a population of aggregating cells. Therefore, it is not surprising that a mutant with a disruption of the adenylyl cyclase gene shows normal chemotaxis to applied cAMP (4). On the other hand, many experiments suggest that activation of guanylyl cyclase is essential for chemotactic signal transduction. The activation of this enzyme is relatively rapid, resulting in a 7–10-fold increase of intracellular cGMP at 10 s after the addition of chemoattractant to sensitive cells (5–7). This rapid increase of cGMP appears to be important for chemotactic movement. Mutants have been isolated that do not respond to the chemoattractants cAMP and folic acid. Since a cell detects these chemoattractants using different surface receptors, it was expected that the mutations affect components of the signal transduction cascade that are shared by different chemoattractants. Out of nine of these KI mutants, two mutants were found that show no cGMP response; mutant KI-8 has no basal guanylyl cyclase activity, whereas mutant KI-10 has basal guanylyl cyclase activity that is not stimulated by chemoattractants (8). Furthermore, characterization of mutants with a defective cGMP phosphodiesterase, designated as *stm*F, also suggests the importance of the cGMP for the processing of chemotactic signals (9–11).

The actomyosin complex may function as a central system regulating locomotion during chemotaxis (for review, see Ref. 12). In actomyosin-dependent cell locomotion, assembly of conventional myosin into filaments is required for the effective interaction with actin fibers, thereby providing optimal motile force on the plasma membrane (13, 14). The assembly of conventional myosin can be inhibited by phosphorylation of three threonines in the tail region (15, 16), and as a consequence, actin-stimulated ATPase activity decreases (17). Analyses of the non-chemotactic mutants KI-8 and KI-10 and of mutant *stm*F indicate that the second messenger cGMP mediates myosin phosphorylation. The receptor-stimulated phosphorylation of conventional myosin was abolished in KI-10 (18) and KI-8,[1] whereas phosphorylation was enhanced in *stm*F in accordance with its prolonged cGMP accumulation (19). Furthermore, osmotic stress has been shown to result in the activation of guanylyl cyclase and phosphorylation of conventional myosin (20). In mutant KI-8, both osmotic stress-mediated responses are absent, and the cell-permeable cGMP analogue, 8-Br-cGMP, restores osmotic stress-induced myosin phosphorylation in KI-8, indicating a connection between cGMP and

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[1] H. Kuwayama and P. J. M. Van Haastert, unpublished observations.

myosin phosphorylation (20). For chemotaxis, this could imply that the local regulation of myosin phosphorylation by cGMP could be a key mechanism to control the direction of motile force.

A cGMP-binding protein is expected to accept the cGMP signal leading to myosin phosphorylation since myosin has no cGMP binding activity (21). The cytosolic fraction of *Dictyostelium* lysates contains a cGMP-binding protein with high affinity and specificity (22–24). The binding protein exists in two conformations, one with relatively low affinity and fast dissociation of the ligand (F-form) and the other with high affinity and slow dissociation (S-form); DNA promotes the conversion of the F-form to the S-form (25). The role of this cGMP binding activity in chemotaxis has long been undetermined. Recently, however, biochemical analyses of nine non-chemotactic KI-mutants revealed that at least four mutants possess an aberrant cGMP binding activity, suggesting that this cGMP-binding protein participates in chemotaxis (26).

We found, unexpectedly, that these non-chemotactic mutants with altered cGMP binding activity also show aberrant cGMP accumulations in response to cAMP stimulation (8, 26). The cGMP-binding proteins in mutant KI-4 and KI-5 appear to be locked in the S-form; these mutants show a very small cGMP response. On the other hand, the cGMP-binding proteins in mutants KI-2 and KI-7 show fast dissociation while the chemoattractant-stimulated cGMP response is prolonged. Genetic analysis suggests that the two phenotypes, an altered cGMP-binding protein and an altered cGMP response, are not provided by multiple mutations (8). These observations provoke the intriguing hypothesis that *in vivo* the activity of guanylyl cyclase is regulated by the target of its product, a cGMP-binding protein.

It has been observed that magnesium-dependent guanylyl cyclase is located exclusively in the particulate fraction of a cell lysate (27, 28) but requires a cytosolic protein for its optimal activity (29). Furthermore, conditions that promote protein kinase activity (addition of ATP/Mg^{2+}) appear to inhibit guanylyl cyclase activity (30). In this paper, we describe experiments investigating the regulation of guanylyl cyclase by the cGMP-binding protein in wild type and mutants KI-4 and KI-7. Our results suggest that the cGMP-binding protein is the factor that activates guanylyl cyclase. Furthermore, once the cGMP-binding protein is occupied with cGMP, it inhibits guanylyl cyclase in an ATP-dependent manner. The importance of this dual regulation of guanylyl cyclase for the correct processing of chemotactic signals is suggested by defective regulation in the chemotactic mutants KI-4 and KI-7. The role of this dual regulation in maintaining spatial information will be discussed.

EXPERIMENTAL PROCEDURES

Materials—cAMP, cGMP, ADPNHP,[2] and GTP were purchased from Boehringer Mannheim. [8-^{3}H]cGMP (910.2 GBq/mmol) was obtained from Amersham Corp. Polycarbonate and nitrocellulose filters were from Nuclepore (Costar, Badhoevedorp, The Netherlands) and Schleicher & Schuell, respectively. DEAE-Sepharose was obtained from Pharmacia Biotech Inc.

Strains and Culture Condition—KI-4, KI-7, and the parental strain XP55 (8) were grown on 1/3 SM plates (0.3% glucose, 0.3% bactopeptone, 40 mM KH$_2$PO$_4$/Na$_2$HPO$_4$ buffer, pH 6.0, and 1.5% agar) with *Escherichia coli* B/r. Cells were harvested in the late logarithmic phase with 10 mM KH$_2$PO$_4$/Na$_2$HPO$_4$ buffer, pH 6.5 (PB). Bacteria were removed by repeated centrifugations at 300 × g for 3 min. Then cells were starved for 5 h by shaking in PB at a density of 10^7 cells/ml at 21 °C.

cGMP Production by cAMP Stimulation—The accumulation of cGMP on stimulation with 1 μM extracellular cAMP was measured in the isotope dilution assay as described previously (7).

Cell Lysis and Preparation of the Cytosolic Fraction—The preparation of a high speed supernatant from cell homogenates was carried out as described previously with a few modifications (26). Starved cells were resuspended to a density of 2 × 10^8 cells/ml in cGMP binding assay buffer (20 mM Hepes/NaOH, 2.2 mM MgSO$_4$, 1 mM EGTA, and 10% glycerol (v/v), pH 7.5). Cells were then homogenized at 0 °C by passing the suspension through a polycarbonate filter (pore size of 3 μm). The homogenate was centrifuged at 4 °C for 1 h at 48,000 × g. The 48,000 × g supernatant was used for the cGMP binding experiments, for partial purification of the cGMP binding activity, and for guanylyl cyclase reconstitution experiments.

Partial Purification of the cGMP Binding Activity—The prepared 48,000 × g supernatant from XP55 or KI-4 was subjected to ion-exchange chromatography on a 10-ml column of DEAE-Sepharose as described previously (25). Proteins were eluted from the resin using a 120-ml linear gradient of NaCl (0–400 mM) in cGMP binding assay buffer. The resulting fractions (1.5 ml) were used in a cGMP binding assay and in a reconstitution assay with particulate guanylyl cyclase. All procedures were carried out at 4 °C within 1 day because the guanylyl cyclase-reconstituting activity and cGMP binding activity are not very stable ($t_{1/2} \simeq 1$ day).

cGMP Binding Assay—The cGMP binding activity of the 48,000 × g supernatant or the partially purified fractions was measured as described previously (23, 25, 26) using 10 nM [^{3}H]cGMP. These binding assays were performed with or without 100 μg/ml denatured herring sperm DNA (boiled for 3 min and immediately cooled on ice).

Guanylyl Cyclase Assay—The activity of guanylyl cyclase in total lysates, particulate fractions, and particulate fractions mixed with cytosolic fractions (reconstituted) was measured as described previously (29) using a lysis buffer without GTPγS (20 mM Hepes/NaOH, 2.2 mM MgSO$_4$, and 1 mM EGTA, pH 7.5). Briefly, cells at a density of 10^8 cells/ml in lysis buffer were lysed by rapid elution through a polycarbonate filter. When ATP, ADPNHP, or 8-Br-cGMP were present, these compounds were added to the homogenate immediately after cell lysis. When azide was present, it was added to the cell suspension 5 min before lysis.

To prepare a particulate fraction, this cell homogenate was diluted 7-fold in lysis buffer and centrifuged at 14,000 × g for 1 min at 4 °C. To determine guanylyl cyclase activity of the particulate fraction, the pellet was resuspended to the volume of the original lysate in either fresh lysis buffer (Fig. 2) or cGMP-binding buffer (Fig. 3). To determine reconstituted guanylyl cyclase activity, the pellet was resuspended either in lysis buffer, in the 14,000 × g supernatant or in partial purified 48,000 × g supernatant (see figure legends).

The guanylyl cyclase reaction was started by mixing these prepared subcellular fractions at 20 °C with an equal volume of reaction mixture (20 mM Hepes/NaOH, 2.2 mM MgSO$_4$ and 1 mM EGTA, 10 mM dithiothreitol, 1 mM GTP, pH 7.5); 100 μg/ml denatured herring sperm DNA was added when indicated. The reactions were terminated after 20, 40, 60, 80, 100, and 120 s by the addition of an equal volume of 3.5% (v/v) perchloric acid, and the cGMP content was measured in the isotope dilution assay described above. The NaCl present in the partial purified supernatant had no detectable influence on guanylyl cyclase activity.

Protein Determination—The protein content of all cell and column fractions was measured with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

RESULTS

Activation of Particulate Guanylyl Cyclase Activity by Cytosolic Proteins from XP55 and Mutants KI-4 and KI-7—In *Dictyostelium* wild-type cells, strain NC4, magnesium-dependent guanylyl cyclase resides in the particulate fraction of a cell homogenate; the cytosol has no detectable activity but stimulates the activity in the pellet (28, 29). We have investigated the regulation of guanylyl cyclase in the wild-type XP55 and the non-chemotactic mutants KI-4 and KI-7. Fig. 1 shows that guanylyl cyclase activity in the homogenate (*T*) of XP55 and mutant KI-7 cells is higher than that in the particulate fractions (*P*). The re-addition of the cytosolic fraction to the particulate fraction enhances guanylyl cyclase activity in XP55 and mutant KI-7 (Fig. 1). The cytosol does not possess guanylyl cyclase activity (data not shown). These results suggest that the cytosol derived from both strains possesses a functional

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[2] The abbreviations used are: ADPNHP, adenosine 5′-(β,γ-imido) triphosphate; 8-Br-cGMP, 8-bromoguanosine 3′,5′-monophosphate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.
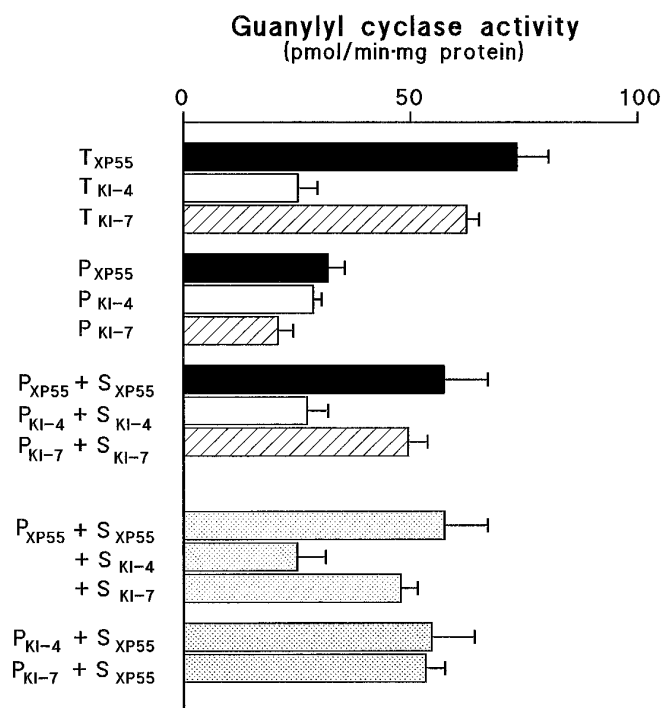
FIG. 1. **Guanylyl cyclase activity in cell homogenates (*T*), particulate fractions (*P*), and in reconstituted mixtures of particulate and cytosolic fractions (*P* + *S*) from the wild-type strain XP55 and mutants KI-4 and KI-7.** Cell homogenates and particulate and cytosolic fractions were prepared as described under "Experimental Procedures." The particulate fraction was resuspended in the original volume with buffer or with the cytosolic fraction from the strain indicated. Guanylyl cyclase activity is expressed as picomoles of cGMP formed per min per mg of protein present in the original homogenate from which the particulate fraction was obtained. Data are the means ± S.D. of two independent experiments with triplicate determinations.

FIG. 2. **Ion-exchange chromatography of the cGMP binding activity and guanylyl cyclase-stimulating activity from the cytosolic fraction of the parental strain XP55 (*A*) and KI-4 (*B*).** The high speed supernatant obtained upon centrifugation of a homogenate at 48,000 × *g* was loaded on a DEAE-Sepharose column. Proteins were eluted by means of a linear NaCl gradient (0–400 mM in 120 ml of cGMP binding assay buffer; *dotted line*). The cGMP binding activity and the ability to activate the particulate guanylyl cyclase from XP55 were determined for each fraction as described under "Experimental Procedures." The activity is expressed as picomoles of cGMP formed per min per mg of protein present in the original homogenate from which the particulate fraction was obtained. The data are from a representative experiment repeated twice (XP55) or once (KI-4).

activator of guanylyl cyclase. In contrast to these cell lines, guanylyl cyclase activities in the homogenate and the particulate fraction of mutant KI-4 are essentially identical, and the re-addition of the cytosolic fraction to the particulate fraction does not affect enzyme activity (Fig. 1). The differences in guanylyl cyclase activities between the pellets of these three strains are much smaller than those between the total homogenates, indicating that basal guanylyl cyclase activity is not affected in the mutants.

To further explore the defect in mutant KI-4, heterologous reconstitution experiments were performed (Fig. 1, *stippled bars*). Guanylyl cyclase in the pellet of XP55 can be stimulated by the cytosol derived from either wild-type XP55 or mutant KI-7 but not by the cytosol from mutant KI-4. However, guanylyl cyclase in the pellet of both KI-7 and KI-4 is stimulated by the cytosol from XP55. These results allow three conclusions: (i) guanylyl cyclase activity in the particulate fractions of both mutants is essentially identical to that of the wild type; their susceptibility to activation by the wild-type cytosolic fraction is also essentially identical; (ii) the cytosolic activator of mutant KI-7 shows normal functional properties; and (iii) the cytosol of mutant KI-4 does not activate the particulate guanylyl cyclase.

Column Chromatography of the Guanylyl Cyclase-stimulating Activity—The proteins in the cytosol of wild-type XP55 were separated by ion-exchange chromatography. Immediately after elution of the column, the ability of each fraction to stimulate particulate guanylyl cyclase from XP55 was tested. As shown in Fig. 2, the guanylyl cyclase-stimulating activity eluted as a single peak at approximately 100 mM NaCl in fraction 41. In the same fractions, we also measured the cGMP binding activity and observed that the guanylyl cyclase-stimu-
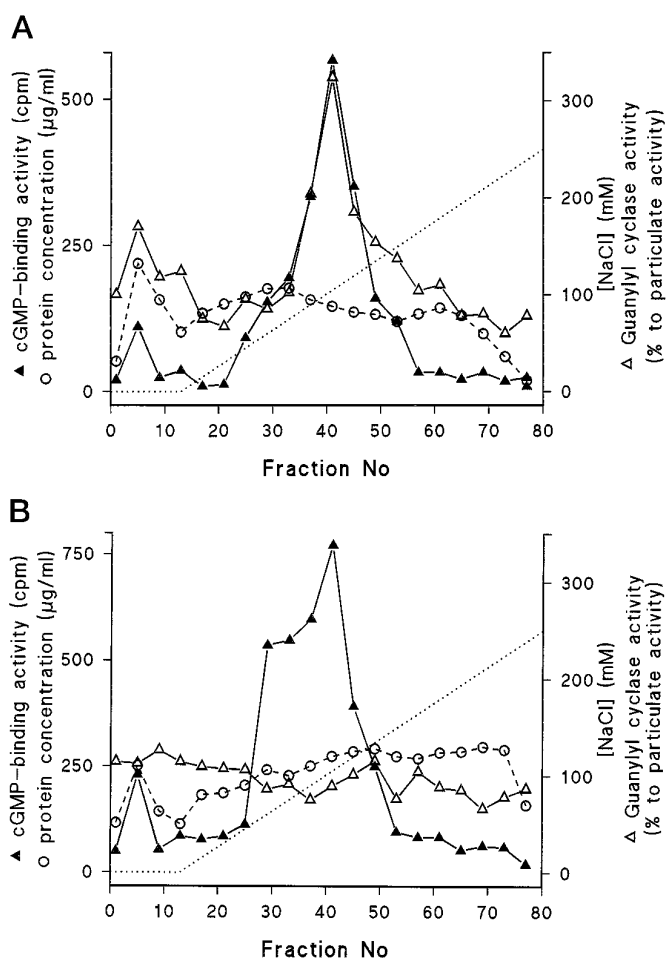
lating activity exactly co-eluted with the cGMP binding activity. Unfortunately, the guanylyl cyclase-stimulating activity is not stable ($t_{1/2}$ of approximately 1 day), preventing purification of the protein. In support of the identity of the cGMP-binding protein as the guanylyl cyclase-stimulating activity, we observed that both activities showed a similar half-life (data not shown).

The cytosolic proteins from KI-4 cells were also separated by ion-exchange chromatography, and guanylyl cyclase-stimulating activity was tested using the enzyme in the particulate fraction of XP55. A large peak of cGMP binding activity was eluted from the column; however, none of the fractions could stimulate guanylyl cyclase activity (Fig. 2*B*). This result shows that KI-4 has the cGMP-binding protein but lacks guanylyl cyclase-stimulating activity.

Effect of DNA on cGMP Binding Activity and Guanylyl Cyclase Activation—DNA fragments strongly enhance the binding of cGMP to the cGMP-binding protein (25, 26). The effect of DNA on the cGMP binding activity and on guanylyl cyclase-stimulating activity was tested in the wild type and in the KI mutants. As shown in Fig. 3*A*, denatured herring DNA en-

hances cGMP binding activity in the cytosol of wild-type XP55 and mutant KI-7, but has no effect on the cGMP binding activity from mutant KI-4. The addition of DNA causes a dramatic increase of guanylyl cyclase activity in the homogenates of XP55 and KI-7 (Fig. 3*B*). In accordance with the lack of effect of DNA on cGMP binding, DNA fragments do not alter guanylyl cyclase activity in KI-4 homogenates.

To summarize the previous experiments, we obtained evidence supporting the hypothesis that the cGMP-binding protein is identical to the cytosolic activator of particulate guanylyl cyclase and that the aberrant cGMP-binding protein in KI-4 lacks the functional effect to stimulate guanylyl cyclase. Although the cGMP binding activity of mutant KI-7 is not completely normal, the cytosol from this mutant stimulates particulate guanylyl cyclase in a manner that is indistinguishable from that of wild-type cells.

In the next series of experiments, we pursue the observation by Schoen (30) that conditions favoring protein phosphorylation lead to inhibition of guanylyl cyclase and investigate the hypothesis that the cGMP-binding protein again mediates this effect.

Reduction of Guanylyl Cyclase Activity by Enhancement of Protein Kinase Activity—Previously, it was shown that enhancement of protein kinase activity by adding ATP/Mg^{2+} resulted in reduced guanylyl cyclase activity (30), and conversely, inhibition of protein kinase activity by the protein kinase inhibitor ADPNHP increased guanylyl cyclase activity in cell homogenates from wild-type cells (27, 28).

Fig. 4 shows that the observations mentioned above are reproduced for the wild-type XP55; 1 mM ATP strongly reduces guanylyl cyclase activity whereas 0.1 mM ADPNHP enhances guanylyl cyclase activity. Because the concentration of ATP in the cell homogenate is estimated to be about 10 μM, which may already partly inhibit guanylyl cyclase, the homogenate was prepared from cells treated with 10 mM azide for 5 min, which is expected to reduce the intracellular ATP concentration 10-fold (31). Pretreatment with azide leads to a significant increase of guanylyl cyclase activity to nearly the same level as that in the presence of ADPNHP.

Guanylyl cyclase activity from KI-4 is not altered by the addition of ATP or ADPNHP or by the pretreatment of the cells with azide (Fig. 4). Guanylyl cyclase activity in the homogenate of KI-4 is low because the enzyme is not activated by the cytosolic factor. Guanylyl cyclase activity of mutant KI-7, which is relatively high due to its activation by the cytosolic factor, is also not affected by ATP, ADPNHP, or azide treatment. Furthermore, ATP, ADPNHP, and azide treatment have no effect on guanylyl cyclase activity of the particulate fraction from wild-type XP55 (Fig. 5). ATP-dependent inhibition of guanylyl cyclase can be reconstituted by adding the cytosolic fraction, indicating that inhibition acts via a cytosolic factor (data not shown). The cytosol was chromatographed as described in Fig. 2*A*, and the fractions were reconstituted with the particulate fraction from azide-treated XP55 cells. ATP-dependent inhibition of guanylyl cyclase was observed only when the enzyme was supplemented with column fractions eluting at 100 mM NaCl (Fig. 5). These are exactly the same fractions that stimulate guanylyl cyclase in the absence of ATP.

The Effect of a cGMP Analogue on Stimulation and ATP-dependent Inhibition of Guanylyl Cyclase—The previous sections suggest that the soluble cGMP-binding protein may activate guanylyl cyclase and that this stimulation is lost by ATP-dependent inhibition of particulate guanylyl cyclase. In the last section, we investigate whether occupancy of this binding protein with cGMP modulates these stimulatory and inhibitory effects. The cGMP analog 8-Br-cGMP was used since it has a
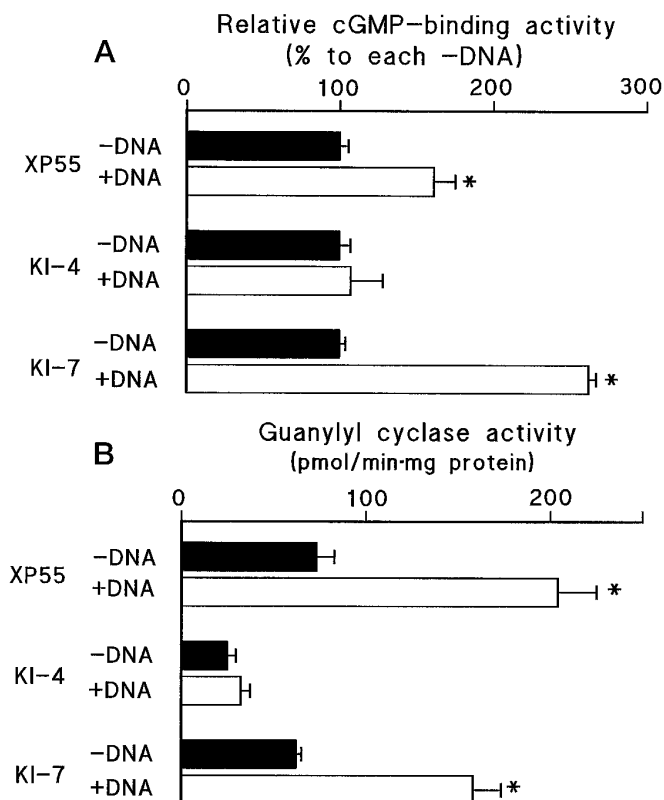

FIG. 3. **Effect of DNA fragments on cGMP binding activity (*A*) and guanylyl cyclase activity (*B*) in cell homogenates of XP55, KI-4, and KI-7.** cGMP binding activity and guanylyl cyclase activity were measured as described under "Experimental Procedures." Assays were performed in the absence or presence of 100 μg/ml denatured herring sperm DNA. Data are the means ± S.D. of two independent experiments with triplicate determinations. *, significantly different from control without DNA ($p < 0.01$).

high affinity for the cGMP-binding protein but does not strongly interfere with the determination of cGMP levels in isotope dilution assay. The experiments have some intrinsic problems since the reaction product cGMP may also bind to the binding protein. Furthermore, the GTP that is present as substrate may be converted to ATP by nucleoside diphosphate kinase present in the cytosol and pellet (32), which then acts as an inhibitor of guanylyl cyclase. Finally, modulation of guanylyl cyclase activity by ATP and 8-Br-cGMP may not be instantaneous. These collective difficulties were anticipated by measuring guanylyl cyclase between 20 and 120 s after the onset of the reaction, during which time minimal amounts of ATP and cGMP are formed from GTP.

The addition of 50 nM 8-Br-cGMP to the particulate fraction has no effect on basal guanylyl cyclase activity, either in the absence or presence of 1 mM ATP (Fig. 5). The cytosol (see total homogenate in figure) or the partially purified cGMP-binding protein stimulates particulate guanylyl cyclase activity as shown before; the occupancy of the cGMP-binding protein with 8-Br-cGMP has no additional effects on guanylyl cyclase activity (Fig. 5). ATP inhibits guanylyl cyclase activity in the homogenate or when it is reconstituted from the particulate and the partially purified cytosolic fraction. The addition of 50 nM 8-Br-cGMP significantly potentiates this ATP-mediated inhibition (Fig. 5). These combined results suggest that cGMP does not participate in the activation of guanylyl cyclase, or in other words, the guanylyl cyclase is stimulated by the cGMP-binding protein whether it is occupied with cGMP or not. On the other hand, ATP-mediated inhibition of guanylyl cyclase by the cGMP-binding protein is potentiated by cGMP.
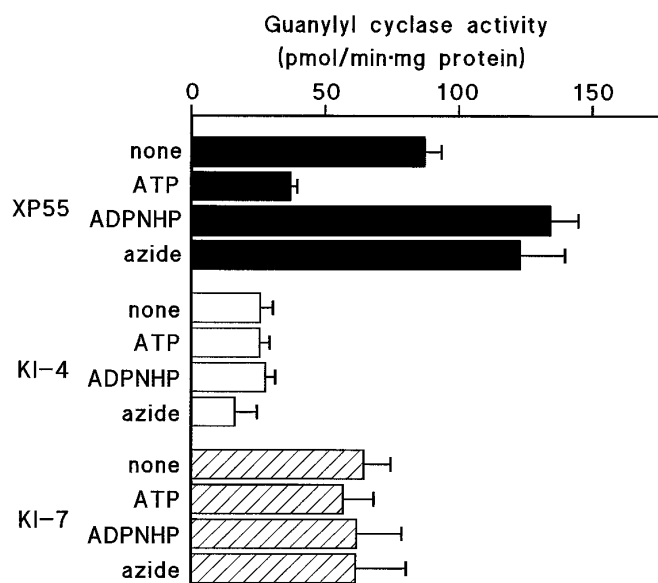
Guanylyl cyclase activity
(pmol/min·mg protein)

FIG. 4. **Effect of ATP, ADPNHP, and azide pretreatment on guanylyl cyclase activity in cell homogenates from XP55, KI-4, and KI-7.** Guanylyl cyclase activity was measured in cell homogenates in the presence or absence of 1 mM ATP or 0.1 mM ADPNHP. The activity was also measured in homogenates that were prepared from cells pretreated with 10 mM azide for 5 min to deplete intracellular ATP levels. Data are the means ± S.D. of three (ATP and ADPNHP) or two (azide) independent experiments with triplicate determinations.

Guanylyl cyclase activity
(pmol/min·mg protein)

FIG. 5. **Effect of the cGMP analogue 8-Br-cGMP on the activation or ATP-mediated inhibition of guanylyl cyclase.** Guanylyl cyclase activity from XP55 was measured in a cell homogenate (T), particulate fraction (P), or after reconstitution of the particulate fraction with the partially purified cGMP-binding protein (*fra*; mixture of fractions 40, 41, and 42 in Fig. 2A). Guanylyl cyclase activity was measured with or without the indicated ATP concentrations. Where indicated, 50 nM 8-Br-cGMP was present during the reaction ($8Br$ +). The concentration of ATP in the homogenate and the washed pellet is calculated to be less than 14 and 2 μM, respectively. Guanylyl cyclase activity is expressed as picomoles of cGMP formed per min per mg of protein present in the original homogenate from which the particulate fraction was obtained. The *asterisk* indicates that the addition of 8-Br-cGMP induces a significant reduction of guanylyl cyclase activity relative to the incubation without 8-Br-cGMP ($p < 0.01$). *cond.*, condition.

DISCUSSION

Activation of guanylyl cyclase by extracellular signal molecules is an important intracellular signaling pathway in many biological systems. In *Dictyostelium discoideum*, mutant analysis revealed that regulation of guanylyl cyclase plays a central role in chemotactic signal transduction. Therefore, understanding the detailed regulation of this enzyme may help to clarify the mechanism of chemotaxis.

The present study started with the observation that non-chemotactic mutants KI-4 and KI-7 have an aberrant cGMP binding activity, and at the same time, show a strongly altered cGMP response. These observations are intriguing, especially from a genetic point of view, because the aberrant cGMP binding activity and altered cGMP response cannot be segregated by parasexual analysis, suggesting that they are caused by the same mutation or by closely linked mutations (8, 26). Particulate fractions from wild-type XP55 and mutant KI-4 and KI-7 cells contain comparable guanylyl cyclase activities. Therefore these mutants are not defective in guanylyl cyclase itself but in its regulation. The simplest explanation for this observation would be that the cGMP-binding protein regulates guanylyl cyclase activity. Since guanylyl cyclase from wild-type cells was previously shown to be activated by a cytosolic protein (29) and inhibited at conditions favoring protein kinase activity (30), we have analyzed the regulation of guanylyl cyclase in mutants KI-4 and KI-7.

The experiments investigating the stimulation of particulate guanylyl cyclase by a cytosolic protein revealed the following results. The cytosolic fraction from mutant KI-4 does not stimulate particulate guanylyl cyclase activity. At the same time, this mutant has an aberrant cGMP binding activity (abnormally low K_d) and shows a very small chemoattractant-mediated cGMP response. DNA fragments, which are known to increase cGMP binding activity (25), also stimulate guanylyl cyclase. In mutant KI-4, both cGMP binding activity and guanylyl cyclase activity are not influenced by DNA. Finally, the cGMP binding activity and guanylyl cyclase-stimulating activity co-elute upon chromatography of cytosolic p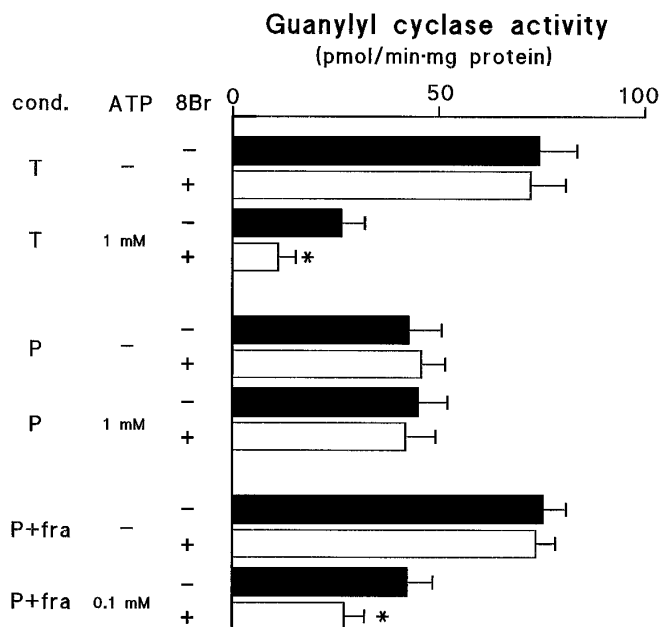roteins. These results strongly support the hypothesis that guanylyl cyclase is activated by a cytosolic cGMP-binding protein. Since the addition of 8-Br-cGMP, a potent ligand for the cGMP-binding protein, does not affect the stimulation of guanylyl cyclase by the partially purified activator, this implies that the cGMP occupancy of the cGMP-binding protein is not a prerequisite for its stimulating activity.

The addition of ATP to a cell homogenate inhibits guanylyl cyclase activity, whereas the addition of a kinase inhibitor ADPNHP or depletion of ATP by azide pretreatment enhances guanylyl cyclase activity. The role of the cGMP-binding protein in this ATP-mediated inhibition is suggested by the following observations; guanylyl cyclase activity in the pellet is not affected by ATP and inhibition by ATP requires the cytosol. Furthermore, the inhibitory activity in the cytosol co-purifies with the cGMP-binding protein. The cytosol of mutant KI-7, which has an aberrant cGMP binding activity with abnormally high K_d, does stimulate particulate guanylyl cyclase activity, but ATP does not inhibit the enzyme. Interestingly, mutant KI-7 shows a prolonged chemoattractant-mediated cGMP response. Finally, the partially purified cGMP-binding protein induces an ATP dose-dependent decrease of guanylyl cyclase that is enhanced by the cGMP analog 8-Br-cGMP. This suggests that cGMP stimulates ATP-mediated inhibition of guanylyl cyclase.

In mammalian cells, two classes of guanylyl cyclase regulators have been isolated. Guanylin regulates intestinal salt and water transportation via the activation of guanylyl cyclase (for review, see Ref. 33), whereas Ca^{2+}-sensitive proteins of the recoverin family (guanylyl cyclase activating protein) activate
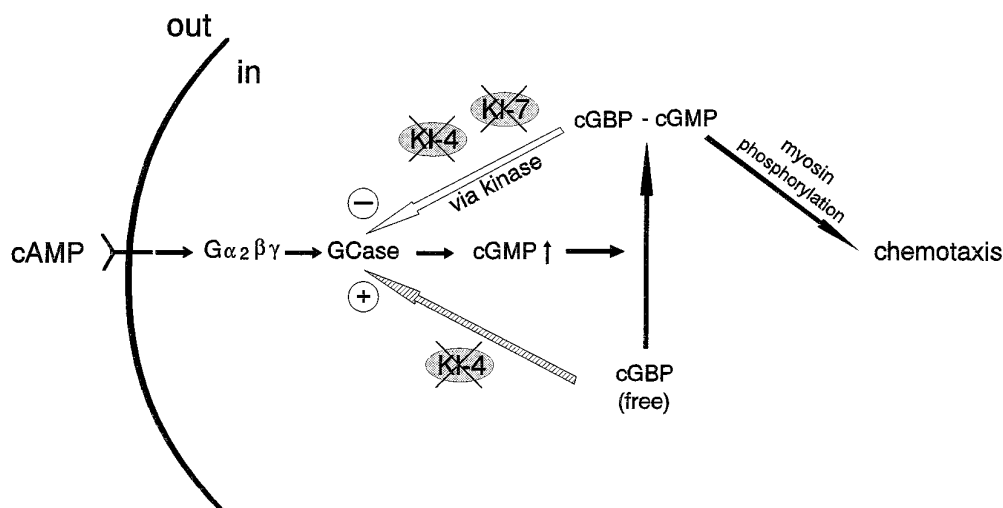
FIG. 6. **Model of the regulation of guanylyl cyclase by a cGMP-binding protein.** Guanylyl cyclase (*GCase*) is activated via a G-protein-coupled cAMP chemoreceptor. The unoccupied form of the cGMP-binding protein (*cGBP*) is required for the optimal activation of guanylyl cyclase. Upon binding of the produced cGMP to this binding protein (*cGBP-cGMP*), the binding protein negatively regulates guanylyl cyclase in an ATP-dependent manner, possibly via a protein kinase. KI-4 is defective in both the positive and negative pathways, whereas KI-7 shows normal activation but no ATP-dependent inhibition of guanylyl cyclase. The occupied cGMP-binding protein mediates the phosphorylation of conventional myosin, finally leading to the formation of a local pseudopodium. In this model, the location of guanylyl cyclase is not properly drawn since it is located on plasma membrane.

guanylyl cyclase and are inhibited by Ca^{2+} ions (34, 35). These activators are not likely to be related to the cGMP-binding protein in this study because they do not contain a consensus cGMP-binding domain, and the estimated molecular mass of the cGMP-binding protein (>80 kDa) is different from the two activators (guanylin peptide consists of 15 amino acids; recoverin and guanylyl cyclase activating protein are 23 kDa). *Dictyostelium* guanylyl cyclase is regulated by Ca^{2+} ions in a way that is very similar to the mammalian enzyme (28, 36). Mutants KI-4 and KI-7 show normal inhibition by Ca^{2+} (8), suggesting that a functional cGMP-binding protein is not required for the regulation of guanylyl cyclase by recoverin-like calcium-binding proteins.

The combined results of the properties of the cGMP-binding protein and the regulation of guanylyl cyclase in *Dictyostelium* mutants KI-4 and KI-7 are complementary. The cGMP-binding protein in KI-4 shows a very high affinity for cGMP, probably because it is locked in a slow dissociating form of the protein. In contrast, the cGMP binding activity in KI-7 has low affinity and fast dissociation. The cytosol of mutant KI-4 can neither stimulate nor inhibit guanylyl cyclase activity, whereas the stimulation of guanylyl cyclase by the cytosol of mutant KI-7 is normal and only inhibition is absent. These observations are in perfect agreement with the chemoattractant-stimulated cGMP responses in these mutants: the absence of guanylyl cyclase-stimulating activity in KI-4 is associated with a very small cGMP response, whereas the absence of the inhibitory activity in KI-7 is correlated with a prolonged cGMP response.

Taken together, we propose that both the stimulation and ATP-mediated inhibition of particulate guanylyl cyclase are mediated by the cytosolic cGMP-binding protein. The results are summarized in a model for the regulation of guanylyl cyclase *in vivo* (Fig. 6). Chemoattractants stimulate guanylyl cyclase via G-protein-coupled surface receptors. The association of guanylyl cyclase with a cGMP-binding protein is necessary for the maximal activation of the enzyme. This cGMP-binding protein does not have to be occupied with cGMP; therefore activation can start before guanylyl cyclase produces cGMP. Once guanylyl cyclase is activated, the produced cGMP binds to the binding protein and the occupied cGMP-binding protein transduces the signal downstream to induce myosin

phosphorylation. The occupied cGMP-binding protein also reduces the activity of guanylyl cyclase in an ATP-dependent manner, possibly via a protein kinase reaction. At this point, two possibilities remain open: the stimulatory activity of the cGMP-binding protein is converted to an inhibitory activity upon occupancy of the cGMP-binding protein and presence of ATP, or the stimulatory activity of the cGMP-binding protein is lost in the presence of ATP. The observation that, in the presence of the partially purified cGMP-binding protein, ATP inhibits guanylyl cyclase activity to a level that is lower than the enzyme activity in the particulate fraction favors the first possibility. The cGMP-binding protein may interact directly with guanylyl cyclase, but it is also possible that other proteins bind to the cGMP-binding protein and regulate both cGMP binding activity and guanylyl cyclase activity. Such a more complex regulation could explain the isolation of several mutants with altered cGMP binding activity, which according to parasexual genetics do not complement each other and are supposedly mutated in different genes.

The model may explain how cells are able to locally retain the information of a diffusible second messenger. For chemotaxis, a cell needs a sensing and transduction mechanism capable of discriminating between the sides of a cell with high and low concentrations of chemoattractant. The present model provides fast activation of guanylyl cyclase when associated with the cGMP-binding protein, followed by the rapid inactivation of the enzyme when the produced cGMP binds to the binding protein. The local activation of the cGMP-binding protein may induce the local phosphorylation of conventional myosin, leading to local pseudopodium formation. The absence of chemotaxis in mutants KI-4 and KI-7 could be caused either by the defective regulation of guanylyl cyclase by the cGMP-binding protein dislocalizing the intracellular cGMP signal or by the inability of the cGMP-binding protein to transduce the signal to downstream effectors, such as the phosphorylation of conventional myosin.

Random mutagenesis of *Dictyostelium* cells has identified nine chemotaxis mutants. Two mutants lack the chemoattractant-stimulated cGMP production, whereas four mutants show an altered cGMP binding activity and aberrant fine regulation of guanylyl cyclase. These mutants identify guanylyl cyclase

and the cGMP-binding protein as two key components in the processing of chemotactic signals in *Dictyostelium*.

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