A Chemoattractant Receptor Controls Development in *Dictyostelium discoideum*

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During the early stages of its developmental program, *Dictyostelium discoideum* expresses cell surface cyclic adenosine monophosphate (cyclic AMP) receptors. It has been suggested that these receptors coordinate the aggregation of individual cells into a multicellular organism and regulate the expression of a large number of developmentally regulated genes. The complementary DNA (cDNA) for the cyclic AMP receptor has now been cloned from Agt-11 libraries by screening with specific antisera. The 2-kilobase messenger RNA (mRNA) that encodes the receptor is undetectable in growing cells, rises to a maximum at 3 to 4 hours of development, and then declines. In vitro transcribed complementary RNA, when hybridized to cellular mRNA, specifically arrests in vitro translation of the receptor polypeptide. When the cDNA is expressed in *Dictyostelium* cells, the undifferentiated cells specifically bind cyclic AMP. Cell lines transformed with a vector that expresses complementary mRNA (antisense) do not express the cyclic AMP receptor protein. These cells fail to enter the aggregation stage of development during starvation, whereas control and wild-type cells aggregate and complete the developmental program within 24 hours. The phenotype of the antisense transformants suggests that the cyclic AMP receptor is essential for development. The deduced amino acid sequence of the receptor reveals a high percentage of hydrophobic residues grouped in seven domains, similar to the rhodopsins and other receptors believed to interact with G proteins. It shares amino acid sequence identity and is immunologically cross-reactive with bovine rhodopsin. A model is proposed in which the cyclic AMP receptor crosses the bilayer seven times with a serine-rich cytoplasmic carboxyl terminus, the proposed site of ligand-induced receptor phosphorylation.

Upon starvation, *Dictyostelium amoebae* synchronously aggregate into a multicellular organism and cells in specific positions differentiate into stalk or spore cells (1, 2). The aggregation of individual cells into the multicellular structure is coordinated by a developmentally regulated cyclic AMP signaling system. At aggregation centers, cyclic AMP is periodically synthesized and secreted at 6-minute intervals. The oscillations in cyclic AMP initiate chemical waves that are propagated through the cell monolayer. The leading edge of each of these waves provides a gradient that directs the migration of chemotactically responsive cells into the aggregation center (3). The oscillations are required for early gene expression as well as proper morphogenesis. Mutants with defective oscillators fail to differentiate, but can be rescued by periodic application of exogenous cyclic AMP. A constant level of extracellular cyclic AMP does not rescue these mutants and suppresses early gene expression in wild-type cells. Late gene expression requires continuous extracellular cyclic AMP. Pharmacologic studies indicate that the effects of cyclic AMP on both early and late gene expression are mediated via the chemoattractant receptor (2, 4).

The cyclic AMP oscillator includes the surface receptor, adenylate cyclase, and cyclic AMP phosphodiesterase (Fig. 1). Binding to the surface receptor triggers activation of adenylate cyclase and newly synthesized cyclic AMP is secreted, completing a positive feedback loop that amplifies the initial stimulus and relays the chemotactic signal to other cells. Within a few minutes, the response ceases as the cells adapt to persistent stimulation. Adaptation is reversible; when the stimulus is removed the cells resensitize, and within a few minutes the next cycle is triggered.

The cyclic AMP receptor, identified by photoaffinity labeling (5), oscillates between two interconvertible forms designated R (44 kilodaltons) and D (43 kilodaltons) in parallel with the oscillations in cyclic AMP synthesis (6). Stimulation with cyclic AMP converts the R form to the D form. The kinetics and dose-dependence of this conversion are similar to those of adaptation (7). Adaptation and the shift in electrophoretic mobility are closely correlated with a fivefold increase in phosphorylation of the receptor, suggesting that phosphorylation of the receptor plays a central role in adaptation (8). Receptor phosphorylation has also been correlated with deactivation of rhodopsin and the β-adrenergic receptors (9).

Recent evidence, including in vitro stimulation of adenyl
cyclease with guanosine triphosphate (GTP), cyclic AMP–stimulated binding of GTP to membranes, and modulation of cyclic AMP binding affinity by GTP and guanosine diphosphate (GDP), suggests that the cyclic AMP receptor is coupled to a GTP-dependent regulatory protein analogous to mammalian G proteins and transducin (1, 10, 11). The cDNA's for two G protein α subunits and a β subunit have been cloned from Dictyostelium and show considerable identity to mammalian G protein subunits (12, 13).

Thus, the chemotactic signaling system in Dictyostelium appears to be a progenitor of vertebrate signaling systems. The opsins, the adenrenergic receptors, and other receptors coupled to G proteins contain multiple hydrophobic regions, and it has been suggested that they traverse the membrane seven times (14). We present the amino acid sequence of the cyclic AMP receptor and propose that it and other eukaryotic chemoattractant receptors are structurally related to the growing class of G protein–linked receptors. We also provide evidence that the receptor plays a central role in the developmental program of Dictyostelium.

**Isolation of receptor cDNA.** We have purified the cyclic AMP receptor and raised, in rabbits, a monospecific antisem that reacts with both the R and D forms (15, 16) (Fig. 2). We screened two λgt11 cDNA libraries with the antisem and obtained seven cDNA clones. These clones produced β-galactosidase fusion proteins (118 kD to 150 kD) that reacted strongly with antisem in immunoblots (Fig. 2). A library prepared from late aggregation stage NC-4 cells yielded two clones in an initial screen of 60,000. The second library, prepared from 3-hour stage AX-3 cells, yielded five clones in an initial screen of 160,000. The AX-3 library was again screened with cDNA probes to obtain 16 additional clones, some of which included the entire coding sequence.

That the cDNA clones encode the cyclic AMP receptor was verified by multiple criteria. Antibodies affinity purified from several of the fusion proteins specifically stained the R and D forms of the receptor in immunoblots of crude membranes. Antibodies affinity purified from two small fusion proteins (A and C) representing nonoverlapping amino acid sequences did not cross-react with each other in immunoblots, but did react with the receptor and a large fusion protein (B) that encompassed both of the shorter sequences, showing that the B cDNA encodes multiple receptor epitopes (Fig. 2).

Furthermore, each of these fusion proteins (A, B, and C) generated antiserum (in rabbits) that specifically recognizes the cyclic AMP receptor in cell extracts.

In vitro translation of mRNA from early aggregation stage cells and subsequent immunoprecipitation of the expressed polypeptide has established characteristics of the mRNA encoding the cyclic AMP receptor. (i) The receptor mRNA encodes a nascent 37-kD polypeptide (16); (ii) the receptor mRNA is undetectable in growing cells, is maximally expressed 3 to 4 hours after starvation, and then declines (16); (iii) the receptor mRNA is approximately 2 kbp, as determined by in vitro translation of size-fractionated poly(A)+ RNA. Each of these characteristics was confirmed with the cDNA clones. Thus, RNA blot analysis with a cDNA probe identifies a 2-kb band absent in vegetative cells (0 hours), rising to a maximum at 4 hours of development, and then declining (Fig. 3A). Furthermore, RNA transcribed in vitro from a cDNA encompassing the entire coding region (cDNA 6B) translates into a 37-kD polypeptide, and complementary RNA generated from cDNA 6B arrests translation of the cyclic AMP receptor mRNA when hybridized to cellular RNA (Fig. 3B).

To test whether the cDNA encoded a cyclic AMP binding protein...
protein, we constructed a cell line that expressed the gene product of cDNA 6B in undifferentiated cells. Wild-type cells at this stage displayed several cyclic AMP binding sites and expressed very low levels of receptor mRNA and 40-kd protein (Fig. 3A). Complementary DNA 6B was cloned into a vector that uses the actin 15 promoter to direct RNA synthesis (17). The actin 15 promoter is maximally active in “0-hour” stage cells (17). At the 0-hour stage, the cells transformed with the construct containing cDNA 6B expressed a 40-kd protein as detected with receptor antiserum, while those transformed with the vector alone did not. Table 1 shows binding of 3H-labeled cyclic AMP to the wild-type and transformed cell lines. The wild-type and the control transformed cell lines display only 1.5 x 10^4 sites per cell at this stage. Those transformed with the construct containing cDNA 6B display 1 x 10^5 to 8 x 10^5 sites per cell (18).

The cyclic AMP receptor controls development. To explore the role of the cyclic AMP receptor in morphogenesis and development, we constructed a cell line that failed to express receptors. Cells were transformed with a construct designed to express RNA complementary to the entire coding region of the cyclic AMP receptor mRNA. The cDNA 6B was cloned into a plasmid vector such that the noncoding strand would be synthesized as a fusion to the neomycin phosphotransferase mRNA (19). The noncoding strand of 6B arrests in vitro translation of endogenous receptor mRNA when hybridized to cellular mRNA (Fig. 3B). A control set of cells was transformed with the vector lacking receptor sequences. During starvation of the cells, the antisense transformants remained as a cell monolayer, without aggregating; the control cells aggregated and formed streams in parallel with wild-type cells (Fig. 4A). The cyclic AMP receptor was undetectable in the antisense transformants, as determined by immunoblot analysis (Fig. 4B). Furthermore, in the antisense transformants the endogenous 2-kb mRNA for the receptor was undetectable by RNA blot analysis and antisense transcripts were detectable (20). These data show that the receptor is required for aggregation and for cells to progress through the developmental program.

Sequence analysis. The nucleotide sequence of cDNA encompassing the entire coding region is shown in Fig. 5. The largest open reading frame encodes a polypeptide of 392 amino acids with a calculated molecular size of 44,243 daltons. The NH2-terminal two-thirds of the coding region is enriched in hydrophobic residues in clusters of 21 to 25 amino acids, while the last 130 amino acids are predominantly hydrophilic and are particularly enriched in serines and threonines. A single consensus sequence for N-linked glycosylation is present near the NH2-terminus (21). The higher mobility of the nascent polypeptide (37 kD) relative to the mature R form (40 kD) suggests a cotranslational modification step such as glycosylation (16). However, the electrophoretic mobility of the receptor in polyacrylamide gels is not altered by treatment of the partially purified receptor with endoglycosidase or by inhibitors of glycosylation, including tunicamycin (even at doses in excess of that needed to inhibit glycosylation of other Dictyostelium glycoproteins (22)). Both the nascent and mature polypeptides migrate more rapidly than would be expected from the deduced sequence (44,243 daltons), as observed for other highly hydrophobic membrane proteins (23).

The hydropathy profile of the cyclic AMP receptor determined by the method of Kyte and Doolittle (24) reveals six strongly hydrophobic regions (M1 to M6) and a seventh domain (M7), which is less hydrophobic (Fig. 6). The pattern is remarkably similar to the profile of bovine rhodopsin, the β-adrenergic receptor, and several other recently identified receptors, although the lengths of the loops and the COOH-terminal regions vary widely (14, 25–28). For rhodopsin, assignment of the hydrophobic regions as membrane-spanning domains is supported by extensive biochemical data, including accessibility to proteases, peptide antibodies, and lipophilic modifying reagents (29). The cyclic AMP receptor and bovine rhodopsin share 22 percent amino acid identity over 270 residues in the NH2-terminal regions of the two receptors (Fig. 7), and the percentage of matched residues increases to 32 percent with conservative replacements. The identities are distributed in the hydrophobic and hydrophilic domains, and the optimal alignment matches each of the putative transmembrane domains and the hydrophilic

<table>
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<th>pBS18</th>
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<tr>
<td>I</td>
<td>15</td>
<td>18</td>
<td>475, 555, 275</td>
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<td>II</td>
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<td>N.D.</td>
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<td>IV</td>
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Fig. 4. Antisense transformation mimics receptor mutation. (A) Morphology of transformed cells. AX-3 cells were transformed with the control vector (19) or with the antisense construct described below. Under starvation conditions, the control transformants behaved as wild-type cells, aggregating with streams of cells entering the aggregation centers. Each of the four independent clones of antisense transformants remained as a uniform cell monolayer. The phenotype of one of these clones and the control of the 7.5-hour stage of development are shown. After 36 hours, the antisense transformants began to show weak signs of morphogenesis. (B) Immunoblot analysis. Cells starved in suspension were removed at 6-hour stage of starvation and subjected to immunoblot analysis with the antisense to the cyclic AMP receptor as described in the legend to Fig. 2. The receptor was expressed in the control transformants (C) at the same level as wild-type cells but was undetectable in the antisense transformants (A) and the antisense transfectants (D).

16 SEPTEMBER 1988 RESEARCH ARTICLES 1469
joining regions in order, suggesting conserved structural features that extend beyond the hydrophobic character of the two proteins. For example, the hydrophilic regions joining the first and second and the fifth and sixth hydrophobic domains share several identities and both are especially enriched in positively charged side chains. Although the identities do not persist in the hydrophilic COOH-terminal regions of the two receptors, both have an abundance of serines and threonines.

There are also identities with other receptors in this family, such as the α- and β-adrenergic, muscarinic acetylcholine, and substance K receptors (14, 27, 28). Certain groups of residues distributed throughout the sequences appear to be highly conserved in many of these receptors (Fig. 7). Domain M6 of many of these receptors contains a cluster of identities within the sequence FXXCWXXPO (30), where X represents an aliphatic residue and O represents phenylalanine or tyrosine. The identities between the cyclic AMP receptor and bovine rhodopsin are particularly numerous here: five out of nine residues are identical, and eight out of nine match with conservative replacements. There appears to be no sequence homology to other known cyclic AMP-binding proteins such as the catabolite activator protein (CAP) of Escherichia coli or the regulatory subunit of cyclic AMP-dependent protein kinase from Dictostylium or from mammals (31, 32).

The structural relation of the cyclic AMP receptor to bovine rhodopsin is supported by immunological data. Antiserum to bovine rhodopsin recognizes the cyclic AMP receptor in immuno-

![Image](image-url)

**Fig. 5.** Nucleotide and deduced amino acid sequence of the cyclic AMP receptor. The sequence of cDNA 5A, which encompasses the entire coding region, was determined from both strands. In addition, nine independent overlapping cDNAs that comprised the entire coding region were partially sequenced. The 1.3-kb sequence shown was identical in the multiple, independent cDNA sequences. In the deduced amino acid sequence, the hydrophobic domains are underlined and numbered M1 to M7. The asterisk (*) indicates the single consensus site for N-linked glycosylation, although the receptor has not been shown to be glycosylated. Exonucleases deletions of various lengths were prepared (49) from cDNA 5A, which had been subcloned into Bluescript as described in the legend to Fig. 2. DNA sequence was determined by means of the dideoxy-nucleotide chain termination method (50), with modified T7 DNA polymerase (Sequenase, United States Biochemical Corp.). Several cDNAs were obtained from the two libraries that were identical in the 1.3-kb region shown above but diverged in either the 5′ or 3′ ends. These divergent sequences did not hybridize to the receptor mRNA in blots or to genomic restriction fragments that included the coding region. The origin of these terminal sequences is unclear.
the binding of cyclic AMP. On the basis of binding studies with
cyclic AMP analogs, Van Haastert and Kien have proposed that
activation of the cyclic AMP receptor involves the formation of a
covalent bond between a nucleophilic residue of the receptor and
the phosphate of the cyclic AMP molecule (34); the polar face of
this proposed helix could offer one of several nucleophiles as well as
a positive charge (histidine) to balance the resulting negative charge
on the phosphoryl oxygen.

The cyclic AMP receptor also has two adjacent, charged residues
(Lys99 and Asp60) in the second hydrophobic domain and an
aspartic acid in the first domain. Pairs of oppositely charged residues
occur in domain M3 of all of the opsin sequences, and there is a
highly conserved aspartic acid within the second transmembrane
domain of many of the opsins, the β-adrenergic, muscarinic,
and substance K receptors (14, 25–28, 35). These charged residues occur
in contexts of predominantly hydrophobic residues, significantly
lowering the average hydrophy value of these domains (24). In the
opsins, intramembrane charged residues have been proposed to
interact with the retinal moiety (26, 35) and in the β-adrenergic
receptor, they have been proposed to play a role in ligand binding
(36). The charged residues within the hydrophobic domains of the
cyclic AMP receptor could similarly interact with cyclic AMP in the
binding site. Thus, the seven transmembrane domains could be
arranged like the staves of barrel, forming a central core cradling the
ligand binding site, as has been proposed for rhodopsin and the β-
adrenergic receptor (26, 35), with charged amino acid side chains
facing into the binding site.

The last 132 amino acids of the cyclic AMP receptor have 18
serine and 6 threonine residues. In our model, this cytoplasmic tail is
the site of stimulus dependent phosphorylation, which occurs
predominantly on serine (16). A particularly interesting stretch of
serines lies from residue 360 to 368, with eight serines interrupted
by one leucine, and bounded on each side by two to three acidic
residues. Since the cyclic AMP receptor appears to be multiply
phosphorylated in response to stimulus, these serines may represent
the specific site of stimulus dependent phosphorylation.

**Novel roles for G protein–linked receptors.** Although many of
the genes required for prokaryotic chemotaxis have been identified
(37), the biochemical mechanisms of eukaryotic chemotaxis are less
well understood. On the basis of the primary sequence presented,
we propose a model for the cyclic AMP receptor, which structurally
relates a chemoattractant receptor to other G protein–linked recep-
tors. Since chemotaxis in Dictyostelium is a primitive sensory
process involved in cell-cell interactions, it is not unreasonable to
suppose that the cyclic AMP receptor and those involved in sensory
and endocrine systems of higher organisms evolved from a common
ancestor. Furthermore, structural similarities are likely to be found
among other eukaryotic chemoattractant receptors when their pri-

![Fig. 6.](image)

![Fig. 7.](image)

The cyclic AMP chemoattractant receptor is believed to play a
central role in the differentiation of Dictyostelium, regulating the
expression of genes in both early and late developmental stages.
Recent studies with mutants defective in signal transduction have

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16 SEPTEMBER 1988

RESEARCH ARTICLES 1471
suggested that the effect of cyclic AMP on early gene expression is mediated through a G protein–coupled receptor (38). The control of gene expression in a developing organism is a novel role for G protein–coupled receptors. This could be a general theme in development: embryonic inducers doubling as chemoeffectors and acting through G protein–coupled receptors. In this way, short-term stimuli could be integrated into long-term changes in the organism. Similar mechanisms may also play a role in neural plasticity. In Aplysia, a mechanism has been proposed for long-term information storage whereby transient stimuli, correlated with G protein–mediated activation of adenylate cyclase, effect long-term changes in synaptic junctions that are dependent on gene expression (39).

The recent advances in the transformation of Dictyostelium have expanded the possibilities of genetic manipulation of these relatively simple eukaryotic cells (19, 40, 41). In our experiments, transformation of Dictyostelium with an antisense construct of the cyclic AMP receptor cDNA has yielded cells that are severely impaired in the aggregation process. These data now raise the question of whether the disruption of receptor expression blocks development by inhibiting the expression of cyclic AMP–regulated genes required for early development or whether the failure to develop is because the cells are unable to respond to a chemoeffect or stimulator. Disruption of the cyclic AMP receptor, by antisense transformation and by homologous recombination, as well as replacement with receptor genes carrying defined mutations, can now be used to further elucidate the role of the receptor in both chemotaxis and development.

REFERENCES AND NOTES
17. The PAB18 vector was a gift of A. Kunita and R. Firth.  
30. Single letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; 
E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, 
R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.  
Acad. Sci. U.S.A. 84, 6 (1987); K. Takalo, S. B. Smith, E. G. Kebbs, A. Walh, 
33. A very strong amphiphilic stretch of sequence also exists downstream of G 
protein–mediated activation of adenylate cyclase, effect long-term changes in synaptic junctions that are dependent on gene expression (39).  
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38. T. Manni, S. F. Schatz, J. Sambrook, Molecular Cloning (Cold Spring Harbor 
York, 1982), vol. 15B, p. 61.  
44. Gift of B. Knox and G. Kharaz.  
45. We thank J. Natherns and D. Shortle for critical reading of the manuscript, R. Doolittle and P. Hargrave for helpful discussions. C. H. Su for providing the NC 
dNA library, D. Knecht and R. Firth for providing transformation vectors, and 
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