CAR2, a prestalk cAMP receptor required for normal tip formation and late development of Dictyostelium discoideum

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Extracellular cAMP serves as a primary signaling molecule to regulate the development of Dictyostelium discoideum. It is required for chemotaxis, aggregation, cytodifferentiation, and morphogenetic movement. The receptors for cAMP are members of the family of cell-surface receptors that are linked to G proteins and characterized by seven putative transmembrane domains. Previously, we have isolated the gene for the cAMP receptor subtype 1 (CAR1) from Dictyostelium and suggested that several genes related to CAR1 were present in the genome. Here, we describe a family of cAMP receptor genes of Dictyostelium and the isolation and function of the gene for the cAMP receptor subtype 2, CAR2. CAR2 is structurally similar to CAR1. Overall, their transmembrane and loop domains are ~75% identical in amino acid sequence; however, their carboxyl termini are quite dissimilar; CAR2 possesses homopolymeric runs of histidines and asparagines that are absent from the corresponding region in CAR1. Although CAR1 is maximally expressed during the early stages of development, CAR2 is expressed only after cells have aggregated and, then, preferentially in prestalk cells. Transgenic Dictyostelium that have had their wild-type CAR2 gene replaced by a defective copy using homologous recombination proceed through early development but are detained at the tight mound stage. CAR2 may be required for cAMP-directed sorting of prestalk cells during pattern formation within the aggregation mound. Furthermore, although prestalk genes are expressed normally in aggregates that lack CAR2, they exhibit an enhanced expression of prespore-specific mRNA. Previously, we had shown that there was a requirement for CAR1 during early development. The present results demonstrate that the multiple responses of Dictyostelium to cAMP are regulated by distinct cAMP receptors that are encoded by unique genes.

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The development of Dictyostelium discoideum is initially characterized by a transition from a unicellular, vegetative organism to a multicellular aggregate with accompanying cytodifferentiation (for review, see Kimmel and Firtel 1991; Williams 1991). As cells stream into loose aggregation mounds, distinct prestalk and prespore cell types become evident; these cells are the progenitors to the terminally differentiated stalk and spore cells found in mature fruiting bodies at the culmination of development. During aggregate formation, a cellular pattern is also established as the prestalk cells sort to the dorsal and ventral regions of the aggregate. A tip of prestalk cells then forms on the mound. This tip extends as the aggregate elongates into a migrating pseudoplasmodium, or slug, containing prestalk cells located primarily at the anterior or very posterior of the organism. Aggregation, differentiation, and pattern formation are regulated by response to extracellular cAMP. The extracellular cAMP serves as a signaling molecule to promote the accumulation of the intracellular messengers, cAMP, IP3, Ca+++, and cGMP, by activating, respectively, adenyl cyclase, phospholipase C, Ca+++ transport, and guanylyl cyclase (Dinauer et al. 1980; Europe-Finner and Newell 1987; van Haastert et al. 1989; Milne and Coukell 1991). As a chemoattractant, cAMP mobilizes cells for adhesion and directs cell sorting within the aggregate (Konijn et al. 1967; Traynor et al. 1992). Extracellular cAMP is also required, along with other factors.
Studies using cAMP analogs have indicated that in most instances exogenous cAMP acts initially at surface receptors and not intracellularly (Schaap and van Driel 1985, Gomer et al. 1986, Haribabu and Dottin 1986, Oyama and Blumberg 1986; Kimmel and Carlisle 1986). Dictyostelium development thus appears to be regulated by multiple responses to cAMP acting through different receptor-mediated pathways. One of these pathways cycles through a responsive and desensitized state that is regulated by the low-level oscillations in extracellular cAMP that are observed during aggregation. Another pathway appears to be activated by continuous exposure to higher cAMP levels. It has not been clear whether these various responses result from the interaction of the same cAMP receptor form with multiple intracellular components or of distinct receptor subtypes. We had presented suggestive evidence in support of the latter (Saxe et al. 1991a). Here, we characterize a family of cAMP receptor genes related to CAR1 and, in particular, describe the isolation of a gene, CAR2, that encodes a cAMP receptor expressed after aggregation that plays an important role during the later stages of Dictyostelium development. These results demonstrate that a series of sequentially expressed cAMP receptors regulate the development of Dictyostelium.

**Results**

**The cAMP receptor gene family of Dictyostelium**

Previously, we have described the isolation of CAR1, a cAMP receptor gene from Dictyostelium that is expressed at high levels during the initial stages of development (Klein et al. 1988, Saxe et al. 1991a). Under stringent conditions, CAR1 probes hybridize with a simple pattern to Dictyostelium genomic DNA blots indicative of a single-copy gene. When CAR1 is hybridized at reduced stringency (25% formamide), a more complex pattern of bands is detected (see Fig. 1). From such data we suggested that multiple CAR genes are present in the genome of D. discoideum. To examine this further, we screened genomic and cDNA libraries at low stringency using a CAR1 cDNA probe and isolated recombinants that hybridized relatively poorly to CAR1. When probes CAR2 and CAR3 from different presumptive cAMP receptor sequences were hybridized at high stringency (50% formamide) to genomic blots of Dictyostelium DNA, they detected a subset of the bands that had originally hybridized only weakly to CAR1 (see Fig. 1).

Figure 1 also shows that all of the bands that cross-hybridize with CAR1 are not accounted for by CAR2 and CAR3, suggesting the presence of an additional, related cAMP receptor gene. In a separate communication we discuss the characterization and function of the final member of this gene family, CAR4 [J. Louis, G. Ginsburg, and A. Kimmel, unpubl.]. Although, these results limit the size of this family of receptors, they do not exclude the presence of more distantly related receptor species.
**Figure 1.** Hybridization of CAR Probes to *D. discoideum* genomic DNA. Genomic DNA was digested with *XbaI* plus *SstI* or *Scal* plus *BstXI* restriction enzymes, separated by gel electrophoresis, and blotted for hybridization with probes from CAR1, CAR2, or CAR3 DNAs. Hybridization reactions were incubated at 37°C in 0.8 M Na+ with either 25% formamide (25%F) or 50% formamide (50%F), as indicated. Arrows indicate restriction fragments for the additional cAMP receptor gene CAR4.

**Sequence analysis of CAR2**

To determine the structure of CAR2 we sequenced both genomic and cDNA copies. Figure 2 shows the nucleic acid sequence of the entire protein-coding region and a portion of the 5'- and 3'-untranslated regions. Except for a small (101-bp), A+T-rich intron located within the protein-coding region at the position equivalent to amino acid 100, the genomic and cDNA sequences are colinear. The deduced amino acid sequence of CAR2 indicates a protein of 375 amino acids. A most striking characteristic is the extended homopolymeric runs of histidines and asparagines located in the carboxy-terminal region. These derive from repeating runs of the codons CAT and AAT, respectively. We and others have noticed similar homopolymeric runs of repeating trinucleotides in the coding as well as noncoding regions of several *Dictyostelium* genes (Kimmel and Firet, 1985; Shaw et al. 1989).

Although the cross-hybridization data strongly suggest that CAR2 is closely related to CAR1, they are, nonetheless, insufficient to confirm that CAR2 is a cAMP receptor. We have shown that vegetatively growing *Dictyostelium* that ectopically express CAR2 newly acquire several thousand cAMP-binding sites on their cell surfaces (Johnson et al. 1992), and Johnson et al. [this issue] have demonstrated the photoaffinity labeling of expressed CAR2 protein by cAMP and its in vivo phosphorylation in cAMP-stimulated cells. Thus, CAR2 encodes a surface cAMP receptor.

**CAR2 is structurally similar to G protein-coupled receptors**

The family of G protein-linked receptors are characterized by a common structural organization (see Klein et al. 1988). All of these receptors have seven regions that are enriched in hydrophobic amino acid residues. It is suggested that these regions form helices that span the lipid bilayer. We had shown previously that the deduced amino acid sequence of CAR1 included seven such regions that shared 20% amino acid identity with the seven putative transmembrane domains of bovine rhodopsin, another cell-surface receptor that couples to G proteins (Klein et al. 1988).

Figure 3 shows an amino acid alignment of CAR2 with CAR1. The regions are compared according to the putative structural domains of CAR1. A 2-residue gap in the first extracellular loop is the only spacing difference required to align the two proteins. The regions with greatest amino acid identities are located within the transmembrane domains, indicating that CAR2 is structurally similar to G protein-coupled receptors.
membrane and cytoplasmic loop domains. Overall, these regions are >70% identical in amino acid sequence, although certain regions are clearly more related than others. When conservative amino acid comparisons are also considered, CAR1 and CAR2 are ~85% similar in the transmembrane and loop domains. Although considerable sequence identity extends partially into the carboxyl terminus, the ~70 amino acids at the carboxyl terminus and the entire amino terminus of CAR2 do not share sequence identity with CAR1. The homopolymeric runs of histidines and asparagines observed in the carboxyl terminus of CAR2 are absent from CAR1. Although seryl residues are distributed throughout the third cytoplasmic and carboxy-terminal domains of CAR2, the clusters of seryl residues characteristic of CAR1 are not observed. Two potential phosphorylation sites in this region are shared by CAR1 and CAR2 (see Fig. 3). The single potential target (Arg-X-Ser) for protein kinase A phosphorylation located in the third cytoplasmic loop of CAR2 is absent in the corresponding region of CAR1 but is similarly localized in CAR3 (see Fig. 3).

Because the transmembrane domain regions of CAR2 and CAR1 were not entirely identical, it was necessary to show that the CAR2 domains were nevertheless sufficiently enriched in hydrophobic residues to theoretically span a lipid bilayer. When analyzed in conjunction with CAR1, the hydropathicity plots are nearly identical. Predominant hydrophilic regions of each protein represent cytoplasmic domains.

Structural similarities among the cAMP receptor gene family

In Johnson et al. [this issue], we describe the sequence and structural analysis of CAR3. CAR2, CAR1, and CAR3 comprise distinct subtypes of the G protein-linked cAMP receptor family of *D. discoideum* and are predicted to form substantially identical structures. Their close similarity is evident, although certain transmembrane and loop domains are more conserved than others. Conceivably the conserved or diverged regions are involved, respectively, in their shared or distinct functions.

Additional relationships among the CAR family members are seen with comparisons among their overall genomic organizations. Figure 4 combines genomic and protein structural information. Boxes indicate transmembrane domains, and lines indicate intra- and extra-
Figure 4. Comparative gene and protein structures of CAR2, CAR1, and CAR3. The single-line backbones represent lengths of the receptor proteins; boxes indicate positions of the putative transmembrane domains. Also shown are the site and size of the conserved intron in transmembrane domain 3 and the relative positions of several shared and diverged restriction enzyme sites.

Cellular domains. In addition, major shared (or diverged) restriction enzyme sites, as well as a conserved intron site within the protein-coding regions, are shown. But for the differences in lengths of the amino- and carboxy-terminal regions, the transmembrane and loop domains are spaced nearly identically among all receptor forms. The single intron within CAR2, CAR3, and CAR1 interrupts an equivalent codon of each gene located within the putative transmembrane domain 3 (Fig. 5). Although the introns differ in sequence, they are similar in size, A+T content (~95%), and splice junctions. The three genes are approximately equally diverged from one another and appear to derive from a common ancestral sequence.

Gene disruption of CAR2

The Dictyostelium marker THY1 (Dynes and Firtel 1989), which permits growth of cells in the absence of thymidine, was inserted into a genomic fragment of CAR2. The site of integration interrupted an amino acid sequence identically conserved in CAR1-4 [see Fig. 6A]. Such disrupted cAMP receptor genes cannot produce a functional receptor protein (Sun and Devreotes 1991). The Dictyostelium strain JH10 (Hadwiger and Firtel 1992) that is auxotrophic for thymidine was transformed with the linearized CAR2 fragment carrying the THY1 marker; cells were selected that were able to grow in the absence of exogenous thymidine. A polymerase chain reaction (PCR) screen was used to preliminarily identify transformants that had had their endogenous copy of CAR2 replaced by the disrupted construct. Confirmation was achieved by DNA blot hybridization [Fig. 6B]. Genomic DNA was isolated from the parental JH10 cells and from two PCR-selected cell lines. The DNAs were digested with the restriction enzyme XbaI, separated by gel electrophoresis, and blotted for hybridization to a CAR2-specific probe. CAR2 hybridizes to a wild-type-size, 2.4-kb DNA band derived from JH10 cells. This band is completely absent in the transformants. Instead, a new band is detected at 5.4 kb, which includes the CAR2 sequence and the 3′-kb thymidine marker, THY1 does not contain any XbaI sites. Thus, the transformants car2-ko1 and car2-ko6 have had their endogenous CAR2 gene substituted by a disrupted copy. The results indicate that no additional copies of the disrupted CAR2 gene were inserted into the genome and that the phenotypes of car2-ko mutants are solely the result of CAR2 disruption.

Previous data based on RNA blot hybridization, PCR amplification, and library screening suggest that the initial accumulation of CAR2 mRNA takes place at the approximate time of tip formation [Saxe et al. 1991b]. RNAs from the less dense, prespore cell fraction of slugs are highly enriched (~10-fold) in CAR2 mRNA relative to RNAs from the denser, prespore cells [Saxe et al. 1991b]. Furthermore, cytological staining for β-galactosidase activity using CAR2 promoter-lacZ fusions indicates CAR2 expression in prestalk cells (C. Saxe, unpublished).

We examined the expression pattern of CAR2 during development of parental and CAR2 disrupted cell lines [Fig. 7]. Poly[A]+ mRNA was isolated from Dictyostelium at major morphological stages, separated by gel electrophoresis under denaturing conditions, and hybridized at high stringency to a CAR2 probe. No CAR2 mRNA is detected in growing cells (0 hr), in aggregating cells (5 hr), or during early formation of multicellular aggregates (10 hr). After tip formation (15 hr), a single 2-kb CAR2 mRNA is expressed in the parental JH10. Similar levels of expression persist through the early stages of culmination (20 hr). Similar analyses of expression of CAR2 were performed using car2-ko1 and car2-ko6 cell lines.

Figure 5. Splice junction identities among CAR2, CAR3, and CAR1. Nucleotide and amino acid sequence comparisons surrounding the intron that interrupts the region encoding transmembrane domain 3. The nucleotide sequence is listed for CAR2, only those nucleotides that differ in CAR3 or CAR1 are indicated. The intron junction and remaining nucleotide lengths are in boldface type and underlined.
Prestalk cAMP receptor gene of Dictyostelium appear by 16 hr (see Fig. 8C, D). Greater than 90% of the original JH10 aggregates are able to complete development and form mature fruiting bodies by 29 hr. car2− cell lines exhibit abnormal patterns, with development arresting after tight aggregates form. Tips that are normally seen after 12 hr do not appear in car2− cells (Fig. 8E). After an extended period of time many car2− aggregates extend a finger-like projection, and eventually (>150 hr) produce a number of deformed fruiting bodies. Thus, the prestalk-specific, cAMP receptor CAR2 plays a key role during tip formation and late development, although it is possible to proceed through this stage, albeit abnormally, in its absence.

Figure 6. Procedure for generating the CAR2 disruption by homologous recombination. (A) Diagram of the CAR2 gene with sequences located upstream and downstream of the single XbaI site within CAR2 indicated by open bars and solid bars, respectively. Transformation vector containing the THY1 gene within CAR2 is indicated as are the regions predicted to undergo a double crossover. The resulting disrupted car2-ko containing the THY1 marker is also shown. (B) Blot hybridization of DNA from JH10 parental and car2-ko1 and car2-ko6. DNAs were digested with XbaI and hybridized with an upstream CAR2 probe (see Fig. 8A). The endogenous XbaI band of JH10 migrates at 2.4 kb; the disrupted car2-ko band is 5.6 kb.

Cells were developed in parallel with parental JH10 cells and RNAs isolated at identical times; however (see below), morphological structures were not equivalent. In neither car2− cell line could we detect the expression of a CAR2 mRNA species. Thus, the cell lines disrupted for CAR2 are completely defective in CAR2 production.

Aggregates of car2− cells overexpress prespore but not prestalk mRNA

We examined the expression of cell-type specific genes in developed car2− cells to determine whether the inability to form tip structures resulted from an inability to undergo cytodifferentiation. We selected markers with distinct spatial and temporal patterns of expression. The prestalk markers ecmA and ecmB are induced by DIF and initially appear in aggregated cells [Williams et al. 1987]. The ecmA gene is primarily expressed in the prestalk A cells that migrate apically during tip formation (see Williams 1991). The prestalk B cells sort to the base of the aggregation mound. During culmination, the levels of ecmB mRNA increase as prestalk A cells enter the stalk tube and begin to express ecmB. The 2H-6 and the prespore 14E-6 genes are induced by cAMP in shaking cultures but are maximally expressed at different developmental times (Mehdy et al. 1983; Ginsburg and Kimmel 1989).

Figure 9A shows developmental expression patterns for ecmA, ecmB, 2H-6, and 14E-6 mRNAs in the parental JH10 and car2−ko1 cell lines. The results for JH10 are typical for these genes. The prestalk markers ecmA and ecmB appear to induce normally in car2−ko1 cells. This would suggest that the prestalk A and B cells differentiate in the car2− cells but do not sort properly. Although ecmA reaches equivalent levels of expression in JH10 and car2−ko1 cells, ecmB is only expressed at low levels

Figure 7. Developmental expression of CAR2 mRNA in control and car2− Cells. JH10 and car2−ko1 cells were harvested in logarithmic phase [V], washed, plated on an agar surface, and allowed to proceed synchronously through development. At 5-hr intervals, cells were collected and RNA was isolated. The RNAs were size separated on denaturing gels and blotted for hybridization to CAR2.

Dictyostelium cells with a defective CAR2 develop abnormally and cannot form tips

JH10 cells and car2− cells were developed on agar plates, and their morphologies were monitored. car2−ko1 development precisely parallels control JH10 through the first 11 hr (Fig. 8A,B). Cells stream and form tight aggregation mounds with appropriate kinetics. Tip structures of JH10 are first observed at ~11 hr, and migrating slugs
Figure 8. Developmental morphologies of control and car2− cells. JH10 and car2−ko1 cells were harvested in logarithmic phase, washed, plated on filter pads, and allowed to proceed synchronously through development. (A) JH10 cells at 9 hr of development; (B) car2−ko1 cells at 9 hr of development; (C) JH10 cells at 12 hr of development; (D) JH10 cells at 19 hr of development; (E) car2−ko1 cells at 19 hr of development.

Discussion

Aggregation and differentiation of Dictyostelium are in part regulated by extracellular cAMP acting as a chemoattractant and first messenger [see Kimmel and Firtel 1991]. Many of the effects of extracellular cAMP are mediated through cell-surface receptors linked to G proteins. On the basis of low-stringency hybridization of Dictyostelium genomic DNA with a probe for cAMP receptor CAR1, we had suggested previously the presence of a family of cAMP receptor genes, but it had been unresolved whether the multiple roles of extracellular cAMP were regulated by different receptor subtypes with separate functions [Saxe et al. 1991a]. In this paper and in Johnson et al. [this volume] we describe the isolation of CAR2 and CAR3, two new members of that gene family and assign to them distinct roles for cAMP regulation during the development of D. discoideum.

Ectopic expression of CAR1, CAR2, and CAR3 has established them as bona fide cAMP cell-surface receptors, and hydropathy analysis suggests that they similarly span the membrane seven times and couple to G proteins [Johnson et al. 1992]. A comparison of the deduced amino acid sequences of these receptors shows ~75% identity through the transmembrane and loop domains and partially into the cytoplasmic carboxyl termini. The carboxy- and amino-terminal regions, however, are
CAR2, CAR3, and CAR4 but not CAR1 [Klein et al. 1988; Johnson et al. 1992; J. Louis, G.T. Ginsburg, and A.R. Kimmel, unpubl.]. Similarly, CAR2, CAR3, and CAR4 contain homopolymeric amino acid clusters in their carboxy-terminal region [Klein et al. 1988; Johnson et al. 1992; J. Louis, G.T. Ginsburg, and A.R. Kimmel, unpubl.]. Such homopolymers are seen in a number of other Dictyostelium and non-Dictyostelium proteins [Kimmel and Firtel 1985]. The significance, if any, of these is unknown.

CAR2 and CAR1 have somewhat complementary patterns of expression. CAR1 is maximally expressed at 5–10 hr of development. CAR1 mRNA levels then decline dramatically, although CAR1 mRNA is still detected at culmination. In contrast, CAR2 is only expressed in postaggregation stages. Nonetheless, the levels of CAR2 and CAR1 mRNAs are similar at these late stages of development after cytodifferentiation and pattern formation have occurred. CAR1 mRNA is expressed throughout development, whereas CAR2 is a prestalk marker.

The expression patterns of CAR1 and CAR2 would suggest different developmental roles for each. CAR1 is maximally expressed during aggregation, when chemotaxis and cAMP relay and signaling are maximal [Saxe et al. 1991a]. Consistent with this pattern of expression, disturbance of CAR1 expression by either antisense RNA or gene disruption causes development to arrest before aggregation [Klein et al. 1988; Sun and Devreotes 1991]. CAR2 RNA, on the other hand, is expressed only after aggregation is completed and loss of CAR2 expression has no effect on the ability of the organism to aggregate, but starting with tip formation, postaggregative development and morphogenesis are strongly affected.

A surprising phenotype of car2− cells is the capacity to overexpress the prespore-specific gene 14E-6. Because only ~20% of differentiated cells are normally prestalk, this phenotype cannot be explained simply by an alteration in cell proportioning and a recruitment of prestalk cells into a prespore pathway. Furthermore, prestalk genes appear to be expressed similarly in both parental and car2− cell lines. Although data would indicate otherwise, it is possible that only a small percentage of prespore cells express 14E-6 mRNA. This population may be larger in car2− cell lines. It would also seem that this phenotype cannot be attributed to a difference in extracellular cAMP metabolism because overexpression of 14E-6 is observed in shaking cultures treated with saturating concentrations of exogenous cAMP. Localization studies of CAR2 gene expression would suggest that the modulation of prespore gene expression by CAR2 must be mediated through prestalk cells. Overexpression of prespore genes may result indirectly from aberrant positioning of prestalk cells within the aggregation mound, although such an effect must be mimicked in agglomerates that form during differentiation in shaking cultures. DIF will inhibit prespore expression, and it is possible that DIF levels are affected in car2− cells [Berks and Kay 1990]. However, such an effect cannot be sufficient to diminish the positive role DIF plays in the induction of

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**Figure 9.** Expression of developmentally regulated genes in control and car2− cells. (A) JH10 and car2−ko1 cells were harvested in logarithmic phase [V], washed, plated on an agar surface, and allowed to proceed synchronously through development. At 5-hr intervals, cells were collected and RNA was isolated. The RNAs were size separated on denaturing gels and blotted for hybridization to probes for the prestalk genes ecmA and ecmB, the prespore gene 14E-6, and the cAMP-regulated gene 2H-6. (B) JH10 and car2−ko1 cells were harvested in logarithmic phase [V], washed, and differentiated in liquid culture for 10 hr in the absence (−) or presence (+) of cAMP. The RNAs were size separated on denaturing gels and blotted for hybridization to a probe for the prespore gene 14E-6.

Largely distinct both in overall length and sequence, the extremely close similarity in gene organization among these receptors suggests a relatively recent divergence from a common ancestor.

CAR2, CAR3, and CAR1 can undergo ligand-induced phosphorylation. The carboxy-terminal region of CAR1 contains clusters of seryl residues that have been implicated in ligand-induced phosphorylation of CAR1 [Klein et al. 1988]. Of these, only serines at positions 298 and 303 are also present in CAR2 [see Fig. 3]. It is noteworthy that these two serines are conserved as well in CAR3 and in CAR4, although any ascribed function is speculative [Klein et al. 1988; Johnson et al. 1992; J. Louis, G.T. Ginsburg, and A.R. Kimmel, unpubl.]. A weak consensus [Arg-X-Ser] sequence for the cAMP-dependent protein kinase is also present in the third cytoplasmic loop of
ecmA. Alternatively, an extremely low-level expression of CAR2 in prespore cells may directly regulate prespore gene expression. It is also clear that CAR2 is not essential for the induction of expression of the cAMP-regulated genes 2H-6, 14E-6, and 10C-3 by exogenous cAMP [Mehdy et al. 1983; Ginsburg and Kimmel 1989]. Data suggest that such cAMP regulation is mediated by cell-surface cAMP receptors [Schaap and van Driel 1985; Gomer et al. 1986; Haribabu and Dottin 1986; Kimmel and Carlisle 1986; Oyama and Blumberg 1986; Kimmel 1987]. Because car3– cells are capable of completing development [Johnson et al., this issue] it appears that receptors other than CAR2 and CAR3 are required for the observed regulation of gene expression by extracellular cAMP.

The differences in phenotypes between CAR1 and CAR2 gene disruptions underscore additional points about cAMP effects on development. Mutation of either gene causes an impairment of development. The receptors are clearly mediating significant and separate decisions for Dictyostelium development. The binding of cAMP to both of these receptors, therefore, is critical for proper developmental progress. Because CAR1 and CAR3 are expressed at similar, albeit low, levels in control and car2– cells, they must not be interchangeable with CAR2.

The level of extracellular cAMP varies dramatically during aggregation. In the first few hours extracellular concentrations remain relatively low. However, by the time tight mounds form, cAMP levels have risen >10-fold [Abe and Yanagasawa 1983]. At that stage, maximal signaling is restricted to the upper surface of the aggregate, a site to which the prestalk cells are primarily chemotactic [Siegert and Weijer 1991, 1992]. In parallel with the increase in extracellular cAMP, cells alter their pattern of receptor sensitivity to cAMP. The relative affinity of CAR1 for cAMP is >10 times higher than is observed for CAR2 [Johnson et al. 1992]. Consistent with these observations it is possible that the low-affinity, prestalk-specific receptor CAR2 is involved in cAMP-directed patterning of prestalk cells as they sort before and during tip formation. Conversely, cAMP levels at this time may be saturating for both CAR1 and CAR3; thus, they would be unable to monitor a cAMP gradient necessary for directed movement. It is interesting that aggregates that overexpress PDE have slightly reduced levels of cAMP and are unable to form normal tipped structures [Traynor et al. 1992]. Yet, these prestalk cells retain their ability to migrate toward an ectopic cAMP source.

The three cAMP receptors may interact with different intracellular components and/or activate different signaling pathways. Alternatively, CAR1, CAR2, and CAR3 may activate similar pathways but only at restricted sensitivities to extracellular cAMP. The close structural and sequence similarities among the three receptor forms suggest common interactions with G proteins. Recently, genes for the α subunits of G proteins have been isolated from Dictyostelium [Pupillo et al. 1989; Hadwiger et al. 1991; Wu and Devreotes 1991]. Some of their expression patterns specifically overlap that of CAR2. It will be of interest to establish whether any of these G protein components interact specifically with CAR2, CAR1, and/or CAR3 during postaggregative Dictyostelium development or whether they share a common pathway. The cell lines disrupted for each of the reported CAR genes can be used to address such questions.

Materials and methods

Growth and development of cells

D. discoideum wild-type strain was grown on bacteria and developed on filters as described previously [Ginsburg and Kimmel 1989]: thy1– parental and THY1 selected strains were grown in axenic liquid media with or without 100 μg/ml of thymidine [Dynes and Firtel 1989; Hadwiger and Firtel 1992]. Cells were developed on agar plates at a surface density of 107 cells/cm2 or differentiated in shaking culture at a density of 106 cells/ml in 10 mm sodium phosphate [pH 6.4], 2 mm MgCl2, 0.2 mm CaCl2. For cAMP treatment, shaking cultures received 20-nM pulses of cAMP at 6-min intervals for 3 hr; subsequently, the cultures were adjusted to and maintained at 300 μM cAMP for the remainder of differentiation [Kimmel 1987].

DNA-mediated transformation of Dictyostelium

The thy1– cell line JH10 was transformed to THY1 essentially as described [Hadwiger and Firtel 1992]. Greater than 50% of the transformants isolated exhibited homologous recombination within the CAR2 locus.

Isolation and hybridization of RNA and DNA

Total and poly[A]+ RNA were prepared from cells as described previously [Kimmel 1987]. RNA was size separated on formaldehydeagarose gels and transferred to nitrocellulose [Kimmel 1987]. Genomic DNA was isolated as described [Kimmel and Firtel 1985]. DNA was size separated on agarose gels, transferred to nitrocellulose, and hybridized to radiolabeled probes. Probes were radiolabeled by the random primer method using [α-32P]-dCTP and hybridized to DNA blots, RNA blots, genomic libraries, and cDNA libraries. Hybridizations were performed at 37°C in 0.8 mM Na+ . High stringency buffers additionally contained 50% formamidc, and reduced stringency buffers contained 25% formamide.

DNA sequencing

Sequencing was done by the dideoxy chain-termination method [Saxe et al. 1991a] as applied to double-stranded DNA.

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Note added in proof

The nucleotide and amino acid sequence data for CAR2 have been submitted to the EMBL/GenBank data libraries.

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