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.

[*Key Words*: cAMP; receptors; gene expression; *Dictyostelium*]

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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, IP_3, Ca^{++}, and cGMP, by activating, respectively, adenylyl 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

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[e.g., differentiation inducing factor (DIF)], for developmentally regulated gene expression and cytodifferentiation (Schaap and van Driel 1985; Gomer et al. 1986; Haribabu and Dottin 1986; Kimmel and Carlisle 1986; Oyama and Blumberg 1986; Kimmel 1987; Williams et al 1987).

Appropriate cell density and depletion of amino acids from growth media trigger the initial phases of development and secretion of cAMP (see Gomer et al. 1991; Rathi et al. 1991). Cells begin to accumulate surface receptors that bind extracellular cAMP specifically and are linked through G proteins to the downstream effectors, including adenylyl cyclase (Schaap and Spek 1984; Theibert et al. 1986). Intracellular cAMP levels increase in response to receptor stimulation, and cAMP is secreted to relay the cAMP signal. With continued stimulation, receptors become desensitized and concomitantly hyperphosphorylated. Extracellular cAMP is degraded by membrane-bound and secreted phosphodiesterase (PDE), which attenuates the signal (see Franke et al. 1991). Receptor phosphorylation levels decrease, receptors regain sensitivity, and a new wave of cAMP signal–relay ensues (Klein et al. 1985, 1987; van Haastert 1987). These oscillating waves appear with periodicities of ~6 min. Providing shaking cultures of *Dictyostelium* with exogenous 20 nM pulses of cAMP at 6-min intervals to mimic normal oscillations promotes the early developmental events that are regulated by cAMP receptors. These include adenylyl cyclase activation and cGMP synthesis. If, however, cAMP is presented as a nonfluctuating stimulus during early development, these responses adapt and receptors down-regulate.

Secreted cAMP is also essential for cytodifferentiation and pattern formation (Schaap and van Driel 1985). Certain responses to cAMP in postaggregated cells may be fundamentally different than those during aggregation. Although many genes require pulses of cAMP for their appropriate developmental regulation early during development, genes expressed specifically in prespore cells may respond to a continuous, exogenous cAMP stimulus (Schaap and van Driel 1985; Gomer et al. 1986; Haribabu and Dottin 1986; Kimmel and Carlisle 1986; Oyama and Blumberg 1986; Kimmel 1987).

cAMP also directs the differential sorting of prestalk and prespore cells during aggregate formation (Traynor et al. 1992). Initially, both cell types appear scattered throughout the aggregation mound. Prestalk cells then begin a spiral migration toward the center of the mound surface in a pattern similar to that observed during aggregation (Esch and Firtel 1991; Siegert and Weijer 1991). This is consistent with prestalk cell movement to the presumptive tip in response to cAMP pulses (Traynor et al. 1992). The differences in movement of the prestalk and prespore cells are also pronounced within the migrating slug (Siegert and Weijer 1991, 1992). Prestalk cells continue to move in a spiral-like pattern within the slug anterior and perpedicular to the longitudinal axis. The periodic movement and shape change of these cells is, again, indicative of a role for cAMP in patterning. In contrast, the prespore cells move parallel to the anterior–posterior axis.

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 1987). *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.

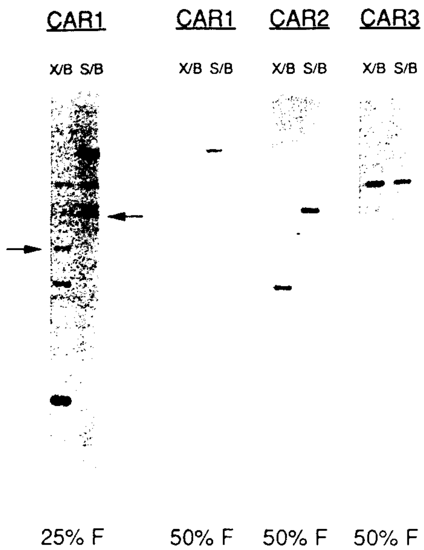

Figure 1. Hybridization of CAR Probes to *D. discoideum* genomic DNA. Genomic DNA was digested with *XbaI* plus *BstXI* (X/B) or *ScaI* plus *BstXI* (S/B) restriction enzymes, separated by gel electrophoresis, and blotted for hybridization with probes from *CAR1*, *CAR2*, or *CAR3* DNAs. Hybridization 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 trinucleotide sequences in the coding as well as noncoding regions of several *Dictyostelium* genes (Kimmel and Firtel 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 hundred 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 trans-

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                      AAAATAAATAAATAAAATCGAAAATAAATAATAATTAAAATATAAATATAAAATTTTATATATTTGTATA

GAAAAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAATAATAATTTTTTATTATTATTTAAATATATATAGAATTAAAAA

ATG ACT ATT ATG TCA GAT ATT ATC GCA CAA AGA ACA ATC TTG CTA ATC GCG GAT TTT TCA TCG ATT
 M   T   I   M   S   D   I   I   A   Q   R   T   I   L   L   I   A   D   F   S   S   I

ATT GGA TGT TCT TTG GTT TTA ATT GGA TTT TGG AGG TTA AAA CTT TTA CGT AAT CAC ATT ACA AAA
 I   G   C   S   L   V   L   I   G   F   W   R   L   K   L   L   R   N   H   I   T   K

ATT ATA TCA TTA TTT TGC GCA ACT TCT CTA TTT AAA GAT GTA ATA TCA ACA ATT ATC ACA TTA CTG
 I   I   S   L   F   C   A   T   S   L   F   K   D   V   I   S   T   I   I   T   L   L

TAT AAA CCC GAC CAA ACA GAA TCT GGT TTT CCT TGT TAT CTT CAT GCA ATA GTA ATT ACT TTT GGT
 Y   K   P   D   Q   T   E   S   G   F   P   C   Y   L   H   A   I   V   I   T   F   G

TCA TTA GCA TTG TGG CTT TGG ACT CTT ATG TTA T  GTATGTTTTTTTATTTTTTTATTTTCCTTATAAAAAAAA
 S   L   A   C   W   L   W   T   L   M   L  S

AAAAAAAAAAAAAATTAAAAAAAATTAATATTAATATTAAAATTTTTTTTTTTTAATTAAAATAG  CA TTT AGT ATT TAT AAT
                                                                  (S) F   S   I   Y   N

TTA ATA GTA AGA AGA GAA CCA GAA CCA GAA AGA TTT GAA AAA TTT TAT TTT TGT CTT TGT TAT GGA
 L   I   V   R   R   E   P   E   P   E   R   F   E   K   F   Y   F   C   L   C   Y   G

TTA CCA TTG ATT AGT ACG ATT GTT ATG TTA TCA ACA CAT ATT ATT CAA CCA GTT GGT GGT TGG TGT
 L   P   L   I   S   T   I   V   M   L   S   T   H   I   I   Q   P   V   G   G   W   C

TGG ATT GGT GAT AAC TAT GAT GGG TAT AGA TTT GGA TTA TTT TAT GGG CCA TTC TTT TTC ATT TGG
 W   I   G   D   N   Y   D   G   Y   R   F   G   L   F   Y   G   P   F   F   F   I   W

GGA ACA TCA GCA ATT TTG GTT GGA TTA ACA TCG AAA TAT ACA TAC TCT GTA ATA CGT AGT AGT GTA
 G   T   S   A   I   L   V   G   L   T   S   K   Y   T   Y   S   V   I   R   S   S   V

TCT GAT AAT AAG GAT AAA CAT ATG ACC TAT CAA TTT AAA TTG ATA AAT TAT ATC GTT GTA TTT TTG
 S   D   N   K   D   K   H   M   T   Y   Q   F   K   L   I   N   Y   I   V   V   F   L

GTT TGT TGG GTA TTT GCA ATC GTT AAT CGT ATT CTA AAT GGT TTA AAT CAA TTT CCA ACG GTT CCA
 V   C   W   V   F   A   I   V   N   R   I   L   N   G   L   N   Q   F   P   T   V   P

AAT GTT TTA CAC ACC TAT TTC AGT GTT TCC CAC GGT TTC TAT GCT TCC ATC ACT TTT ATT TAC AAT
 N   V   L   H   T   Y   F   S   V   S   H   G   F   Y   A   S   I   T   F   I   Y   N

AAT CCA TTG ATG TGG CGT TAC TTT GGT GCA AAA TTC TTA TTG ATC TTT AGT AAA TTT GGT TTA TTT
 N   P   L   M   W   R   Y   F   G   A   K   F   L   L   I   F   S   K   F   G   L   F

GTT CAA GCT CAA CAA CGT TTG GAA TTA AAT AAA AAT AAT AAT AAT CCA TCA CCA ATT ATG AGA TCA
 V   Q   A   Q   Q   R   L   E   L   N   K   N   N   N   N   P   S   P   I   M   R   S

AAG AAT GCT CTA GAT AAT GGT GCC GAC TCT TCG GTT GTA GAA TTA CCT TGT CTC TCA AAA GCT GAT
 K   N   A   L   D   N   G   A   D   S   S   V   V   E   L   P   C   L   S   K   A   D

TCT TTA TCA CTT GAT GCT GAA AAT AAT ATT GAA ACT CCA AAA GAA AAT GAA AAT CAA AAT CAT CAT
 S   L   S   L   D   A   E   N   N   I   E   T   P   K   E   N   E   N   Q   N   H   H

CAT CAT CAT CAT CAT CAT CAT CAT CAT AAT CAT TAT AAT AAT AAT AAC AAT AAT AAT AAT ATA AAT
 H   H   H   H   H   H   H   H   H   N   H   Y   N   N   N   N   N   N   N   N   I   N

AAT AAA AAT GAT ATG ATT  TAAAAGTGATAATAAAATAAATAATAAATAATCAATAATCAATAATAAGGTTCAATATATTC
 N   K   N   D   M   I

ACACTAAACGGATTACTTTTTTTTGAAAAAAAAAAAAAAAAAAAAAAGAAAAAAAAAAAAAAAAG
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Figure 2. Nucleotide and derived amino acid sequences of *CAR2*. The entire protein-coding region of *CAR2* is indicated along with 5'- and 3'-untranslated sequences. The homopolymeric runs of histidyl and asparginyl residues at the carboxyl terminus of the *CAR2* protein are indicated by underline. The single intron of 101 bp is in boldface type and underlined.

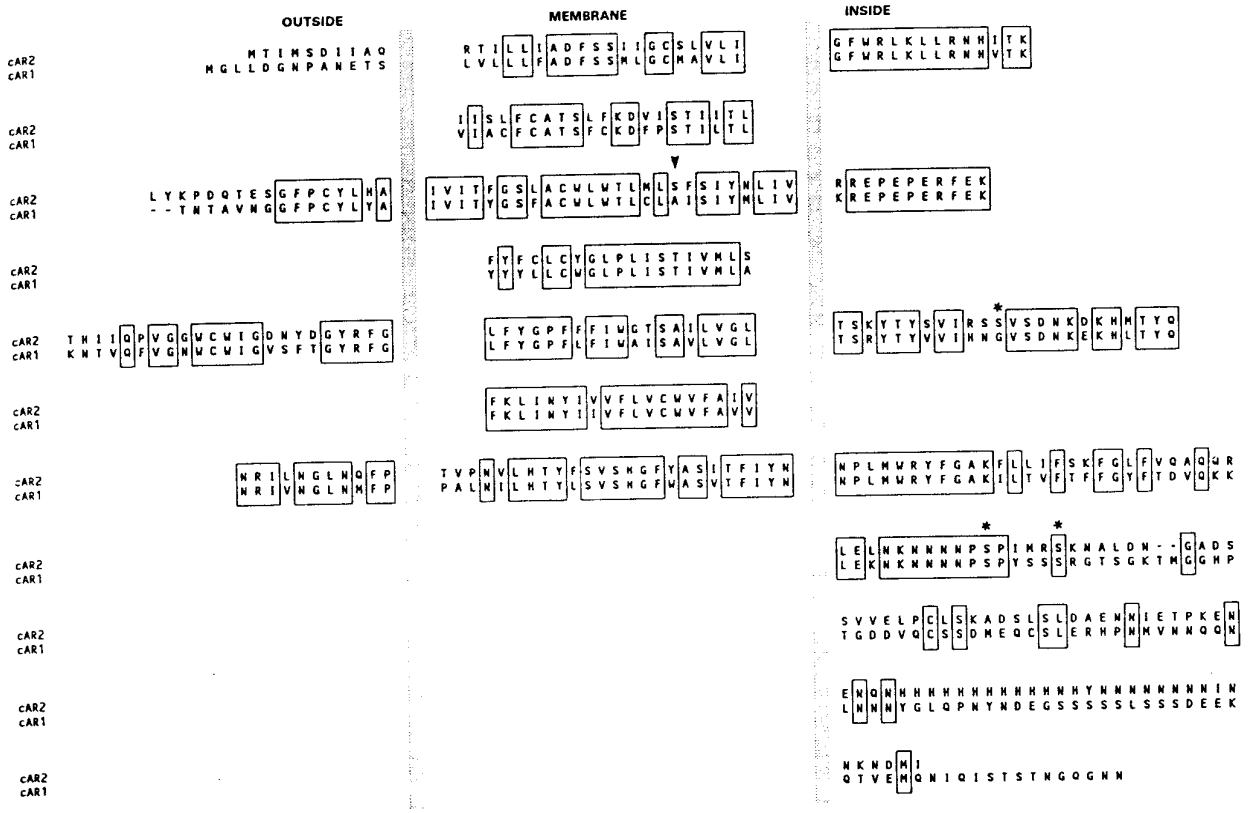

Figure 3. Amino acid sequence comparison between CAR2 and CAR1. Amino acid identities are enclosed in boxes. Sequences are displayed with extracellular (outside) loops, intracellular (inside) loops, and transmembrane domains according to the topological structure predicted for CAR1 (Klein et al. 1988). The splice junction conserved in both genes is indicated by the downward arrowhead; an asterisk (*) indicates potential seryl phosphorylation sites for CAR2.

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-

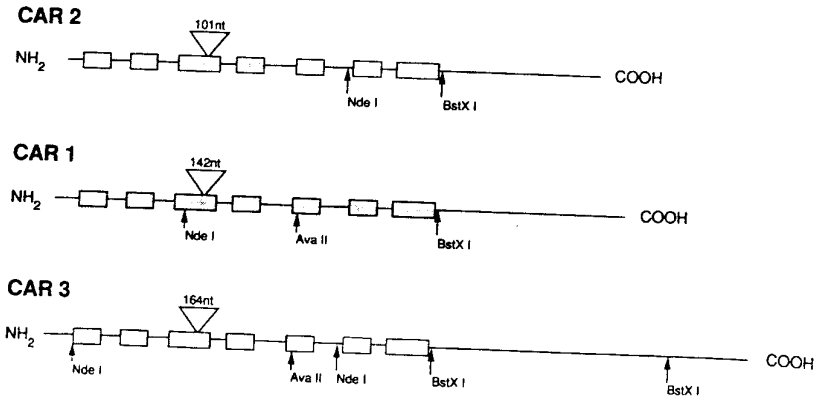

Figure 4. Comparative gene and protein structures of CAR2, CAR1, and CAR3. The single-line backbones represents lengths of the receptor proteins; boxes indicate positions of the putative transmembrane domains. Also shown are the site and size of the conserved intron in transmembrane domain 3 and the 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 is 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, prestalk 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, unpubl.).

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.

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CAR2  Leu  Met  Leu  Ser
      CTT  ATG  TTA  T     GTATGT - - - 92bp - - - TAG   (Ser) Phe  Ser  Ile
                                                        CA    TTT  AGT  ATT

CAR3  Leu  Cys  Leu  Ser
      T A  TGT                GTAAGT - - - 155bp - - - CAG   (Ser) Phe  Ser  Ile

CAR1  Leu  Cys  Leu  Ala
      T A  TGT  C T  G       GTAAGT - - - 133bp - - - TAG   (Ala) Ile  Ser  Ile
                                                        A
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Figure 5. Splice junction identitites 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.

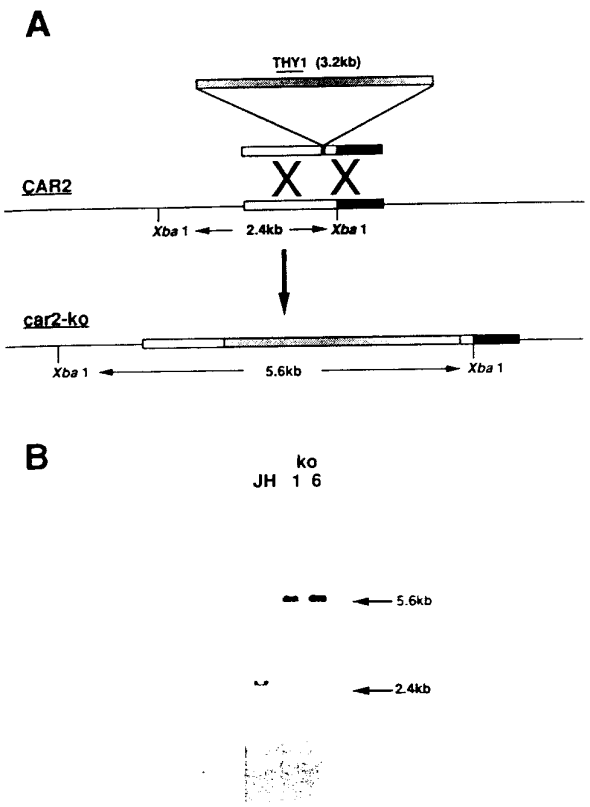

Figure 6. Procedure for generating the *CAR2* disruption by homologous recombination. (*A*) Diagram of the *CAR2* gene with sequences located upstream and downstream of the single *Xba*I site within *CAR2* indicated by open bars and solid bars, respectively. Transformation vector containing the *THY1* 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 *Xba*I and hybridized with an upstream *CAR2* probe (see Fig. 8A). The endogenous *Xba*I band of JH10 migrates at 2.4 kb; the disrupted *car2–ko* band is 5.6kb.

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.

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 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.

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* apppear to induce normally in *car2–ko1* cells. This would suggest that the prestalk A and B cells differentiate in the *car2*$^-$ cells b o not sort properly. Although *ecmA* reaches equivalent levels of expression in JH10 and *car2–ko1* cells, *ecmB* is only expressed at low levels

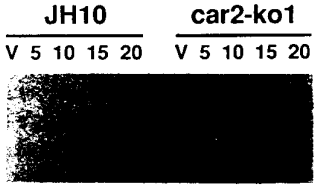

Figure 7. Developmental expression of *CAR2* mRNA in control and *car2*$^-$ Cells. JH10 and *car2–ko1* cells were harvested in logarithmic phase (V), washed, plated on an agar surface, and allowed to proceed synchronously through development. At 5-hr intervals, cells were collected and RNA was isolated.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

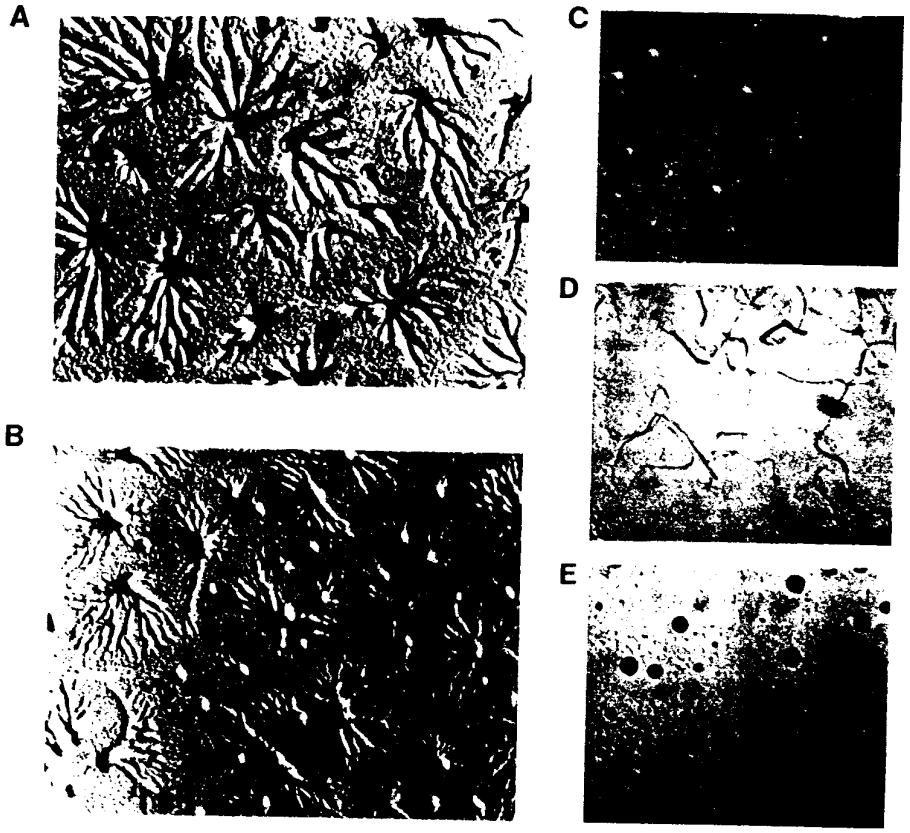

Figure 8. Developmental morphologies of control and *car2*$^-$ cells. JH10 and *car2–ko1* cells were harvested in logarithmic phase, washed, plated on filter pads, and allowed to proceed synchronously through development. (*A*) JH10 cells at 9 hr of development; (*B*) *car2–ko1* cells at 9 hr of development; (*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.

in *car2*$^-$ lines. Because the *car2*$^-$ lines do not culminate, it seems likely that high levels of expression are not acheived because of the inability to further increase *ecmB* expression during stalk tube formation.

2H-6 expression is regulated comparably in both cell lines. The slightly diminished expression observed in *car2–ko1* is probably not significant. Conversely, a dramatic difference in expression level is observed for the prespore gene 14E-6. Here, 14E-6 mRNA accumulates to levels that are ~10-fold greater in *car2–ko1* cells than in parental cells, although its temporal regulation is similar in both strains.

To determine whether the regulation of 14E-6 was similarly affected in shaking culture, we differentiated *car2–ko1* cells in the presence of a saturating concentration (300 μM) of exogenous cAMP. Again, we observe a significant overexpression of 14E-6 in *car2*$^-$ cells compared with JH10. No differences in expression were seen for the cAMP-regulated genes 2H-6 or 10C-3 that are not prespore specific.

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

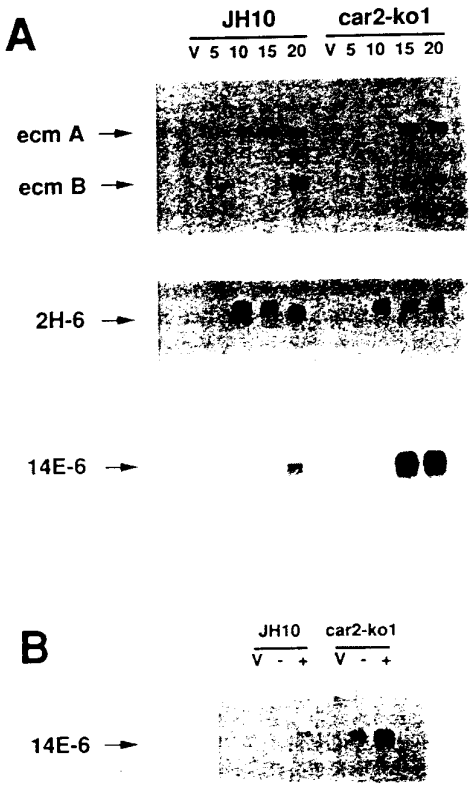

Figure 9. Expression of developmentally regulated genes in control and *car2*$^-$ cells. (*A*) JH10 and *car2–ko1* cells were harvested in logarithmic phase (V), washed, plated on an agar surface, and allowed to proceed synchronously through development. At 5-hr intervals, cells were collected and RNA 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 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

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 10^7 cells/cm^2 or differentiated in shaking culture at a density of 10^7 cells/ml in 10 mM sodium phosphate (pH 6.4), 2 mM $MgCl_2$, 0.2 mM $CaCl_2$. 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^{32}P]-dCTP and hybridized to DNA blots, RNA blots, genomic libraries, and cDNA libraries. Hybridizations were performed at 37°C in 0.8 M Na^+. High stringency buffers additionally contained 50% formamide, and reduced stringency buffers contained 25% formamide.

DNA sequencing

Sequencing was done by the dideoxy chain-termination method (Saxe at 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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