

Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development

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Extracellular cAMP acts through cell-surface receptors to coordinate the developmental program of *Dictyostelium*. A cAMP receptor (cAR1), which is expressed during early aggregation, has been cloned and sequenced previously. We have identified a new receptor subtype, cAR3, that has ~56% and 69% amino acid identity with cAR1 and cAR2, respectively. cAR1, cAR2, or cAR3 expressed from plasmid in growing *Dictyostelium* cells can be photoaffinity labeled with 8-N₃[³²P]cAMP and phosphorylated when stimulated with cAMP. cAR3 RNA was not present during growth but appeared during late aggregation. Its expression peaked at 9 hr and then fell to a reduced level that was maintained until culmination. The expression of cAR3 protein followed a similar pattern, but with a 3-hr lag, and reached a maximum at the mound stage. In contrast, cAR1 protein was expressed predominantly during early aggregation and at low levels during later stages. At their respective peaks of expression, there were ~5 × 10³ cAR3 sites per cell compared with ~7 × 10⁴ cAR1 sites per cell. The cAR3 gene was disrupted by homologous recombination in several different parental cell lines. Surprisingly, the *car3*⁻ cell lines display no obvious phenotype.

[Key Words: cAMP; receptors; *Dictyostelium*; morphogenesis]

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Extracellular cAMP, binding to cell-surface receptors, is critical to the developmental program of *Dictyostelium*. During early development, oscillations of cAMP move outward from organization centers in concentric or spiral waves to coordinate the aggregation of many thousands of single amoebas. cAMP acts during aggregation both as a chemoattractant and as a cell-cell signaling molecule. After ~30 oscillations, cells form a tight aggregate and differentiation begins with the formation of prestalk and prespore cell types. Development results in the formation of a fruiting body, which consists of a stalk structure that holds aloft a mass of spores (Devreotes 1982).

A cAMP receptor, cAR1, has been identified and, characteristic of G-protein-linked receptors found in yeast and vertebrates (Dohlman et al. 1991), its sequence consists of seven potential transmembrane domains and a cytoplasmic carboxyl terminus (Klein et al. 1988). Present within its carboxy-terminal region are serine res-

idues, which are the sites of ligand-induced phosphorylation (D. Hereld, R. Vaughan, and P. Devreotes, in prep.). cAMP stimulation of intact cells causes cAR1 to shift in size from 40 to 43 kD and to acquire ≥4 moles of phosphate (Klein et al. 1987). The kinetics and dose dependence of cAR1 phosphorylation and dephosphorylation closely correlate with that of adaptation and deadaptation of several cAMP-induced responses. This regulation probably underlies the oscillatory behavior of the cells in early aggregation (Vaughan and Devreotes 1988).

Cell-surface cAMP-binding sites are present throughout development (Schaap and Spek 1984). cAR1 accounts for the sites detected during early aggregation (Klein et al. 1987), but the molecular basis of a second smaller peak measured during early culmination is unknown. The cyclic nucleotide specificity of these binding sites is similar to that of cAR1 and affinities of cAMP binding to membranes of these cells can be inhibited by GTPγS, indicating that there are receptor-G protein interactions (Schaap and Van Driel 1985; Schenk et al. 1991).

Cells that lack cAR1 by gene disruption (*car1*⁻ cells) do not enter the developmental program but remain as a monolayer of single cells (Sun and Devreotes 1991). Al-

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though these cells have almost no detectable surface cAMP-binding sites, several lines of evidence suggest that another cAR is present. After prolonged periods of starvation *car1*⁻ cells are weakly responsive in assays that measure chemotaxis to cAMP. When mixed with wild-type cells, some *car1*⁻ cells can develop to form spores, albeit at a very low efficiency (Sun and Devreotes 1991). Finally, adenyl cyclase activity is detectable in *car1*⁻ cells, but the kinetics and magnitude of this response differs from that attributed to cAR1 (M. Pupillo et al. 1992).

As reported in the accompanying article, cAR2 is a receptor subtype expressed in prestalk cells predominantly during slug formation (Saxe et al. 1992). Cells that lack cAR2 by gene disruption (*car2*⁻ cells) are delayed in development at the mound stage and fail to readily form tips. cAR2 appears to facilitate the continuation of morphogenesis into later development (Saxe et al., this issue). In this report we describe the identification of a third cAMP receptor subtype, cAR3, that shares ~65% amino acid identity with the other members. The peak expression of cAR3 is at the mound stage, which follows cAR1 but precedes cAR2. We demonstrate that all three receptors are photolabeled with 8-N₃[³²P]cAMP and undergo ligand-stimulated phosphorylation. Unlike *car1*⁻ and *car2*⁻ cells that are blocked at different developmental stages, cells that lack cAR3 by homologous recombination do not display an overtly altered pattern of development. This phenotype suggests that cAR3 may control a subtle aspect of morphology during late aggregation, but cAR1 and cAR2 are able to compensate for its loss.

Results

Isolation of the *cAR3* gene

About 10⁵ colonies from a partial *Sau3A* genomic library were screened using a full-length cAR1 cDNA as a probe (Klein et al. 1988). Of the four clones isolated, three had a restriction map corresponding to the genomic organization of the cAR1 locus, whereas the fourth clone, designated GR6, hybridized more weakly and yielded a different restriction pattern. After removal of an intron according to its consensus splice sites (see Saxe et al., this issue), the sequence contained a 1470-bp open reading frame (ORF) encoding a putative protein of 490 amino acids. There were two in-frame AUGs at the 5' end of the coding region (Fig. 1). Translational initiation probably begins at the first AUG because in-frame stop codons were 5' of this location (data not shown). The ORF encoded a protein with a calculated molecular mass of 56,174 daltons.

Using GR6 as a probe, four cDNAs were obtained in a screen of 5 × 10⁵ phage of a λgt11 library constructed from pooled RNA isolated from 2-, 3-, and 4-hr cells (Klein et al. 1988). The cDNA sequences, which encompassed almost the entire coding region of GR6, confirmed the genomic nucleotide sequence and the placement of the intron. On the basis of its extensive homol-

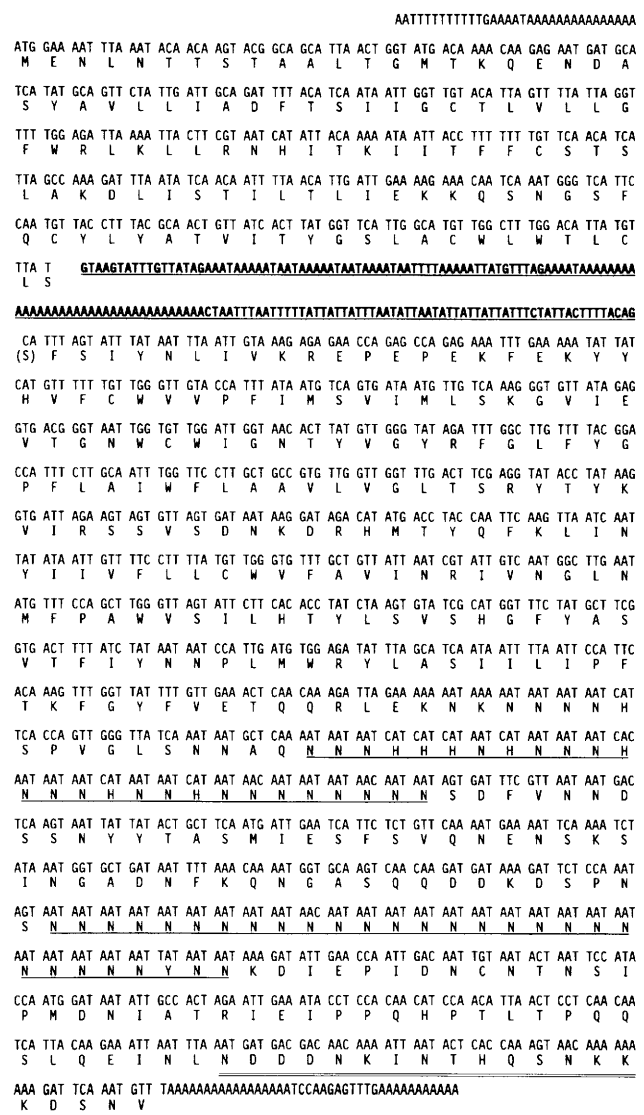


Figure 1. Sequence of cAR3. Translation of the nucleotide sequence of GR6, after removal of the intron, results in a protein of 490 residues. The intron is shown in boldface type and underlined. Strings of asparagine repeats are underlined. The epitope used to generate cAR3 antiserum is double underlined.

ogy with cAR1 and cAR2 (Saxe et al. 1992), this ORF was designated cAR3.

cAR3 sequence

The major features of the predicted cAR3 protein were similar to those of cAR1 (Klein et al. 1988). A hydropathy plot revealed six strongly hydrophobic domains, and a seventh amphipathic region that could form seven transmembrane domains of ~20–25 residues each (Kyte and Doolittle 1982). The final 220 residues of the protein, ~45% of the sequence, are primarily hydrophilic. This topology is characteristic of the superfamily of G-protein-coupled receptors, such as the adrenergic receptors

(O'Dowd et al. 1989) and the rhodopsins (Findlay and Pappin 1986; Hargrave 1986). Similar to the proposed structure of these receptors, cAR3 would have an extracellular amino terminus and a long cytoplasmic carboxyl terminus.

The extracellular portions of cAR3 contain two potential amino-linked glycosylation sites (Asn-X-Ser/Thr; Hubbard and Ivatt 1981). One site is located at asparagine 5 near the amino terminus and the other at asparagine 204 in the loop between the second and third putative transmembrane domains (Fig. 2). However, attempts to show that cAR3 is glycosylated were unsuccessful; treatment of the protein with peptide N-glycosidase F (PNGase F) did not alter its apparent molecular weight on SDS-PAGE (data not shown).

Sequence comparison

A search of the NBRF protein data bank, using only the sequence of the transmembrane and loop regions, revealed limited identity between the transmembrane re-

gions of cAR3 and the following G protein-coupled receptors: turkey β -adrenergic receptor (24% identity over 55 amino acids), pig muscarinic acetylcholine receptor III (21% identity over 132 amino acids), and human green opsin (18% identity over 120 amino acids). Using the FASTA alignment program (Lipman and Pearson 1985), cAR3 has 56% and 69% sequence identity with cAR1 and cAR2, respectively (Klein et al. 1988; Saxe et al. 1991). As shown in Figure 2, the identity between cAR3 and cAR1 begins at the first transmembrane domain I, extends to the initial portion of the carboxy-terminal domain, and is contiguous with the exception of a single-residue gap in the first extracellular loop. The sequence identity between the three cARs is highest among transmembrane domains and intracellular loops and is less extensive in the extracellular loops. Little similarity exists between the three receptors before the first transmembrane domain and beyond residue 311 of the carboxyl terminus of cAR3.

Both the amino and carboxyl termini of cAR3 are longer than cAR1 by 10 and 87 amino acids, respectively.

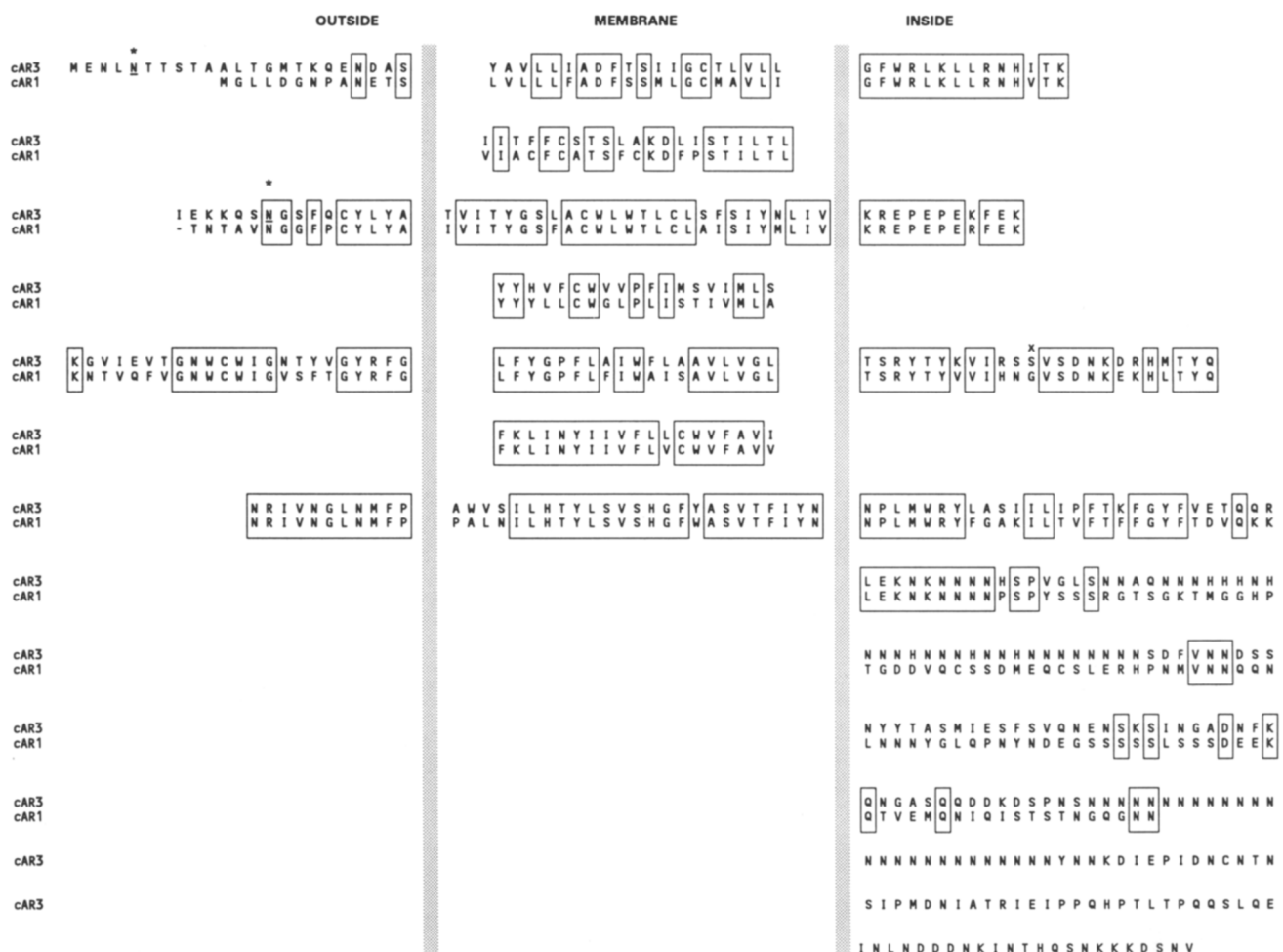


Figure 2. Sequence comparison of cAR3 and cAR1. The amino acid sequences of cAR3 (top) and cAR1 (bottom) share 56% identity when aligned by the DFASTP program (Lipman and Pearson 1985). Two amino-linked glycosylation sites (*) above asparagine residues and a protein kinase A site (X) are also shown.

Identification of a cAMP receptor subtype

lated at multiple sites and because comparable levels of each receptor are being expressed (cAR1, 3.7×10^5 sites/cell; cAR2, 2.1×10^5 sites/cell; cAR3, 6.4×10^5 sites/cell; Johnson et al. 1992), both cAR2 and cAR3 must also acquire multiple phosphates with stimulation. Amino acid analysis of cAR3 showed that threonine residues were phosphorylated in the basal state, whereas serine residues were targeted upon cAMP stimulation (data not shown).

Developmental and cell-type expression of cAR3

To examine the developmental regulation of cAR3, wild-type AX-3 cells were developed on agar plates and RNA was isolated every 3 hr until fruiting bodies were formed (Fig. 4A). A RNA blot hybridized with a cAR3 probe revealed a doublet of ~2 kb that was not present in growing cells. The amount of cAR3 RNA increased to a maximum at the mound stage (~9 hr), dropped to a lower plateau, and then increased slightly at 15 hr.

In contrast to cAR2, which is expressed preferentially in prestalk cells, cAR3 RNA does not appear to be enriched in either prestalk or prespore cells. Migrating pseudoplasmodia were disrupted into single cells and then separated by density in Percoll gradients. RNA was prepared from light and dense fractions, which are enriched in prestalk and prespore cells, respectively (Ratner and Borth 1983), and blotted for hybridization to cAR3 (Fig. 4B). The cAR3 RNA is present in equal amounts in both fractions.

The developmental expression of cAR1 and cAR3 pro-

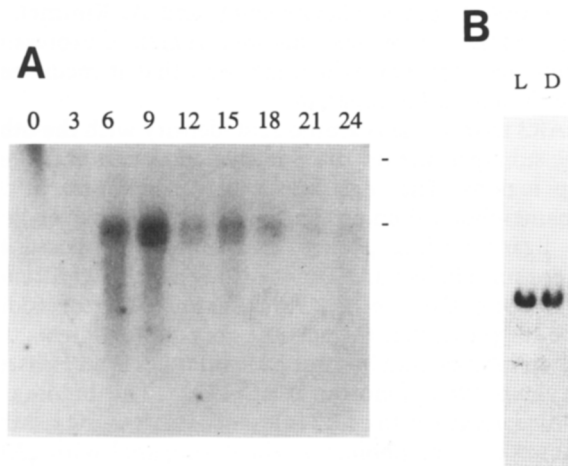


Figure 4. Developmental and cell-type expression of cAR3 RNA. (A) RNA was prepared from wild-type cells at 3 hr intervals throughout development and blotted for hybridization to GR6. A 2-kb doublet of cAR3 RNA was induced during aggregation (6 hr), was maximal at the mound stage (9 hr), and persisted at reduced levels throughout later development. Bars at right indicate the position of RNA (2 and 4 kb). (B) RNA was isolated from cells that were separated by density in Percoll gradients and blotted as in (A). Light (L) and dense (D) fractions are enriched in prestalk and prespore cells, respectively.

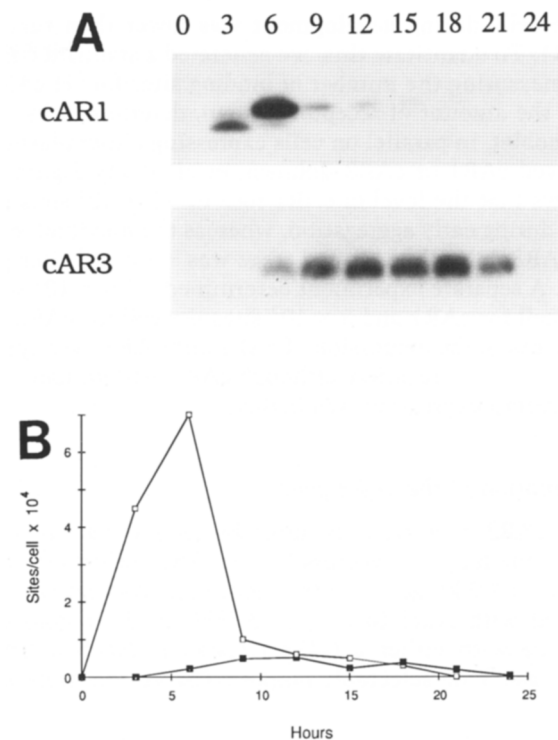


Figure 5. (A) Developmental expression of cAR1 and cAR3 proteins. Protein samples were collected at 3-hr intervals throughout the development of wild-type cells and immunoblotted with cAR1 or cAR3 antisera. cAR1 migrated at 40 and 43 kD and was expressed mainly during aggregation (3–6 hr). cAR3 appeared as a protein of ~65 kD that was expressed at later stages of development beginning at the mound stage (9 hr). (B) Developmental expression of cAR1- and cAR3-binding sites. cAMP-binding sites of cAR1 (□) and cAR3 (■) were measured during the development of wild-type cells (see Materials and methods). cAR1 was expressed during early aggregation to $\sim 7 \times 10^4$ sites per cell, whereas cAR3 was induced later at 10-fold lower levels (5×10^3 sites per cell).

teins were compared. At 3-hr intervals throughout development, cells were extracted with SDS sample buffer and prepared for immunoblotting (Fig. 5A). A cAR3 antiserum was generated against a synthetic peptide corresponding to the final 20 amino acids of the cAR3 carboxyl terminus (see Fig. 1). cAR1, which migrated at 40 and 43 kD, was present in low amounts during growth, peaked earlier during aggregation, and subsided rapidly at later time points (Klein et al. 1987). cAR3 migrated predominantly at an apparent molecular mass of 65 kD. Consistent with the time course of its RNA expression, cAR3 was not present in growing cells, was induced at early aggregation (6 hr), and was maximally expressed at the mound stage (9–12 hr). The level of expression then fell briefly, peaked again during the slug stage (18 hr) and declined at culmination. Occasionally, a higher molecular mass form of cAR3 of 67 kD was observed. Separate experiments have shown that the amount or appearance of this larger form did not follow a consistent pattern.

The number of cAR3-binding sites expressed in wild-

type cells during development was lower than that of cAR1. To quantitate this, we generated a standard curve by measuring the number of binding sites for ^3H -cAMP and the amount of receptor protein, determined by immunoblot, in parallel on cells expressing either plasmid-derived cAR1 or cAR3 (Johnson et al. 1992). Figure 5B shows that the level of cAR1 rose to $\sim 7 \times 10^4$ sites per cell during early aggregation, whereas the maximal level of cAR3 during the mound stage was $\sim 5 \times 10^3$ sites per cell. A separate experiment determined $\sim 2.5 \times 10^4$ sites per cell for cAR1 and 5×10^3 sites per cell for cAR3. In both cases, the expression of cAR1 and cAR3 overlapped during late aggregation, although cAR1 predominated, as its overall expression was higher.

Disruption of the cAR3 gene

The cAR3 gene was disrupted by recombination with two gene-targeting vectors (Fig. 6). The constructs contained a 2.9-kb genomic fragment of cAR3 that was digested with *NdeI* to replace 0.73 kb of the coding sequence with either a G418 resistance (Neo) or UMP synthase (Ura) selectable marker. The vectors were lin-

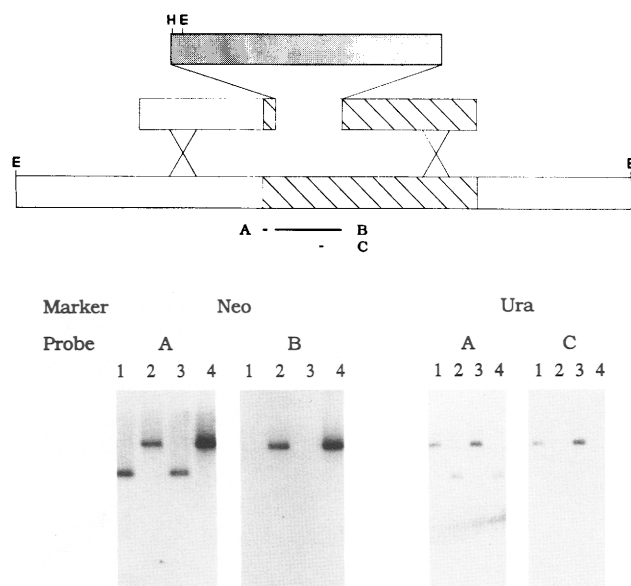


Figure 6. cAR3 gene disruption construct and genomic analysis of transformants. (Top) Diagram of the disruption construct containing either a 2.2-kb neomycin resistance marker or a 3.7-kb UMP synthase gene (shaded region), flanked by 1 kb of 5'-noncoding sequence (unshaded regions) and 1 kb cAR3-coding sequence (hatched region). Genomic DNA was prepared from transformants, digested with *EcoRI* (Neo) or *EcoRI*-*HindIII* (Ura) and blotted for hybridization with several cAR3 probes. Four independent clones from each transformation are shown. Probe A is a 32-bp oligomer corresponding to the amino-terminal sequence of cAR3, probe B is the cAR3 *NdeI* fragment, and probe C is another oligomer that is absent in the disruption constructs, as indicated. In each case (Neo or Ura), the blots on the left, which had been hybridized with probe A, were stripped and rehybridized with probe B or C.

earized and transformed into *Dictyostelium* cells, and clones were selected. Genomic DNA was prepared from transformants, digested with *EcoRI* (Neo) or *EcoRI*-*HindIII* (Ura), and blotted for hybridization with several cAR3 probes. In Figure 6, four independent clones from each transformation are shown. Probe A, a 32-bp oligomer corresponding to the amino-terminal sequence of cAR3, detects two size bands, a 5-kb band representing the intact cAR3 gene and an ~ 2.5 -kb band, indicating a double crossover event with the vector that introduced a new *EcoRI* or *HindIII* site. To confirm this result, the blots were stripped and then probed with either the cAR3 *NdeI* fragment (probe B) or an oligomer (probe C) that is absent in the disruption construct. The clones that had the smaller band did not hybridize with the 0.7-kb fragment or oligomer and confirmed that this cAR3 coding sequence was absent from its genome. These transformants were characterized further.

The *car3*⁻ (*neo*) cells lacked cAR3 protein but expressed normal levels of cAR1 protein with the proper developmental expression (data not shown). Several independent "random" integrants and *car3*⁻ clones (i.e., lanes 1–4 in Fig. 6) have been examined in parallel under our standard developmental conditions. No obvious differences in the timing of development or the morphological features of the aggregates have been observed.

Discussion

We report the identification of a new cAMP receptor subtype, cAR3, that is a member of the cAMP receptor family, which currently includes three other subtypes, cAR1 (Klein et al. 1988), cAR2 (Saxe et al., this issue), and cAR4 (J. Louis, G. Ginsburg, and A. Kimmel, in prep.). Each subtype has a distinct pattern of expression during development, which suggests that it mediates a specific role in the program.

cAR3 shares $\sim 65\%$ amino acid identity with the other cAR subtypes within the putative transmembrane and loop regions. The high degree of conservation in the transmembrane domains suggests that they may enclose the ligand-binding pocket in a manner proposed for rhodopsin (Findlay and Pappin 1986) and the β -adrenergic receptor (Dixon et al. 1987). Pharmacological studies have suggested that the adenine ring of cAMP rests in a hydrophobic pocket in three cAR subtypes (Van Haastert and Kien 1983; Johnson et al. 1992). The binding of cAMP derivatives to cAR2 is less sensitive to the relative changes in hydrophobicity when compared with cAR1 and cAR3. The photolabeling reaction appears more efficient for cAR2 than cAR1 or cAR3 as saturating concentrations of photolabel were used. Although the affinity of cAR2 under the conditions of the photolabeling reaction is similar to the other subtypes, it may bind 8- N_3 [^{32}P]cAMP differently so that the azido group reacts more productively.

The cytoplasmic carboxy-terminal domains of all three subtypes are rich in serine and threonine residues. cAR1 contains 18 serines grouped into four clusters in

its carboxyl terminus, and these residues comprise the sites for ligand-induced phosphorylation (D. Hereld, R. Vaughan, and P. Devreotes, in prep.). cAR2 and cAR3 contain 9 and 26 serine and threonine residues, respectively, in their carboxy terminal regions, which are more evenly distributed. Serines 310 and 315 of cAR3 are conserved in their sequence position between the three cARs. In addition, cAR2 and cAR3 contain a conserved protein kinase A phosphorylation site in the third cytoplasmic loop region.

All three cAR subtypes become phosphorylated in response to cAMP. Because cAR2 and cAR3 are expressed in the $\Delta 208$ cells that lack cAR1, the receptor kinases cannot be acting on these subtypes indirectly by activation by cAR1. Instead, the receptor kinases appear to be activated upon ligand occupation of each receptor. Because of the high degree of sequence identity between the three cARs, it seems likely that a single protein kinase phosphorylates the receptors. In support of this, plasmid-expressed cAR1 and cAR3 can be phosphorylated equally well during early aggregation (Johnson et al. 1991) and the mound stage, respectively (data not shown), suggesting that receptor kinases are present throughout development. The kinetics of phosphorylation differ between the cARs. cAR1 has a half-time of ~ 45 sec (Vaughan and Devreotes 1988), whereas the half-times of cAR2 and cAR3 are slower (data not shown).

The proposed intracellular loops of the three cARs are highly conserved and are rich in charged residues. Structure and function experiments on rhodopsin and the β -adrenergic receptor have shown the cytoplasmic regions nearest the transmembrane domains to be important in receptor-G protein coupling (Strader et al. 1987; O'Dowd et al. 1988; Weiss et al. 1988). Chimeras between the α_2 - and β_2 -adrenergic receptors demonstrate that the loop between transmembrane domains V and VI is the most important region for coupling to G_s (Bouvier et al. 1988). Other studies have indicated that in addition to the V/VI loop, the III/IV loop is needed to switch G protein specificity (Wong et al. 1990). Because the loop regions are highly conserved between the cARs, it seems likely that these receptors couple to the same G protein or class of G proteins. The expression of any of the three cAR subtypes in the *car1*⁻ cells can restore cAMP-induced Ca^{+2} influxes and cAMP-induced phosphorylation of the $G_{\alpha 2}$ subunit, responses that are absent in the parent cells (Johnson et al. 1993; J. Milne and P.N. Devreotes 1993). However, neither cAR2 nor cAR3 was able to completely rescue the aggregation minus phenotype of the *car1*⁻ cells.

The protein kinase A phosphorylation site in the third cytoplasmic loop of cAR2 and cAR3 may be functionally significant. In the β -adrenergic receptor, a peptide that is derived from this loop and contains such a consensus site can stimulate adenylyl cyclase in vitro, and this activity is abolished upon phosphorylation of the peptide by protein kinase A (Okamoto et al. 1991). In *Dictyostelium*, overproduction of the regulatory subunit of protein kinase A blocks development before the mound stage (Simon et al. 1989). Possibly, protein kinase A acts

on cAR2 or cAR3 to block an inhibitory response that allows differentiation to begin.

cAR3 protein appeared as a doublet when expressed either from plasmid during growth or endogenously during development and it migrated at a larger size than predicted from its amino acid sequence. When protein extracts from the $\Delta 208$ /cAR3 cells were immunoblotted with specific antiserum or when intact cells were photolabeled with 8-N₃[³²P]cAMP, cAR3 migrated as a doublet of 57–60 and 62–64 kD. The doublet may have arisen from a number of possible causes: Translational initiation may have occurred at both Met 1 and 15. Protein degradation may have occurred at the carboxyl terminus as both forms of cAR3 were photolabeled evenly while the antiserum generated against the carboxy-terminal 20 amino acids predominantly recognized the larger form. In addition, the asparagine-rich regions in the carboxyl terminus may have caused the anomalous migration.

The maximal number of cAR3 sites measured in mound-stage wild-type cells was $\sim 5 \times 10^3$ sites per cell. In contrast, cAMP-binding sites present during early aggregation, which are mainly comprised of cAR1 (Klein et al. 1987), rise to 5×10^4 to 10×10^4 sites per cell (Green and Newell 1975; Klein and Juliani 1977; Van Haastert 1985). During late aggregation (6–9 hr), both cAR1 and cAR3 were present. We do not know whether cAR3 is expressed in all cells at low levels or in a subset of cells at higher levels. However, Percoll gradient fractionation of slug stage cells showed that both cAR1 and cAR3 RNA distribute similarly in prestalk and prespore cells (Fig. 3B; Saxe et al. 1991). Immunofluorescence staining of developed individual cells or multicellular structures with the cAR3 antiserum should distinguish between these two possibilities.

The lack of an obvious phenotype in the *car3*⁻ cells is intriguing. The presence of a family of highly related surface receptors suggests there is redundancy built into cell-cell signaling strategies in development. cAR3 appears after cAR1 and before cAR2, but its time course of expression overlaps with each. Furthermore, the affinity of cAR3 for cAMP is intermediate between that of cAR1 and cAR2. These observations suggest that cAR1 or cAR2, or both, may substitute for cAR3 to bring about apparently normal development under our standard conditions. We are currently examining the *car3*⁻ cells under a wider variety of developmental conditions that may reveal a specific requirement for cAR3.

Materials and methods

Cell culture and development

AX-3 cells were grown in HL-5 media (Watts and Ashworth 1970) and vector-transformed cells were grown in HL-5 media with 20 μ g/ml of neomycin (G418). All cells were harvested during late log phase growth and washed once in 10 mM PO₄ buffer (pH 6.5) containing 2 mM MgSO₄ and 0.2 mM CaCl₂. Cells were developed on 1.5% agarose plates at 5×10^7 cells per 10-cm petri dish (Devreotes et al. 1987).

Isolation and sequencing of clones

Hybridization conditions for all blots and lifts were as follows. Nitrocellulose filters were hybridized in 50% formamide, 1 M NaCl, 1% SDS, and 10% dextran sulfate overnight at 42°C and washed twice with $2 \times$ SSC, 1% SDS at 60–65°C, for 30 min each.

For library screening, $\sim 10^5$ clones of a partial *Sau3AI Dictyostelium* genomic library (PAT plasmid; library provided by R. Firtel, University of California, San Diego) were probed with 32 P random-primed [Feinberg and Vogelstein 1983] 6B, a full-length cDNA of cAR1 [Klein et al. 1988]. A 1.7-kb clone, GR6, was isolated and contained the entire coding region of cAR3 (Fig. 1). Four cAR3 cDNAs were obtained by screening 5×10^5 clones of a *Dictyostelium* λ gt11 library [Klein et al. 1988] using 32 P-labeled random-primed GR6 as a probe. Three cDNAs (clones 1, 4, and 5) were subcloned into the *EcoRI* site of Bluescript (KS+, Stratagene), whereas 0.5-kb of the fourth (clone 7) was amplified by PCR using a 5' cAR3 primer (5'-CCGAATTCCATTCAC-CAGTTGGGTTATC-3') and a 3' λ primer that flanked the cloning site. Double-stranded sequencing was performed by dideoxy-nucleotide chain termination [Sanger et al. 1977]. Two primers (the above-mentioned and 5'-GGTTCATTGGCATGT-TGGC-3') were used for sequencing.

Vector construction and transformation

To create the cAR3 disruption construct, a modified Bluescript vector (Stratagene), pSL-4, was used. This construct has a 24-bp palindromic oligo, which contains stop codons (TAG) in all three reading frames and a *BglIII* site, ligated into the *EcoRV* site. pSL-4 was digested with *Sall*, filled in, and ligated to remove its *AccI* site. Full-length cAR3 was obtained by digesting GR6 with *XbaI* and *SmaI*. This insert was filled in, had *BamHI* linkers added, and was cloned into the *BamHI* site of pSL-4. pSL-4/cAR3 was digested with *XbaI*, filled in and digested with *AccI* to remove the 5'-coding region of cAR3. This was replaced by the *AccI*–*EcoRV* fragment from 5PcAR3 (a 2-kb genomic fragment of cAR3 cloned into the *EcoRI* site of Bluescript), which contained 1.8 kb of 5' of the cAR3 *AccI* site. This vector, 5cAR3, was digested with *NdeI* to remove ~ 0.5 kb of cAR3-coding sequence. Markers for G418 resistance (*BamHI*–*BglIII* fragment from Neo1DXTBR, A15TX; Cohen et al. 1986) containing the G418^R cassette [NPT1 gene flanked by the actin 15 promoter and terminator] and for uracil prototrophy (*Clal* fragment of p3B1; Kalpaxis et al. 1991) were blunted and cloned into *NdeI* site of 5cAR3 to create 5cAR3neo and 5cAR3ura vectors, respectively. The constructs were linearized with *EcoRI* and transformed into *Dictyostelium* (uracil auxotroph; gift of W. Loomis, University of California, San Diego) by electroporation as described [Sun and Devreotes 1991]. Transformant clones were selected with 10 μ g/ml of G418 and 0.2 mM uracil (Neo) or FM minimal medium [Franke and Kessin 1977] (Ura). The B18, cAR1, D208/cAR2, and D208/cAR3 cells have been described previously [Johnson et al. 1991, 1992].

DNA and RNA isolation and analysis

Genomic DNA was isolated by removing cells from confluent 10-cm Petri dishes, pelleting and resuspending in 0.5 ml buffer containing 0.32 M sucrose, 5 mM MgCl₂, 1% Triton X-100, and 10 mM Tris (pH 7.5). After pelleting for 10 min at 14K nuclei were resuspended in 0.2 ml of 10 mM EDTA in 10 mM Tris (pH 7.5) to which 0.2 ml of 0.1% SDS in 10 mM Tris (pH 7.5) was added. After gentle mixing, 1 mg/ml final concentration RNase A was added and the DNA was incubated at 65°C for 30 min. Proteinase K (final concentration, 2 mg/ml) was added and the

incubation repeated for 45 min. The DNA was extracted and precipitated as described [Sambrook et al. 1989]. DNA (5 μ g) was restriction digested with *EcoRI* (Neo) or *EcoRI*–*HindIII* (Ura), separated on 0.8% agarose gels, and blotted as described [Sambrook et al. 1989]. Two oligonucleotides, (5'-ATGGAAAATT-TAAATACAACAAGTACGGACC-3' and 5'-CCCGTCACCT-CTATAACACCC-3'), corresponding to nucleotides 1–32 and 453–473 of the cAR3-coding sequence, respectively, and the 0.73-kb *NdeI* fragment of cAR3 were used as probes (Fig. 6).

Total RNA was isolated from AX-3 cells during development as described [Klein et al. 1988]. In brief, 5×10^7 cells were solubilized in 4 ml of 6 M guanidine-HCl, incubated at 65°C for 5 min, and precipitated with one-half volume of ethanol at –20°C for 2 hr. After pelleting RNA for 30 min at 5K, the procedure was repeated two more times using half the starting volumes with the final pellet resuspended in 0.4 ml of water and precipitated. The RNA was pelleted, washed with 80% EtOH, and resuspended in 50 μ l of TE. Five micrograms of each RNA sample was size fractionated on an agarose gel containing formaldehyde, blotted as described [Sambrook et al. 1989], then hybridized with GR6.

Prestalk and prespore RNA were isolated by disrupting migrating pseudoplasmodia and separating cells by density in Percoll gradients as described [Ratner and Borth 1983].

Preparation of antigen and immunization

The peptide NH₂-CNDDDNKINTHQSNNKKDSNV-CO₂H, corresponding to the final 20 amino acids of cAR3, with a cysteine added at the amino terminus for cross-linking purposes, was synthesized. The peptide was attached to keyhole limpet cyanin (KLH) as described [Green et al. 1982] and injected subcutaneously into a rabbit. High titer serum was obtained after a second boost 4 weeks later and used routinely at 1 : 1000 or 1 : 2000 dilution.

Estimation of receptor abundance during development

The number of cAR1 and cAR3 binding sites was measured throughout the development of wild-type cells in the following manner. Total receptor sites per cell were determined by Scatchard analysis of transformed cells expressing plasmid-derived cAR1 or cAR3 (cAR1 and Δ 208/cAR3 cells, respectively; Johnson et al. 1992). In addition, whole-cell extracts were prepared at 5×10^7 cells/ml, and a titration series of twofold dilutions (to 1 : 1024) was made. From wild-type cells, whole-cell extracts were prepared every 3 hr throughout development on agarose plates at 5×10^7 cells/ml. Protein samples of the time course and the cAR1 or cAR3 titration series were separated by SDS-PAGE. These gels were transferred together, immunoblotted together using either cAR1 or cAR3 antisera, and exposed to the same film. After autoradiography, the receptor bands from the titration series were scanned by a densitometer to correlate the intensity of the protein band with the number of receptor sites per cell to generate a standard curve. The developmental time course of each receptor was then compared with its respective standard curve to determine receptor number. This experiment was performed twice with similar results.

In vivo phosphorylation

Growing cells were prepared for in vivo labeling as described [Vaughan and Devreotes 1988] and resuspended at 10^8 /ml. Two milliliters of cells were shaken at ~ 150 rpm and incubated with 0.3 mCi/ml of 32 P-labeled H₃PO₄ and 5mM caffeine for 30 min at room temperature. One milliliter of cells was then trans-

ferred to a second cup containing 1 μ M cAMP and 10 mM DTT for 15 min. Extracts were prepared from 0.5 ml cells and separated as described (Johnson et al. 1992).

Other assays

Intact cells were photoaffinity labeled with 100 nM 8-N₃[³²P]-cAMP using a modification of the previously described procedure (Devreotes et al. 1987). After 3 min of UV radiation, cells were extracted in a lysis buffer containing 1.5% CHAPS (Klein et al. 1985) and separated as described (Johnson et al. 1992). Whole-cell extracts were immunoblotted as described (Klein et al. 1987). To detect cAR1, a polyclonal antiserum generated against the purified receptor, R4, was used at 1 : 1000 dilution (Klein et al. 1987).

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Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of Dictyostelium development.

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