Overexpression of the cAMP Receptor 1 in Growing Dictyostelium Cells†

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ABSTRACT: cAR1, the cAMP receptor expressed normally during the early aggregation stage of the Dictyostelium developmental program, has been expressed during the growth stage, when only low amounts of endogenous receptors are present. Transformants expressing cAR1 have 7-40 times over growth stage and 3-5-fold over aggregation stage levels of endogenous receptors. The high amounts of cAR1 protein expressed constitutively throughout early development did not drastically disrupt the developmental program; the onset of aggregation was delayed by 1-3 h, and then subsequent stages proceeded normally. The affinity of the expressed cAR1 was similar to that of the endogenous receptors in aggregation stage cells when measured either in phosphate buffer (two affinity states with $K_d$'s of approximately 30 and 300 nM) or in 3 M ammonium sulfate (one affinity state with a $K_d$ of 2-3 nM). When expressed during growth, cAR1 did not appear to couple to its normal effectors since these cells failed to carry out chemotaxis or to elevate cGMP or cAMP levels when stimulated with CAMP. However, cAMP stimulated phosphorylation, and loss of ligand binding of cAR1 did occur. Like aggregation stage control cells, the cAR1 protein shifted in apparent molecular mass from 40 to 43 kDa and became highly phosphorylated when exposed to cAMP.

In addition, the number of surface cAMP binding sites in cAR1 cells was reduced by over 80% during prolonged cAMP stimulation. These results define a useful system to express altered CAR1 proteins and examine their regulatory functions.

Dictyostelium discoideum normally live as freely growing amoebae, but when deprived of nutrients, cell division and growth cease, and the cells enter a developmental program that results in the formation of a multicellular structure. During early development, organizing centers arise which secrete cAMP¹ every 6 min. The released cAMP stimulates neighboring cells, which relay the chemical signal outward in the form of concentric or spiral waves (Tomchik & Devreotes, 1981). The propagated waves of cAMP act as chemoattractant gradients which coordinate the migration of cells toward the aggregation center (Devreotes, 1982).

Early aggregation is coordinated by a G-protein-linked signal transduction system. Extracellular cAMP binds to a cell surface receptor, coupled to a G-protein, which leads to activation of adenyl cyclase. The newly synthesized intracellular cAMP is then secreted from the cell. Ligand binding also causes adaptation which uncouples the receptor from its effectors within minutes. The rapid removal of extracellular cAMP by cell surface phosphodiesterases allows the receptors to resensitize, and the cycle is reinitiated (Klein et al., 1985; Janssens & Van Haastert, 1987; Gundersen et al., 1989).

A cAMP receptor (denoted cAR1) has been cloned and its primary sequence determined (Klein et al., 1988). Characteristic of other G-protein-coupled receptors, such as rhodopsin (Hargrave, 1986) and the adrenergic receptors (Dohlman et al., 1987), its predicted sequence encodes a protein consisting of seven transmembrane domains followed by a hydrophilic C-terminal region. This cytoplasmic region contains 18 serines,

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Abbreviations: cAR, cAMP receptor; cAMPS, adenosine cyclic 3',5'-monophosphate; cGMP, guanosine cyclic 3',5'-monophosphate; LBL, loss of ligand binding; DB, developmental buffer; HBS, Hepes-buffered saline; PB, phosphate buffer; AS, ammonium sulfate; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
cAMP Receptor in *Dictyostelium*

![Diagram](image)

**FIGURE 1:** pB18 expression construct. pB18 is a derivative of pUC18 which contains the actin 6 Neo cassette to encode neomycin resistance. cDNAs encoding cAR1 or cAR1 Δ311 were cloned in the sense orientation in the unique BglII site. The strong and constitutively active actin 6 promoter controls transcription.

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some of which are the sites of ligand-induced phosphorylation (R. Vaughan, unpublished data). The kinetics of cAR1 phosphorylation and dephosphorylation correlate strongly with that of adaptation and deadaptation (Vaughan & Devreotes, 1988).

Recently, several other cAMP receptors, cAR2–cAR4, have been cloned. This family of receptor subtypes share about 60% identity in the transmembrane and loop regions but have distinct cytoplasmic C-termini (Saxe et al., 1991). Each receptor has a unique pattern of expression during development. For instance, growing cells have only low amounts of cAR1 protein, but during development, cAR1 expression rises to a maximum in the early aggregate stage and then declines (Klein et al., 1987). The peak expression of other cAMP receptor subtypes is subsequent to that of cAR1 although there is some overlap (Saxe et al., 1991). However, since no cAR is significantly expressed during growth, cAR's expressed exogenously at that time are detectable against a negligible background of other receptor subtypes. Such a system simplifies the examination of binding and regulatory phenomena of each cAR.

We have used this system to examine the biochemical properties of cAR1. cAR1 expressed during growth has a similar affinity to the cAMP binding sites expressed during the aggregate stage. The receptor undergoes at least two of its normal ligand-induced regulatory functions: phosphorylation and loss of ligand binding. Hence the components involved in cAR1-mediated desensitization are present during growth as well as early development. These observations define a convenient means to study these regulatory properties of cAR1 by mutation without regard for the potential effects on development.

**MATERIALS AND METHODS**

**Cell Growth and Development.** AX-3 cells were grown in HL-5 media, and vector-transformed cells were grown in HL-5 media with 20 μg/mL G418. Cells were grown to a density of approximately 5 × 10^9/mL and developed by shaking at 2 × 10^7/mL in development buffer (DB) as described (Devreotes et al., 1987). Cells were prepared for assays by washing in an equal volume and resuspending at 10^9 cells/mL in DB.

**Vector Construction and Transformation.** pB6 (Klein et al., 1988) was digested with BamHI/EcoRV to isolate a full-length cAR1 cDNA (cAR1) or digested with FokI/Nael to create a truncation in the C-terminal region at amino acid 311 (cAR1 Δ311). BglII linkers were added to each and then ligated into the BglII site of the expression vector, pB18 (gift of R. Firtel), in the sense orientation (Figure 1). Transformations were done as described (Nellen et al., 1984) with some modifications. AX-3 cells (5 × 10^7) were grown overnight in 20 mM Bis-Tris HL-5 in Petri dishes, and medium was changed again the following morning. DNA (5–10 μg) was precipitated in 0.125 M CaCl₂ in 1 × HBS 30 min prior to adding to the cells. Four hours later, cells were treated for 5 min with 14% glycerol (w/v) and then incubated in HL-5 overnight. Transformants were cloned the following day and selected with HL-5 containing 20 μg/mL G418. Cell lines were derived from individual clones which appeared after 2–3 weeks.

Stable expression of cAR1 could be maintained only when cAR1 cells were grown on surfaces. When they were maintained in shaking culture for several weeks, cAR1 expression levels fluctuated or plummeted. The basis of this instability is not known, but substrate adhesion can alter actin promoter activity (Knecht & Loomis, 1987). The level of cAR1 expression varied by about 20-fold between individual clones within a transformation while some transformations yielded no expression. cAR1 expression levels stabilized after about a month in culture on plates.

**cAMP Binding Assays.** cAMP binding in phosphate buffer (PB) was performed in the absence or presence of ammonium sulfate (AS) as described (Van Haastert, 1985). In brief, 8 × 10^6 cells were added to PB containing 10 mM DTT, 10 mM (32P)cAMP, and various concentrations of cAMP in a 100-μL volume at 4 °C. Cells were incubated 1 min and then centrifuged for 2 min at 1000g. To determine binding in AS, the above assay included 850 μL of 3 mM AS and, after adding cells, 50 μL of 10 mg/mL BSA. Cells were incubated 5–7 min and then centrifuged for 3 min. For both assays, the supernatants were carefully aspirated and the cells were resuspended in 80 μL of 0.1 M formic acid. One milliliter of scintillation fluid was then added and the radioactivity determined. Nonspecific binding was determined by adding excess cAMP to the incubation mixture at a final concentration of 1 mM (PB) or 0.1 mM (AS). Binding curves were best fit using the computer modeling program LIGAND (Munson & Rodbard, 1980).

**Other Assays.** Whole cells were labeled with 100 nM 8-
N_{2}P-(32P)cAMP for photoaffinity labeling (Devreotes et al., 1987) or with (22P)P, (Vaughan & Devreotes, 1988) for in vivo phosphorylation as described. Loss of ligand binding was assayed as described (Van Haastert, 1987) by shaking 5 × 10^6 cells in 2 mL of phosphate buffer in the presence or absence of 100 μM cAMP and 10 mM DTT for 15 min. Cells were washed in 15 mL of PB three times at 4 °C, and specific binding sites were measured by using 5 nM (3H)cAMP in PB. Whole cells were immunoprecipitated and immunoblotted as described (Klein et al., 1987).

**RESULTS**

**Expression of cAR1 Protein.** To express cAR1 during growth, a full-length cDNA was fused to the *Dictyostelium* actin 15 promoter in the sense orientation in the expression vector pB18 (Figure 1). This promoter is constitutively active during growth and throughout early development (Knecht et al., 1986). This construct or pB18 were transformed into AX-3 cells. Stable transformant clones were selected and screened for cAR1 expression by immunoblotting with a polyclonal cAR1 antiserum. One clone with a high level of expression (designated cAR1 cells) and one control clone (designated B18 cells) were characterized further.

As previously reported, cAR1 cells express 7–40 times more binding sites than control transformed cells (Klein et al., 1988). To verify that the additional binding sites were expressed from
to 10 h and CAR1 protein was examined by immunoblot protein during growth (0 h), which increased slightly al., 1987). CAR1 cells, however, expressed high levels of CAR1 rise at 3 h, peak between 6 and 8, and then decline (Klein et (Figure 3). As in wild-type cells, the endogenous CAR1 in B18 cells is developmentally regulated; protein levels begin to result from plasmid expression and not from induction of was not disrupted. Thus the additional cAMP binding sites and their affinities assigned. Data are shown for the model that statistically fits the data best. Three independent experiments were performed with cAMP binding assayed either on both cells in PB or for one set of cells in both PB and AS. *nd, not determined.

The affinity of CAR1 expressed from plasmid during growth is similar to that of the endogenous cAMP binding sites in developed cells. Two affinity states were detected: a high-affinity state of 25 nM (cAR1) or 40 nM (B18) and a low-affinity state, which comprised most of the binding sites, of 300 nM (cAR1) or 350 nM (B18). A second experiment revealed only one affinity state for both cAR1 and developed B18 cells. Both cell lines have two affinity states of about 30 and sites/cell while CAR1 (lanes 7–9) migrated as a 40-kDa protein, identical in size with the endogenously expressed CAR1 (lanes 4–6) in developed B18 cells. However, cAR1 Δ311 appeared as a 24-kDa protein (lanes 10–12). Darker exposure of the autoradiograph revealed low amounts of full-length CAR1 protein present in the cAR1 Δ311 lane, which indicated that the endogenous cAR1 locus was not disrupted. Thus the additional cAMP binding sites resulted from plasmid expression and not from induction of the endogenous cAR1 gene(s). To examine the developmental expression of the exogenous receptor, cAR1 cells were developed by shaking for times up to 10 h and cAR1 protein was examined by immunoblot (Figure 3). As in wild-type cells, the endogenous cAR1 in B18 cells is developmentally regulated; protein levels begin to rise at 3 h, peak between 6 and 8, and then decline (Klein et al., 1987). cAR1 cells, however, expressed high levels of CAR1 protein during growth (0 h), which increased slightly throughout the first 10 h of development. This level was 3-5-fold higher than the peak expression attained in developed B18 cells. High levels of constitutive expression are consistent with previous studies using the actin 15 promoter (Knecht et al., 1986).

The affinity of CAR1 expressed in growing cells was determined by assaying for surface (²H)cAMP binding in phosphate buffer (PB). As shown in Figure 4 and Table I, the transforming plasmid, we used an identical expression construct, cAR1 Δ311, in which the insert was a truncated form of cAR1. The cAR1 Δ311 cells expressed about one-third as many cAMP binding sites as the CAR1 cells (data not shown). When photoaffinity-labeled with (³²P)-8-N₃-cAMP. Nonspecific labeling was determined by assaying for surface (³H)cAMP binding in the absence or presence of 3 M ammonium sulfate (AS) and used to create Scatchard plots. Binding curves were fitted with the LIGAND program for models with one or two binding sites and their affinities assigned. Data are shown for the model that statistically fits the data best. Three independent experiments were performed with cAMP binding assayed either on both cells in PB or for one set of cells in both PB and AS. *nd, not determined.

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cAMP Receptor in Dictyostelium

by about 30-fold and exposes additional receptor sites (Van Haastert, 1985). For both cAR1 and developed B18 cells, the affinity for cAMP ($K_a = 4$ and 2 nM, respectively) and the number of sites exposed ($3.7 \times 10^4$ and $9.6 \times 10^4$ sites/cell, respectively) in ammonium sulfate were similarly enhanced (Table I).

cAR1 did not appear to couple to its normal effectors when expressed during the growth stage. These cells did not display cAMP-mediated chemotaxis, cGMP increases, or cAMP-induced aggregation by 1-3 h. Once development was in progress, however, it proceeded normally. Aggregation centers, wave patterns, and the typical morphological stages were all present.

Regulatory Properties of Expressed cAR1. To examine the ligand-induced phosphorylation of cAR1 expressed during growth, cells were labeled in vivo with $^{32}$P iP and incubated in the presence or absence of 10 nM cAMP. The receptor was then immunoprecipitated with cAR1 antiserum (Figure 5). The endogenous cAR1 in developed cells migrated as a 40-kDa band (R form) in the basal state (lane 7), and following stimulation with cAMP, it shifted to 43 kDa (D form) and became highly phosphorylated (lane 8). A similar transition and increase in phosphorylation were observed in growing cAR1 cells, and consistent with the increased amount of expression, higher levels of phosphorylation were observed (lanes 1 and 2). The kinetics ($t_{1/2} = 90$ s) and dose dependency ($K_d = 5$ nM) of phosphorylation observed in developed wild-type cells (Vaughan & Devreotes, 1988) appeared to be the same in growing cAR1 cells (unpublished data).

In developed cells, persistent incubation with cAMP causes a loss of surface cAMP binding sites, a process termed loss of ligand binding (LLB) (Klein & Juliani, 1977; Van Haastert, 1987). The transformed cell lines were used to determine whether the cells would similarly undergo LLB during the growth stage. Both growing cAR1 and developed B18 cells were incubated in the presence or absence of 0.1 mM cAMP for 15 min and washed repeatedly, and the number of surface cAMP binding sites were detected with $^{1}$H-cAMP in phos-
from coupling to Ga2 or to a G-protein more abundant during
growth, Ga1.

Van Haastert (1985) has shown that, in developed cells, the
receptor affinity for cAMP increases by 30-fold when 3.4 M
ammonium sulfate is added to the binding assay. This affinity
enhancement is caused by a reduction in the dissociation rate of
cAMP from the receptors. In our studies, cAMP binding in the
presence of ammonium sulfate enhanced receptor affinity and
the number of exposed binding sites similarly in both cell lines.
In B18 and cAR1, ammonium sulfate decreased the apparent
Kᵦ's to 2 and 4 nM, respectively.

Basal and cAMP-induced phosphorylation of cAR1 occurs
similarly whether the receptor is expressed endogenously in
aggregation stage cells or from plasmid in growing cells. A
time course of trypsin or endoproteinase Lys-C digestion of the
32P-labeled receptor or C-terminal domain shows that the
pattern of phosphopeptides generated is similar in both sets
of cells (unpublished data). This indicates that the ligand-
induced receptor kinase acts on the same sites of cAR1 both
during growth and development. Furthermore, the kinetics of
phosphorylation and dephosphorylation are comparable
(unpublished data), suggesting that the cAR1 kinase and
phosphatase are already present in nonlimiting amounts in
growing cells.

When preincubated with ligand, cAMP receptors undergo
a loss of ligand binding (LLB) and no longer bind extracellular
cAMP (Klein & Juliani, 1977; Van Haastert, 1987). Studies in
mammalian systems have shown that, after prolonged ligand
stimulation, β-adrenergic receptors occupy a distinct membrane
compartment which can be separated from the plasma mem-
brane by sucrose density gradient centrifugation (Lohse et al.,
1990). In developed Dictyostelium cells, previous experiments
have demonstrated a maximum loss of 80% of surface binding
sites after stimulation with 1 μM cAMP for 15 min (Van
Haastert, 1987). Our results show both cell lines lose at least
75% of surface cAMP binding. The extent of LLB is inde-
pendent of the level of cAR1 overexpression, which suggests
that this mechanism is not easily saturated.

These results expand our ability to examine the functions of
cAMP receptors. For cAR1, we can now study receptor
phosphorylation and LLB by introducing altered or chimeric
cAR proteins during the growth state and examine these two
regulatory mechanisms. For each cAR subtype, we can study
the properties of cAMP binding; recently both cAR2 and
cAR3 have been expressed and the binding and pharmacolo-
-gical aspects of each cAR compared (unpublished data).
cAR clones can now be expressed in an environment that is
free of their normally associated effectors and G-proteins as
well as in mutant cells that are blocked in development.
Coexpression of G-protein α-subunits with cAR's may enable
signal transduction to occur out of the context of normal
development and allow the examination of receptor/G-protein
coupling. In addition, it may be possible to introduce and
overexpress G-protein-coupled receptors from other eukaryotic
organisms and examine their ability to couple with Dictyo-
stelium signal transduction components.

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