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## Overexpression of the cAMP Receptor 1 in Growing *Dictyostelium* Cells<sup>†</sup>

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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 cAMP<sup>1</sup> 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 adenylyl 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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<sup>1</sup> Abbreviations: cAR, cAMP receptor; cAMP, adenosine cyclic 3',5'-monophosphate; cGMP, guanosine cyclic 3',5'-monophosphate; LLB, 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.

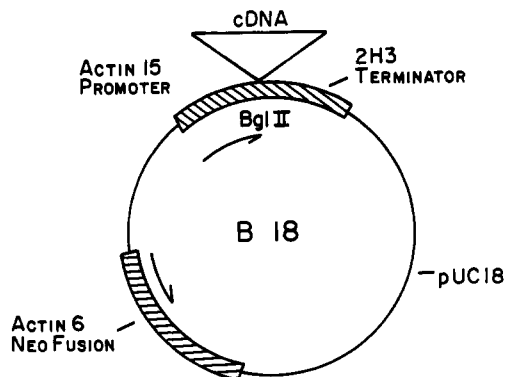


FIGURE 1: pB18 expression construct. pB18 is a derivative of pUC18 which contains the actin 6 Neo cassette to encode neomycin resistance. cDNAs encoding cAR1 or cAR1  $\Delta$ 311 were cloned in the sense orientation in the unique *Bgl*II site. The strong and constitutively active actin 15 promoter controls transcription.

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  $\mu$ g/mL G418. Cells were grown to a density of approximately  $5 \times 10^6$ /mL and developed by shaking at  $2 \times 10^7$ /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  $10^8$  cells/mL in DB.

**Vector Construction and Transformation.** pB6 (Klein et al., 1988) was digested with *Bam*HI/*Eco*RV to isolate a full-length cAR1 cDNA (cAR1) or digested with *Fok*I/*Nae*I to create a truncation in the C-terminal region at amino acid 311 (cAR1  $\Delta$ 311). *Bgl*II linkers were added to each and then ligated into the *Bgl*II site of the expression vector, pB18 (gift of R. Firtel), in the sense orientation (Figure 1). Transfor-

mations were done as described (Nellen et al., 1984) with some modifications. AX-3 cells ( $5 \times 10^7$ ) were grown overnight in 20 mM Bis-Tris HL-5 in Petri dishes, and medium was changed again the following morning. DNA (5–10  $\mu$ g) was precipitated in 0.125 M  $\text{CaCl}_2$  in  $1 \times$  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  $\mu$ g/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,  $8 \times 10^6$  cells were added to PB containing 10 mM DTT, 10 nM ( $^3\text{H}$ )cAMP, and various concentrations of cAMP in a 100- $\mu$ L volume at 4  $^\circ\text{C}$ . Cells were incubated 1 min and then centrifuged for 2 min at 10000g. To determine binding in AS, the above assay included 850  $\mu$ L of 3 M AS and, after adding cells, 50  $\mu$ L 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  $\mu$ L 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- $\text{N}_3$ -( $^{32}\text{P}$ )cAMP for photoaffinity labeling (Devreotes et al., 1987) or with ( $^{32}\text{P}$ ) $\text{P}_i$  (Vaughan & Devreotes, 1988) for in vivo phosphorylation as described. Loss of ligand binding was assayed as described (Van Haastert, 1987) by shaking  $5 \times 10^7$  cells in 2 mL of phosphate buffer in the presence or absence of 100  $\mu$ M cAMP and 10 mM DTT for 15 min. Cells were washed in 15 mL of PB three times at 4  $^\circ\text{C}$ , and specific binding sites were measured by using 5 nM ( $^3\text{H}$ )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

Table I: Summary of Binding Parameters of B18 and cAR1 Cells<sup>a</sup>

expt	cell	phosphate buffer				ammonium sulfate			
		$K_d$ (nM)		sites/cell ( $\times 1000$ )		$K_d$ (nM)		sites/cell ( $\times 1000$ )	
		high	low	high	low	high	low	high	low
I	B18	40 $\pm$ 9	350 $\pm$ 180	17 $\pm$ 12	58 $\pm$ 13	1.8 $\pm$ 0.26		96 $\pm$ 2	
II	B18		110 $\pm$ 14		64 $\pm$ 3	nd		nd	
	cAR1		86 $\pm$ 22		190 $\pm$ 17	nd		nd	
III	cAR1	25 $\pm$ 8	230 $\pm$ 45	75 $\pm$ 38	260 $\pm$ 34	3.5 $\pm$ 0.34		370 $\pm$ 6	

<sup>a</sup>(<sup>3</sup>H)cAMP binding was determined at 9 or 10 different cAMP concentrations in phosphate buffer (PB) 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. <sup>b</sup>nd, not determined.

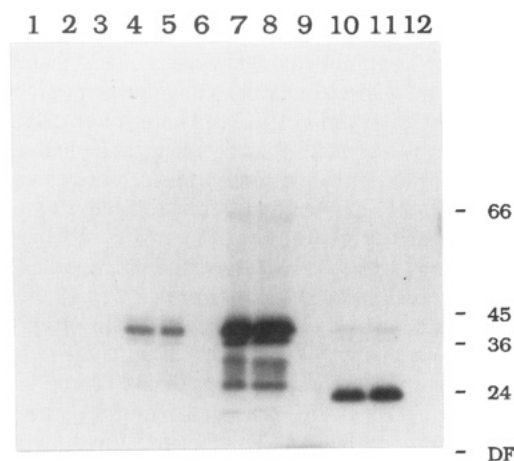


FIGURE 2: Photoaffinity labeling of cAR1 and cAR1  $\Delta 311$  cells. Growing (lanes 1–3) or developed B18 (lanes 4–6), growing cAR1 (lanes 7–9), and growing cAR1  $\Delta 311$  (lanes 10–12) cells were photoaffinity labeled with (<sup>32</sup>P)-8-N<sub>3</sub>-cAMP. Nonspecific labeling was examined by including excess cold cAMP into the assay (lanes 3, 6, 9, and 12). Full-length cAR1 migrates at 40 kDa while cAR1  $\Delta 311$  is approximately 24 kDa.

the transforming plasmid, we used an identical expression construct, cAR1  $\Delta 311$ , in which the insert was a truncated form of cAR1. The cAR1  $\Delta 311$  cells expressed about one-third as many cAMP binding sites as the cAR1 cells (data not shown). When photoaffinity-labeled with (<sup>32</sup>P)-8-N<sub>3</sub>-cAMP (Figure 2), the exogenously expressed 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  $\Delta 311$  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  $\Delta 311$  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 (<sup>3</sup>H)cAMP binding in phosphate buffer (PB). As shown in Figure 4 and Table I,

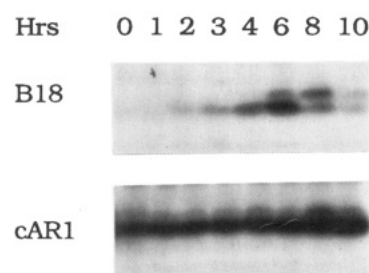


FIGURE 3: Developmental regulation of exogenously or endogenously expressed cAR1 protein. B18 or cAR1 cells were developed and examined for expression of cAR1 protein by immunoblots with cAR1 antiserum. Receptor expression in control cells (B18) was induced at 3 h, peaked at 6–8 h, and then declined in development. cAR1 cells expressed high levels of protein throughout the first 10 h of development.

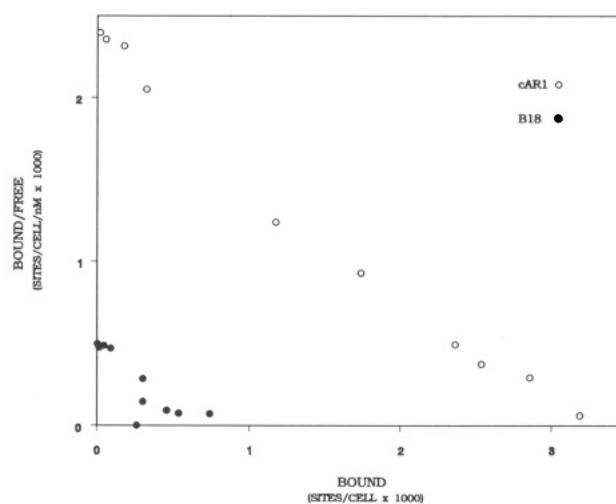


FIGURE 4: Scatchard analysis of cAR1 and developed B18 cells. Receptor affinity was determined by the binding of (<sup>3</sup>H)cAMP to cells in phosphate buffer. Developed B18 cells (●) express about  $7 \times 10^4$  binding sites/cell while cAR1 cells (○) have over  $3 \times 10^5$  sites/cell. Both cell lines have two affinity states of about 30 and 300 nM (see Table I).

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 230 nM (cAR1) or 350 nM (B18). A second experiment revealed only one affinity state for both cAR1 and developed B18 cells of 86 and 110 nM, respectively. The relative number of cAMP binding sites correlates well with relative cAR1 protein levels in each cell line. Control cells which were developed for 4 h expressed about  $7 \times 10^4$  sites/cell, while cAR1 cells expressed approximately  $(2-3) \times 10^5$  sites/cell in the growth stage. cAMP binding assayed in the presence of 3 M ammonium sulfate increases the apparent affinity for cAMP

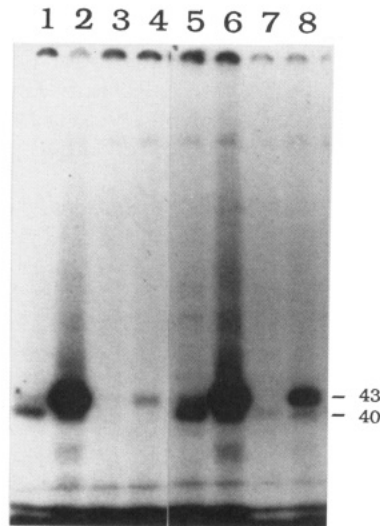


FIGURE 5: Receptor phosphorylation in cAR1 and B18 cells. Growing (lanes 1–4) and developed (lanes 5–8) cAR1 (lanes 1, 2, 5, and 6) or B18 (lanes 3, 4, 7, and 8) cells were labeled *in vivo* with  $(^{32}\text{P})\text{P}_i$  in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of cAMP. Receptor protein was immunoprecipitated with cAR1 antiserum and separated by SDS-PAGE. The apparent molecular mass (kDa) of basal and ligand stimulated receptors is shown on the right.

by about 30-fold and exposes additional receptor sites (Van Haastert, 1985). For both cAR1 and developed B18 cells, the affinity for cAMP ( $K_d = 4$  and  $2$  nM, respectively) and the number of sites exposed ( $3.7 \times 10^5$  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 increases above vector control backgrounds (data not shown). Surprisingly, the high level of constitutive expression of cAR1 had only a modest dominant phenotype; it delayed the onset of 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  $(^{32}\text{P})\text{P}_i$  and incubated in the presence or absence of  $10 \mu\text{M}$  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 ( $K_{50} = 5$  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  $(^3\text{H})\text{cAMP}$  in phos-

Table II: Loss of Ligand Binding<sup>a</sup>

	% cAMP binding sites lost after ligand stimulation
B18	$76 \pm 5.3$
cAR1	$84 \pm 2.3$

<sup>a</sup>Developed B18 or growing cAR1 cells were assayed for surface cAMP binding sites with or without preincubation with unlabeled cAMP. Results are from three experiments.

phate buffer. As shown in Table II, both cell lines lost at least 75% of their surface binding sites even though the growing cAR1 cells expressed over 3 times the number of binding sites compared to developed B18 cells.

## DISCUSSION

cAR1 plays a critical role in development since mutant cell lines that lack cAR1 by antisense mutagenesis (Klein et al., 1988; Sun et al., 1990) or gene disruption (Sun & Devreotes, 1991) fail to differentiate. We have established stable cell lines which express cAR1 up to 40 times over that of the endogenous receptors during growth and 3–5 times the peak developmentally regulated levels. When starved, cAR1 cells are able to differentiate and make fruiting bodies. The initiation of the developmental program was delayed for several hours, with cells remaining as a monolayer before proceeding into development.

We had expected the premature overexpression of cAR1 would severely disrupt the developmental program. The cAMP oscillator, which is responsible for center formation and proper functioning of cell–cell signaling, has been theoretically modeled. To create oscillations, these models require a precise interplay among the activities of surface cAMP receptors, adenylyl cyclase, and phosphodiesterase. Oscillatory behavior is limited to a specific range of these activities, and if any of these ranges are exceeded, the system becomes unbalanced and the oscillations stop (Goldbeter & Segel, 1977; Martiel & Goldbeter, 1987). Hence, overexpression of only cAR1 is predicted to disrupt the oscillator and block development. The relatively normal development of the cAR1 cells contradicts this prediction. It is possible that the receptor is not the limiting factor in adenylyl cyclase activation and thus an increase in cAR1 levels does not correspondingly increase adenylyl cyclase activation. Instead, other components in this signaling pathway, such as  $G\alpha_2$ , the G-protein presumed to couple to cAR1, may be in limiting quantities. In addition, we have noted that the kinetics of receptor phosphorylation proceeds normally in the cAR1 cells. The capacity of this regulatory system may allow the response to adapt with similar kinetics over a wide range of receptor levels and allow proper development.

The cAMP binding affinity of cAR1 expressed in growing cells is similar to that of the endogenous receptors expressed in aggregation. Previous equilibrium binding studies with aggregation stage cells have shown two affinities: a high-affinity site of approximately 10 nM and a low-affinity site, comprising most of the binding, of 150–450 nM (Green & Newell, 1975; van Haastert, 1985). In agreement with these studies, some of our binding data were best fit for two affinity sites of approximately 30 and 300 nM for both B18 and cAR1 cells. In one experiment, however, both cell lines appeared to have an intermediate affinity for cAMP (Table I). High-affinity sites are thought to result from coupling to a G-protein (Birnbaumer et al., 1985). In wild-type cells,  $G\alpha_2$  is coordinately expressed with cAR1 (Kumagi et al., 1989; Pupillo et al., 1989) but has variable low levels present in the growth stage. The high-affinity sites in growing cAR1 cells may result

from coupling to  $G\alpha 2$  or to a G-protein more abundant during growth,  $G\alpha 1$ .

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  $K_d$ '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  $^{32}P$ -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,  $\beta$  adrenergic receptors occupy a distinct membrane compartment which can be separated from the plasma membrane 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  $\mu$ M 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 independent 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 pharmacological 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  $\alpha$ -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 *Dictyostelium* signal transduction components.

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Registry No. cAMP, 60-92-4.

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