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 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 desensitize, 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, 14

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.

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cAMP Receptor in Dictyostelium

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 × 10^7/mL and developed by shaking at 2 × 10^6/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 BamHI/EcoRV to isolate a full-length cAR1 cDNA (cAR1) or digested with FokI/NaeI to create a truncation in the C-terminal region at amino acid 311 (cAR1 A311). BglII linkers were added to each and then ligated into the BglII site of the expression vector, pBl8 (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 varies 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 × 10^7 cells were added to PB containing 10 mM DTT, 10 nM (3H)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 10000 g. To determine binding in AS, the above assay included 850 μL of 3 M 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 (32P)P (Vaughan & Devreotes, 1988) for in vivo phosphorylation as described. Loss of ligand binding was assayed as described (Van Haastert, 1987) by shaking 5 × 10^7 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 pBl8 (Figure 1). This promoter is constitutively active during growth and throughout early development (Knecht et al., 1986). This construct or pBl8 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
CAR1 protein was examined by immunoblot during growth (0 h), which increased slightly with previous studies using the actin 15 promoter (Knecht et al., 1986). CAR1 cells, however, expressed high levels of CAR1 protein 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 (3H)cAMP binding in phosphate buffer (PB). As shown in Figure 4 and 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 × 10^4 sites/cell while cAR1 cells (O) have over 3 × 10^5 sites/cell. Both cell lines have two affinity states of about 30 and 300 nM (see Table I).

Table I: Summary of Binding Parameters of B18 and cAR1 Cells

<table>
<thead>
<tr>
<th>expt</th>
<th>cell</th>
<th>Kd (nM)</th>
<th>sites/cell (×1000)</th>
<th>Kd (nM)</th>
<th>sites/cell (×1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>high</td>
<td>low</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>I</td>
<td>B18</td>
<td>40 ± 9</td>
<td>350 ± 180</td>
<td>17 ± 12</td>
<td>58 ± 13</td>
</tr>
<tr>
<td></td>
<td>cAR1</td>
<td>25 ± 8</td>
<td>230 ± 45</td>
<td>75 ± 38</td>
<td>260 ± 34</td>
</tr>
<tr>
<td>II</td>
<td>B18</td>
<td>110 ± 14</td>
<td>64 ± 3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>cAR1</td>
<td>86 ± 22</td>
<td>190 ± 17</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>1.8 ± 0.26</td>
<td>96 ± 2</td>
<td>3.5 ± 0.34</td>
<td>370 ± 6</td>
</tr>
</tbody>
</table>

(3H)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. *nd, not determined.

The affinity of CAR1 expressed from plasmid 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 × 10^4 sites/cell, while cAR1 cells expressed approximately 2-3 × 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.
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 ($K_d = 4$ and 2 mM, 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 effect when examined 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 $^{32}$P and incubated in the presence or absence of 10 mM 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 in expression, higher levels of 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 $^{3}H$ cAMP in phosphate 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 Go2, 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, Go2 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 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 of phosphorylation and dephosphorylation are comparable in the presence of ammonium sulfate enhanced receptor af-

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-


