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# The Surface Cyclic AMP Receptor in *Dictyostelium*

## LEVELS OF LIGAND-INDUCED PHOSPHORYLATION, SOLUBILIZATION, IDENTIFICATION OF PRIMARY TRANSCRIPT, AND DEVELOPMENTAL REGULATION OF EXPRESSION*

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**A monospecific polyclonal antiserum to the surface cAMP receptor of *Dictyostelium* has been developed by immunization with purified receptor immobilized on particles of polyacrylamide and on nitrocellulose paper. In Western blots, the antiserum displays high affinity and specificity for both the R ($M_r$ 40,000) and D ($M_r$ 43,000) forms of the receptor previously identified by photoaffinity labeling with 8-azido-[$^{32}$P] cAMP. These bands, labeled with the photoaffinity label or with $^{32}P_i$, were quantitatively and specifically immunoprecipitated, supporting co-purification data that all represent the same polypeptide. The R form, found in unstimulated cells, contained at least 0.2 mol of phosphate/mol of receptor. The D form, generated by cAMP stimulation of intact cells, contained at least 4 mol of phosphate/mol of receptor. In the absence of detergents, the receptor was exclusively located on membranes. The receptor was solubilized effectively in Triton X-100 and sedimented as a broad peak of 5–7 S on sucrose velocity gradients. Western blots of membranes isolated at different times after starvation indicate that the appearance of cell surface cAMP binding sites during the aggregation stage of development (5–6 h) is due to *de novo* synthesis of receptor protein. Pulse labeling with [$^{35}$S]methionine indicated that the receptor is most rapidly synthesized during the preaggregation stage of development (1–3 h), prior to its maximal accumulation in membranes. The serum specifically immunoprecipitates a polypeptide of $M_r$ 37,000 from an *in vitro* translation reaction using RNA isolated from preaggregation stage cells. The time course of expression of the mRNA coding for the $M_r$ 37,000 polypeptide parallels the rate of receptor synthesis *in vivo*.**

The aggregation of *Dictyostelium* is mediated by a plasma membrane-bound receptor for extracellular cAMP which has been identified by photoaffinity labeling with 8-$N_3$-[$^{32}$P] cAMP[1] as a doublet ($M_r$ 40,000 and 43,000) (1–3). Binding of cAMP ($K_D$ = 50 nM) induces a set of transient responses that includes chemotaxis, GTP-dependent activation of adenylate cyclase, activation of guanylate cyclase, and phosphorylation of myosin heavy and light chains (4–7). In the presence of a fixed level of cAMP, many of these responses subside (a process termed adaptation) with a half-time of about 2 min (7, 8). When cAMP is removed, the cells become resensitized (9). This reversible adaptation is associated with a shift in electrophoretic mobility of the surface receptor from the R form ($M_r$ 40,000), present in unstimulated cells, to the D form ($M_r$ 43,000), present in adapted cells (10, 11). Stimulation of cells with cAMP is also associated with an extensive reversible phosphorylation of the cAMP receptor which is probably responsible for the shift in its electrophoretic mobility (12, 13).

Similar response-adaptation cycles are seen in other transmembrane signaling systems such as the chemotactic receptors of bacteria (14), the response to light in rod outer segments (15), and the β-adrenergic receptor-mediated activation of adenylate cyclase (16). In these systems, as in *Dictyostelium*, adaptation has been reported to be temporally correlated with reversible modification of the respective receptors. In bacteria, the modification is methylation of chemotactic receptors while in eukaryotic cells, the modification is phosphorylation (13–17). Thus, the cAMP receptor of *Dictyostelium* appears to parallel receptors of higher eucaryotes in being regulated by GTP and in being phosphorylated in response to ligand binding.

The level of cAMP binding to intact cells rises during differentiation to a maximum of 10–20-fold over vegetative cells during the early aggregation stage and then falls to less than 20% of the maximum binding. It has not been determined whether the regulation of expression of binding sites is due to *de novo* synthesis of receptors or mobilization of cryptic receptors (either internal receptors or plasma membrane-bound receptors that do not bind cAMP). The cAMP receptor, in turn, appears to regulate its own expression as well as the expression of several other aggregation stage proteins, including membrane-bound adenylate cyclase and phosphodiesterase activities and several genes of unknown function (reviewed in Ref. 18). This regulation by cAMP requires oscillations in extracellular cAMP with a period of 6–10 min. If endogenous cAMP synthesis is inhibited but exogenous cAMP is added every 6 min, the cells differentiate. If cAMP is absent or present at a fixed level, differentiation is blocked. The negative effect of continued cAMP stimulation may reflect an adaptation to cAMP that is mediated by the same mechanisms that cause adaptation of the chemosensory responses. Recent evidence also suggests that a cAMP receptor regulates cell-type specific differentiation later in development. In contrast to that of the early genes, regulation of the late genes is nonadaptive; continuous levels of cAMP effectively induce the late gene products (19).

Both the R and D forms of the cAMP receptor have now been purified to homogeneity (1). This report describes a

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[1] The abbreviations used are: 8-$N_3$-[$^{32}$P]cAMP, 8-azido-[$^{32}$P]cAMP; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

monospecific antiserum generated against the cAMP receptor which is used to characterize the level of ligand-induced receptor phosphorylation, subcellular localization, and the developmental regulation of receptor expression.

## MATERIALS AND METHODS

*Cell Growth and Development*—AX-3 cells were grown to a density of $5 \times 10^6$/ml in HL-5 medium and developed at $2 \times 10^7$/ml in development buffer (DB) as described (1).

*Preparation of Antigen and Immunization*—The cAMP receptor was purified in the D form as described in the preceding paper. Samples eluted from the preparative L gels from several purifications were pooled and subjected to a second preparative SDS-PAGE using a 1.5-mm H gel. 2 ml of gel eluate with approximately 10–40 μg of cAMP receptor (estimated by comparison of intensity of silver-stained bands with standards) in sample buffer (25 mM Tris, pH 6.8, 1.5% SDS, 2.5% dithiothreitol, 5% glycerol, bromphenol blue) was electrophoresed at 50 V for 16 h. The gel was fixed in MeOH:acetic acid:water (5:1:5) for 30 min and then silver stained (which included a 15-min incubation in 5% glutaraldehyde). The receptor band, identified previously from autoradiographs of analytical gels, was excised with a razor blade, and the gel slice was cut into 4–5-mm pieces. 2 ml of sterile water was added to the gel pieces which were then either macerated further or sonicated intermittently using a Branson sonicator with a mini-probe at a setting of 5 until a homogeneous suspension of particles was achieved. A second gel was run in parallel and transferred for 3 h at 4 °C to 3 sheets of nitrocellulose using a transblot apparatus. Transfer buffer was 25 mM Tris, pH 8, 192 mM glycine, 20% MeOH (20). The nitrocellulose sheets were dried and autoradiographed for 1–3 days. The autoradiographs were aligned with the nitrocellulose sheets, and the radioactive band was excised and dissolved in 1 ml of dimethyl sulfoxide, and mixed 1:1 with Freund's incomplete adjuvant. The gel containing the untransferred protein was then silver stained, and the receptor band was processed by sonication as described above.

One female New Zealand White rabbit was immunized with multiple intradermal and subcutaneous injections of 0.2–0 3 ml over the entire back. The rabbit initially received approximately 30 μg of receptor in macerated gel pieces mixed 1:1 with Freund's complete adjuvant. After 3 weeks the rabbit received a second immunization with 30 μg of receptor in macerated gel pieces mixed 1:1 with Freund's incomplete adjuvant. At 7 weeks the rabbit received 30 μg of receptor, 20 μg in the sonicated suspension of gel pieces and 10 μg which had been transferred to nitrocellulose. At 9 weeks, a low level response was detected in Western blots, and the rabbit was boosted with 30 μg of receptor in sonicated gel pieces and on nitrocellulose, which resulted in high titer antiserum as determined by Western blot analysis.

*Western Blots*—Western blots were done essentially as described (20). Membranes were prepared and solubilized in sample buffer as described (2, 3), run on 1.5-mm 10% SDS-PAGE, and transferred to nitrocellulose for 2 h at 4 °C. Antiserum was diluted 1:500 in most cases. For competition experiments, antiserum was preincubated with purified receptor at 50 ng/ml for 15 h at 4 °C. $^{125}$I-Protein A (specific activity, 20 Ci/μg) was prepared by the chloramine-T method (21) and used at $5 \times 10^6$ cpm/ml. Autoradiographs were exposed for 4–24 h.

*Immunoprecipitation*—Immunoprecipitation of the cAMP receptor was performed from membranes solubilized in sample buffer (1.5% SDS). Samples were diluted to 0.1% SDS with an immunoprecipitation buffer (IP) consisting of receptor buffer (1) diluted 1:10 with 20 mM Tris, pH 7.5, 0.15 M NaCl, 0.5% Nonidet P-40, and 0.5 mg/ml ovalbumin. Anti-receptor antiserum was added to a final concentration of 1:200, and samples were incubated at 4 °C for 2 h with rotation. For competition experiments, antiserum was preincubated with purified receptor at 50 ng/ml for 15 h at 4 °C. Protein A-Sepharose CL-4B beads (Pharmacia) suspended in IP buffer (80 mg/ml) were added (200 μl of beads/ml of sample) and incubated with rotation at 4 °C for an additional 60 min. The samples were centrifuged for 1 min in a Microfuge, the pellets were washed twice with IP buffer, and twice with IP buffer plus 0.1% SDS and 1.0% sodium deoxycholate and without ovalbumin. The washed pellets were then treated with SDS sample buffer at a volume equal to the original volume of solubilized membranes and heated to 95 °C for 10 min, and centrifuged again for 1 min. The resulting supernatants were subjected to SDS-PAGE and autoradiographed.

*Labeling the cAMP Receptor*—Labeling of the cAMP receptor with 8-$N_3$-[$^{32}$P]cAMP (2) and with $^{32}P_i$ (1, 13) has been described. For [$^{35}$S]methionine labeling of intact cells, [$^{35}$S]methionine (specific activity, 1200 Ci/mmol, New England Nuclear) was dried under an $N_2$ stream, resuspended in DB, and added to cells as indicated in the figure legends.

*Estimation of the Number of Mol of Phosphate/Mol of Receptor*—For determining the amount of $^{32}$P incorporated into receptor, $^{32}$P-labeled receptor from stimulated and unstimulated cells was immunoprecipitated, electrophoresed on SDS-PAGE, and autoradiographed as described. The gel was aligned with the autoradiograph, and the bands corresponding to the receptor were excised and counted for Cerenkov radioactivity.

To determine the amount of protein in the immunoprecipitated receptor bands, unlabeled membranes from stimulated or unstimulated cells were prepared and immunoprecipitated in parallel with $^{32}P_i$-labeled receptor. These unlabeled receptor preparations were then electrophoresed, transferred to nitrocellulose, and Western blotted in parallel with purified receptor of known concentrations. The amounts of receptor in the immunopellets were determined from the standard curve of receptor protein concentration obtained from densitometric scans of the Western blots. The protein concentration of the purified receptor sample (prepared as described in the preceding report) was determined by comparison on a silver-stained SDS-polyacrylamide gel to a protein standard curve generated using purified ovalbumin, concanavalin A, and trypsin inhibitor.

To determine the specific activity of the [$\gamma$-$^{32}$P]ATP pools, cells were labeled with $^{32}P_i$ and then R and D forms of the receptor prepared as described. At the end of the labeling period, cells were washed once with DB, and then a volume of cells was removed to an equal volume of 0.1 N perchloric acid. The sample was centrifuged for 5 min in a Microfuge to remove acid-insoluble material, and the supernatant was removed. ATP was resolved from the other soluble components in the supernatant by two-dimensional chromatography on polyethyleneimine cellulose plates (Polygram CEL 300 PEI/$UV_{254}$, Macherey-Nagel). The first dimension was developed in 0.2 M ammonium bicarbonate and the second in 0.7 M ammonium sulfate. The plates were dried and autoradiographed. Parallel samples containing internal standards of ATP, ADP, and AMP were similarly chromatographed, and the internal standards on the polyethyleneimine plate were visualized with UV light. The ATP/[$^{32}$P]ATP spot was easily identifiable by autoradiography and comparison to the plates containing the standards. The area on the plate containing the ATP was scraped off the cellulose backing and treated with 30 mM HCl for 15 min to elute the ATP. The solution was neutralized with Tris, pH 8.0, and aliquots were assayed both for total ATP using the luciferase assay (22) and for Cerenkov radiation.

The amount of [$\gamma$-$^{32}$P]ATP in the ATP was determined by digesting the ATP with glycerol kinase in the presence of glycerol and $MgCl_2$. Aliquots of the sample were chromatographed in two dimensions on polyethyleneimine plates and both ATP and ADP spots were counted for radioactivity. Greater than 98% of the radioactivity disappeared from the ATP spot after this treatment. The increase in the amount of radioactivity in the ADP spot after the reaction was considered to be the amount of [$\alpha$-$^{32}$P]- and [$\beta$-$^{32}$P]ADP released from the glycerol kinase reaction. In two separate determinations this value was found to be about 50%, indicating that 50% of the [$^{32}$P]ATP was labeled in the $\gamma$ position. Data are presented as mean ± S.D. of four determinations.

*Membrane Localization and Solubilization of Receptor in Nonionic Detergents*—Cells at the aggregation stage of development were prepared as described (2) to yield receptor in either the R form or the D form. The cells were then washed and resuspended at $4 \times 10^7$ cells/ml in RBS (25 mM Tris, pH 7.5, 5 mM dithiothreitol, 5 mM EDTA, 250 mM sucrose, and a mixture of protease inhibitors described elsewhere (2)). Cells (5 ml) were cooled to 4 °C and lysed by rapid passage through a 5-μm filter (Nucleopore) attached to a syringe. The lysate was centrifuged in a 15-ml glass Corex tube in a Sorvall SS-34 rotor at 10,000 rpm for 30 min. The pellet was resuspended in RB with 1% Triton X-100, incubated at 0 °C for 45 min, and centrifuged at 18,000 rpm for 30 min. The pellet was solubilized in SDS-PAGE sample buffer (5 ml), and 4 ml of the supernatant was centrifuged in a Beckman Ti-80 rotor at 45,000 rpm for 1 h. Aliquots from each step were subjected to SDS-PAGE and Western blotted as described, and stained with horseradish peroxidase conjugated to goat anti-rabbit IgG.

The Triton-solubilized material (200 μl) was layered onto a 5–20% linear sucrose gradient (4 ml) containing RB with 1% Triton X-100 and centrifuged in a Beckman Ti 50.1 rotor at 45,000 rpm for 12 h.

After centrifugation, 200-μl fractions were collected and subjected to SDS-PAGE and Western blotted as described above. Each lane was scanned, and the optical density was plotted as a fraction of the maximum signal.

*In Vitro Translation*—Total RNA from AX-3 cells was prepared as described (23) at 1-h intervals during development in suspension. *In vitro* translation was done using Promega nuclease-treated rabbit reticulocyte lysate as described in the Promega protocol with total RNA at 200 μg/ml. The translation was carried out at 30 °C for 60 min, and samples were then immunoprecipitated as described above. Incorporation of [$^{35}$S]methionine into total protein was determined by trichloroacetic acid precipitation following alkaline hydrolysis. Samples of the reaction mix prior to immunoprecipitation were subjected to SDS-PAGE and fluorography. The volumes of the immunoprecipitates were normalized to the number of trichloroacetic acid-precipitable cpm and were also subjected to SDS-PAGE and fluorography.

## RESULTS

### *Specific Antiserum to the Surface cAMP Receptor*

*Western Blots*—Fig. 1 shows a Western blot of crude membranes prepared from aggregation stage cells before and after stimulation with cAMP. Although antiserum was generated against the D form, the R form was recognized as efficiently. Prior to cAMP stimulation, greater than 90% of the receptor is in the R form and less than 10% is in the D form (*lane 1*). After 10 min of stimulation with cAMP, 80% of the receptor is in the D form (*lane 2*). The kinetics and concentration dependence of the mobility shift detected in the Western blots are identical to those monitored by photoaffinity labeling (11). Following stimulation with cAMP, the R form disappears and the D form appears concurrently with a $t_{½}$ of 2 min.

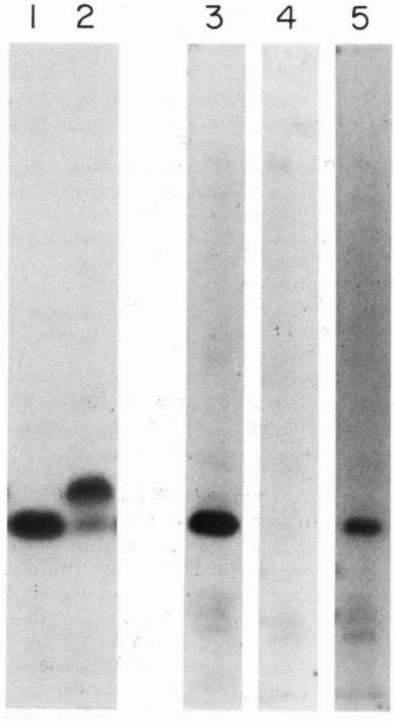


FIG. 1. **Specificity of antiserum in Western blots.** Sets of cells were prepared in which the receptor was in the R or D form, membranes isolated, separated by SDS-PAGE, and transferred to nitrocellulose. Immune serum at 1:500 dilution was applied to membranes containing the R (*lane 1*) or D (*lane 2*) form. *Lanes 3–5* show staining of the R form membranes with immune serum (*lane 3*), preimmune serum (*lane 4*), or immune serum preincubated with 50 ng/ml purified receptor (*lane 5*). The experiment in *lanes 1* and *2* has been repeated over 30 times with similar results; the experiment in *lanes 3–5* has been repeated four times with similar results.

When the stimulus is removed, the R form reappears and the D form disappears, both with a $t_{½}$ of about 5 min. These data strongly suggest that ligand-induced modification of the receptor converts the R form into the D form.

Preimmune serum did not stain the receptor (Fig. 1, *lane 4*). Fig. 1 also shows that the staining of the R form of the receptor was specifically competed by preincubating the immune serum with purified receptor. The residual staining of the receptor band (*lane 5*) was 10% of the control (*lane 3*) as determined by optical scanning of shorter exposures. Equivalent nonspecific staining occurred with immune and preimmune serum and immune serum absorbed with purified receptor (*lanes 3–5*). The half-maximal signal in Western blots for both forms of the cAMP receptor was seen at a 1:500 dilution of antiserum. However, the receptor was readily detected with serum at dilutions greater than 1:500,000. It should be noted that these gels were heavily loaded with membranes; for instance, more than 1 μg of actin is present between the R and D forms of the receptor (estimated to be about 10 ng), yet there is no nonspecific staining of actin.

Faint bands are occasionally seen at approximately $M_r$ 55,000 and $M_r$ 70,000 (Fig. 1, *lanes 1* and *2*). These bands, representing less than 5% of the $^{125}$I-protein A stained material, show a cAMP-dependent shift in electrophoretic mobility and developmental regulation that parallels the cAMP receptor. Thus, it seems that these bands represent specific staining of protein(s) related to the cAMP receptor or an aggregated form of the receptor itself, rather than nonspecific staining (1). We cannot rule out that the actual $M_r$ of the intact receptor is 55,000 or 70,000 and that the bands at $M_r$ 40,000–43,000 are proteolytic fragments.

*Immunoprecipitation*—The cAMP receptor can be specifically immunoprecipitated from SDS-solubilized membranes as shown in Fig. 2. Cells were developed for 5 h, treated with either 5 mM caffeine or 10 nM cAMP, and labeled with 8-$N_3$-[$^{32}$P]cAMP, $^{32}PO_4$, or [$^{35}$S]methionine. Immunoprecipitation from SDS-solubilized membranes was carried out as described under "Materials and Methods." The recovery of receptor was 75–90%, while none was recovered with preimmune serum. The receptor is stable to proteases and to phosphatases during the immunoprecipitation incubations (data not shown). The antiserum immunoprecipitates both the photoaffinity-labeled (*lanes 1–6*) and the $^{32}$P-labeled R and D forms (*lanes 7–12*), offering further support, in addition to co-purification (1), that the phosphorylated proteins are equivalent to the cAMP receptor identified by photoaffinity labeling.

The number of mol of phosphate/mol of receptor was determined from similar immunoprecipitation experiments as described under "Materials and Methods" (Table I). These were at least 0.2 ± 0.1 mol of phosphate/mol of the R form of the receptor. Following cAMP stimulation of intact cells, which generated the D form, the level of phosphorylation rose to 4.0 ± 0.8 mol of phosphate/mol of receptor.

In order to give a better indication of the specificity of the immunoprecipitation, cells were labeled with [$^{35}$S]methionine from 3 to 5 h of development. Membranes were prepared, solubilized in sample buffer, and immunoprecipitated. The cAMP receptor is estimated to be present at 0.02–0.04% of the total membrane protein. As shown in Fig. 2, *lanes 13–18*, after immunoprecipitation, the R and D forms are the major proteins seen in fluorography.

### *Membrane Localization and Solubilization of the Receptor*

All of the cAMP receptor (detected by Western blotting) was present in the membrane fraction (10,000 × *g* pellet from cells lysed either by osmotic shock (1) or by passage through

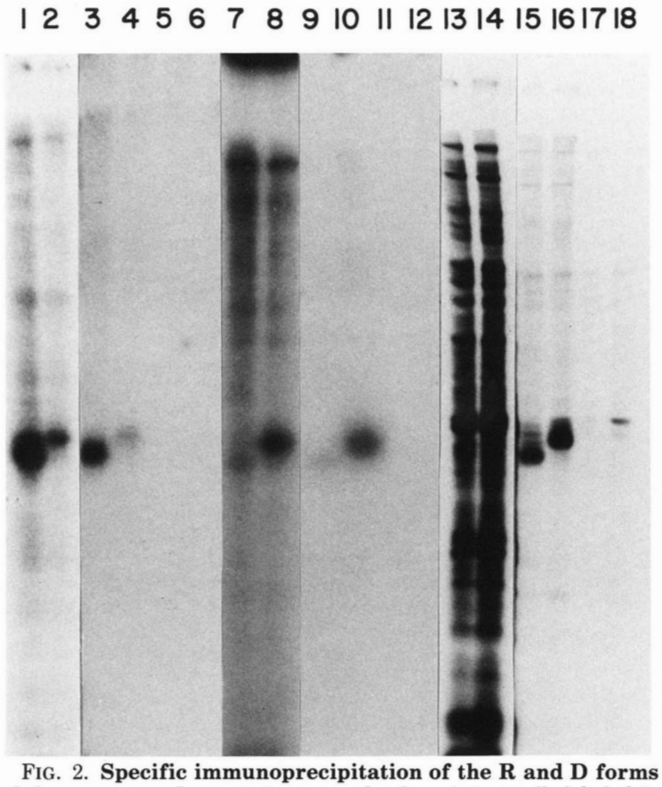


FIG. 2. **Specific immunoprecipitation of the R and D forms of the receptor.** *Lanes 1–6* are samples from intact cells labeled 8-$N_3$-[$^{32}$P]cAMP; *lanes 7–10* are samples from cells labeled *in vivo* with [$^{32}$P]$PO_4$; *lanes 13–18* are samples from cells labeled *in vivo* with [$^{35}$S] methionine. *Lanes 1, 2, 7, 8, 13,* and *14* are crude membrane fractions; *lanes 3, 4, 9, 10, 15,* and *16* are corresponding immunopellets; *lanes 5, 6, 11, 12, 17,* and *18* are corresponding preimmunopellets (see "Materials and Methods"). Odd- or even-numbered lanes contain the R or D forms of the receptor. Experiments have been repeated at least four times with similar results.

TABLE I

*Quantitation of receptor phosphorylation*

| | Sample loaded[a] | $P_i$/band[b] | Protein/band[c] | $P_i$/receptor | $\bar{x} \pm$ S.D. |
|---|---|---|---|---|---|
| | *μl* | *pmol* | *ng* | *mol/mol* | |
| R Form | | | | | |
| Experiment 1 | 75 | 0.3 | 111 | 0.1 | |
| | 50 | 0.2 | 71 | 0.1 | 0.2 ± 0.10 |
| | 25 | 0.3 | 34 | 0.3 | |
| Experiment 2 | 75 | 0.2 | 24 | 0.3 | |
| D Form | | | | | |
| Experiment 1 | 75 | 3.2 | 31 | 4.1 | |
| | 50 | 2.2 | 21 | 4.2 | 4.0 ± 0.8 |
| | 25 | 1.2 | 10 | 4.8 | |
| Experiment 2 | 75 | 0.8 | 12 | 2.7 | |

[a] The $^{32}$P-labeled receptor from stimulated and unstimulated cells was immunoprecipitated and electrophoresed as described under "Materials and Methods."

[b] Receptor bands were localized by autoradiography, excised, and counted for Cerenkov radioactivity. The specific activity of the [$\gamma$-$^{32}$P]ATP pool was determined using the bioluminescence assay as described under "Materials and Methods."

[c] Total receptor protein was determined by Western blotting parallel unlabeled receptor samples as described under "Materials and Methods."

a 5.0-$\mu$m Nucleopore filter), and neither the R form nor the D form was detected in the 10,000 × *g* supernatant from cell lysates. However, 80% of the receptor can be solubilized from membranes using 1% Triton X-100 (not sedimented at 250,000 × *g* for 1 h). The solubilized material was analyzed by velocity sedimentation in a sucrose gradient (containing 1% Triton X-100) followed by Western blotting of the fractions. As shown in Fig. 3, after 12 h the receptor sedimented as a broad peak of 5–7 s and often separated into two peaks or a peak and a shoulder. Receptor was not detected in the pellet although ferritin and thyroglobulin did pellet.

### *Developmental Regulation of Receptor Synthesis and Accumulation*

As shown in Fig. 4*A*, the binding of [$^3$H]cAMP (and 8-$N_3$-cAMP) is regulated during development with a maximum level as cells begin to aggregate. To determine whether the increase in binding sites reflects an increased level of receptor synthesis (as opposed to exposure of cryptic binding sites), cells were taken at various stages of development from 0 to 10 h and treated with 5 mM caffeine or 10 $\mu$M cAMP to generate the R or D forms, respectively. Membranes were prepared and Western blotted. As shown in Fig. 4*B*, the level of cAMP receptor present in total cellular membranes rises during development to a maximum of 17-fold over vegetative cells (during the aggregation stage) and then falls off to less than 20% of the maximum level. The intensity of antibody staining closely parallels the time course of [$^3$H]cAMP binding throughout development (Fig. 4*D*). It is interesting to note that during the time of maximal receptor expression, at 4–6 h of development, receptors shift cleanly from the R to D form when cells are stimulated with cAMP. However, at 0–3 and 7–10 h, receptors appear to less responsive to the cAMP stimulus and consist of mixtures of R and D forms both prior to and following stimulation (Fig. 4*B*, *inset*).

The same sets of cells were pulse labeled with [$^{35}$S]methionine for 15 min, and the receptor was immunoprecipitated. As shown in Fig. 4*C*, the peak rate of [$^{35}$S]methionine incorporation into the receptor occurred from 1.5 to 3 h. By the time the maximal accumulation of receptor occurred at 5–6 h, the rate of synthesis had declined to levels found in undifferentiated cells (Fig. 4*D*).

### *Identification of an in Vitro Translation Product and Developmental Regulation of Translatable mRNA*

RNA from cells developing in suspension was isolated at 1-h intervals from 0 to 8 h. The spectrum of *in vitro* translated

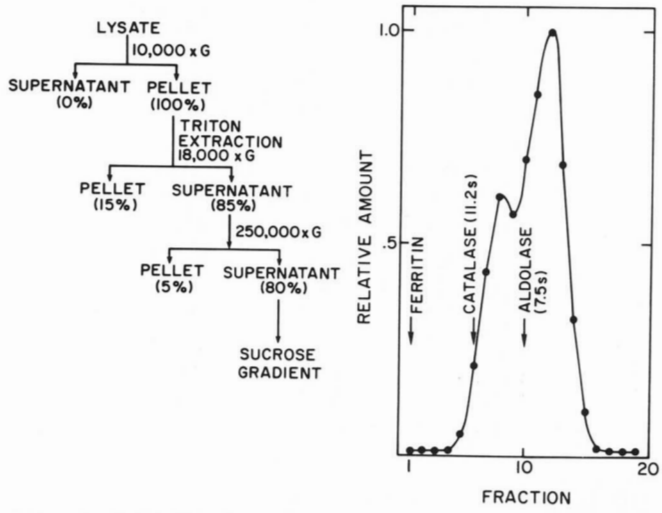


FIG. 3. **Solubilization of receptor in nonionic detergents.** Cells are prepared in which the receptor was in the R form. *Left*, fractionation scheme indicating that receptor is insoluble in the absence of detergents and solubilized by 1% Triton X-100. Quantitations shown were measured by scanning of Western blots of the R form of the receptor. *Right*, sucrose gradient sedimentation of solubilized R form of the receptor. Fractions were analyzed on SDS-PAGE and Western blotted. Shown is the relative amount of receptor in each of the fractions. Recovery of the loaded sample was 91%.

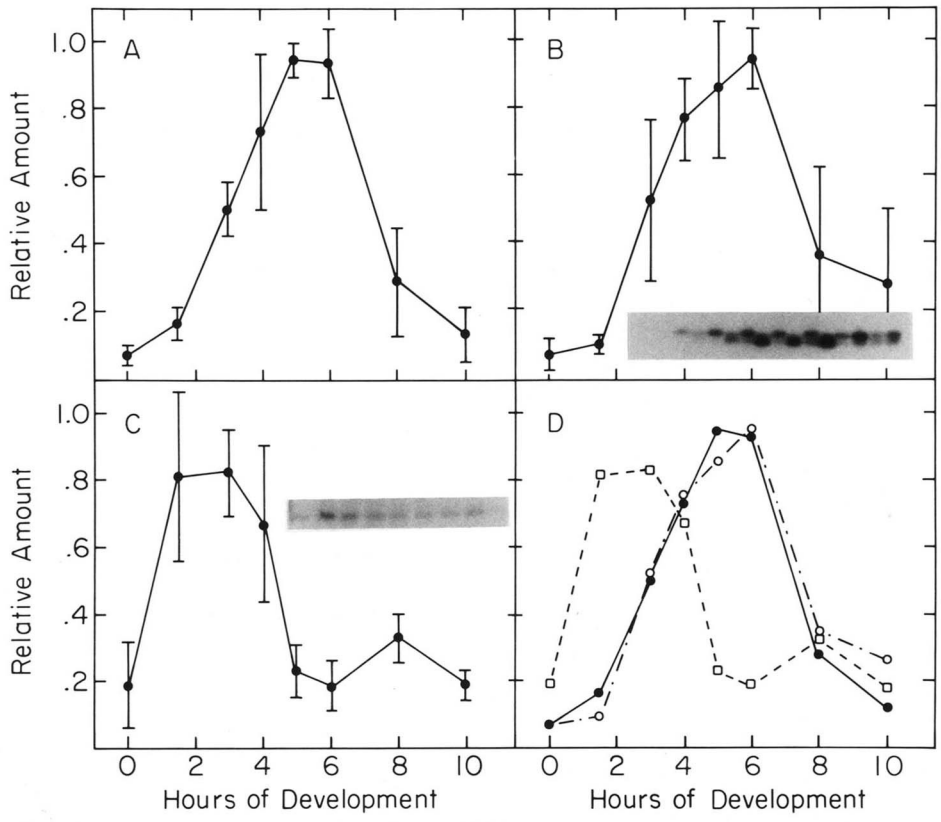


FIG. 4. **Developmental regulation of surface cAMP binding sites, receptor protein, and rate of receptor synthesis.** Cells were placed under conditions for optimal development for the indicated times and treated with 5 mM caffeine or 10 $\mu$M cAMP to generate the R or D forms. *A*, [$^3$H]cAMP binding was determined as previously described. *B*, Western blot, carried out as shown in Fig. 1, is shown in the *inset*. Each pair of lanes from left to right corresponds to cells harvested at 0, 1.5, 3, 4, 5, 6, 8, and 10 h. The *first lane* in each pair corresponds to cells treated with caffeine; the *second lane* of the pair corresponds to cells treated with 10 $\mu$M cAMP. Blots were quantitated by scanning of the caffeine-treated samples. *C*, fluorograph of immunoprecipitates from cells pulse labeled with [$^{35}$S]methionine for 15 min is shown in the *inset*. Major band (R form of the receptor) was scanned to determine the relative rate of incorporation. Three independent 10-h experiments, carried out over a period of several months, were combined. In each experiment, data was normalized to the highest level. This was typically 5 or 6 h for [$^3$H]cAMP binding and Western blotting and 1.5 or 3 h for [$^{35}$S]methionine pulse labeling. Data from all three experiments was averaged. *Error bars* (standard errors) were largely due to a slight difference in the development time courses in the three experiments. *D*, replots of averages of surface cAMP binding sites (●), receptor protein (○), and rate of synthesis (□) data.

proteins was similar. The reaction mix was immunoprecipitated with anti-receptor antiserum and subjected to SDS-PAGE and fluorography. The immunoprecipitation (Fig. 5*A*) reveals an intense band at $M_r$ 37,000 that is developmentally regulated with a peak at 3 h of development. The band is not observed in vegetative cells and falls to undetectable levels by 8 h of development. This pattern of expression parallels the rate of receptor synthesis shown in Fig. 4*C*, as would be expected if the rate of *in vivo* receptor synthesis is proportional to the level of translatable RNA. The immunoprecipitation of this band was completely blocked by preincubation of the antiserum with purified receptor (Fig. 5*B*). The nonspecific bands in the immunoprecipitation were not affected by the preabsorption with purified receptor. It was estimated that the $M_r$ 37,000 polypeptide, at the time of maximal expression, represents about 0.07% of the total *in vitro* translated protein indicating that the relative abundance of receptor mRNA is severalfold higher than the relative abundance of receptor protein.

## DISCUSSION

The cAMP receptor of *Dictyostelium*, or one of its subunits, has been purified and used to generate a monospecific polyclonal antiserum. The following observations indicate that the antiserum recognizes the cAMP receptor. 1) In Western blots of membranes from aggregation stage cells, the antibody stains a protein that comigrates with the R form of the receptor from cells prior to cAMP stimulus and comigrates with the D form following cAMP stimulation. The kinetics of cAMP-induced mobility shift detected by Western blotting agrees with the kinetics of shifting of the photoaffinity-labeled receptor (11). 2) The antibody is very specific for receptor. Preabsorption of the immune serum with purified receptor completely and specifically blocks the immunoprecipitation and competes the staining of receptor in Western blots by 90%. Preimmune serum does not immunoprecipitate or stain the receptor. Although the receptor represents approximately 0.05% of the total membrane protein, it is the major band in Western blots and in the immunoprecipitation. It does not specifically recognize cytosolic proteins and in particular does not cross-react in Western blots with the regulatory subunit of cAMP-dependent protein kinase (gift of Dr. B. Leichtling). 3) The antiserum stains the receptor in Western blots with a high affinity (half-maximal signal at 1:500 dilution, but a signal is detected at greater than 1:500,000 dilution). 4) The antiserum specifically immunoprecipitates the receptor pho-

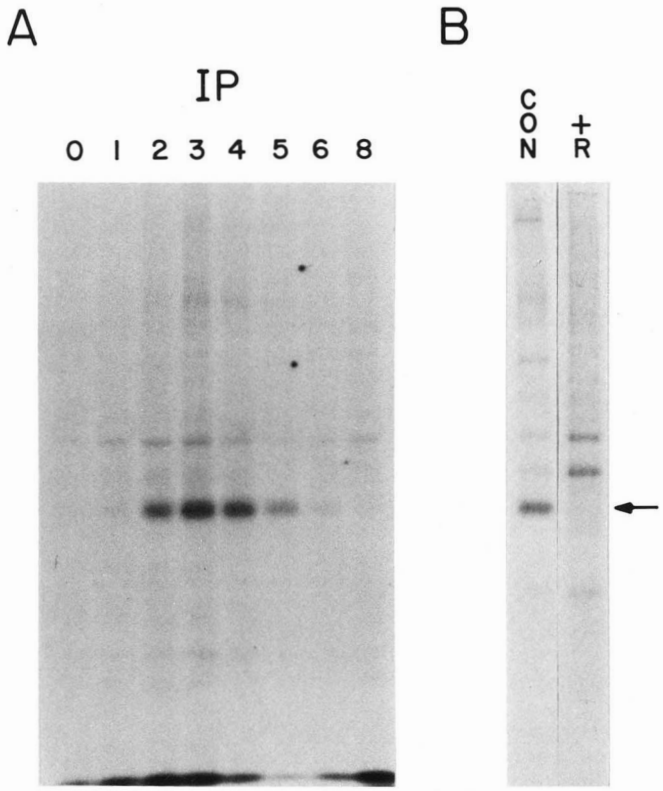


FIG. 5. ***In vitro* translation of stage-specific mRNA.** *A*, identification of *in vitro* translation product. Total cellular RNA was prepared from Ax-3 cells at 0, 1, 2, 3, 4, 5, 6, and 8 h of development, was translated *in vitro*, and immunoprecipitated. *B*, a sample was divided and immunoprecipitated with serum (1:2000 dilution) that had been preincubated with 0.03% SDS (CON) or 50 ng/ml purified receptor (+*R*). Experiments were repeated four times with similar results.

toaffinity labeled on intact cells with 8-$N_2$-[$^{32}$P]cAMP and *in vivo* with $^{32}P_i$ and [$^{35}$S]methionine. 5) The antibody recognizes (in Western blots and by immunoprecipitation) a protein that has the developmental regulation expected of the cAMP receptor.

It has been suggested previously that the disappearance of the R form and the appearance of the D form of the receptor following stimulation of intact cells with cAMP represents a reversible covalent modification of the R form of the receptor which causes a decrease in its electrophoretic mobility (3, 11). However, using 8-$N_3$-[$^{32}$P]cAMP to identify the receptor, it was not possible to rule out that the R and D forms are separate polypeptides and that the binding site on the R form becomes inaccessible to $N_3$-[$^{32}$P]cAMP while the binding site on the D form becomes accessible following stimulation with cAMP. The data from Fig. 1 (*lanes 1* and *2*) and Fig. 2 (*lanes 15* and *16*) strongly support that the R form is converted to the D form since they show that the R form *protein*, rather than a binding site, disappears while the D form *protein* appears following cAMP stimulation.

Although the receptor is a minor cellular protein, it is the major phosphoprotein in crude membrane fractions. It has been previously demonstrated that the majority of phosphorylation occurred on serine residues. Preliminary evidence indicated that the basal and cAMP-induced phosphorylation sites are located on distinct phosphopeptides. The immunoprecipitation studies reported here demonstrate that the receptor contains at least 0.2 mol of phosphate/mol of receptor prior to cAMP stimulation and that the level increases to about 4 mol/mol of receptor following cAMP stimulation. This indicates that there are multiple sites of phosphorylation on the previously identified phosphopeptides. An extensive ligand-induced phosphorylation of serine and threonine residues also occurs on the $\beta$-adrenergic receptor in mammalian and avian cells and rhodopsin in the rod outer segment of the retina (16, 17). The analogy of these systems with the cAMP receptor in *Dictyostelium* may indicate that this is a general property of receptors that interact with G proteins. In the rod outer segment, phosphorylation of rhodopsin leads to termination of the capacity to activate transducin (17). In *Dictyostelium* a similar mechanism may bring about adaptation of an array of physiological responses. As previously reported, the pattern of modulation of the cAMP-induced mobility shift of the receptor closely correlates with the adaptation process (11). It may turn out that the cAMP receptor shares structural homology with rhodopsin and the $\beta$-adrenergic receptors, which are predicted to span the bilayer as many as seven times (24).

The polypeptide we have identified as the surface cAMP receptor contains both the cAMP binding site and sites of internal phosphorylation, suggesting that this polypeptide is a transmembrane protein which could presumably transmit signals across the membrane. The receptor solubilized in nonionic detergents sediments on a sucrose gradient as 5–7 S. This large an S value may reflect association of the receptor with itself, with an additional receptor subunit, or with an interacting component of the transmembrane signaling system which is lost during purification of receptors solubilized in SDS, although the large S value could also be due to nonionic detergent binding to the receptor.

The level of [$^3$H]cAMP binding rises during development to a maximum of 10–20-fold over vegetative cells during the aggregation stage and then falls to less than 20% of the maximum binding. However, it was not known whether the rise in binding sites reflected *de novo* synthesis of cAMP receptors, mobilization of internal receptors present throughout development but not at the surface, or exposure of cryptic binding sites present at the surface throughout development but inaccessible until the appropriate time in development. The Western blot of total cell membranes during development, which shows that the intensity of antibody staining closely parallels the rise in cAMP binding, demonstrates that the rise in the number of binding sites is due to a rise in the amount of receptor protein. Furthermore, the rate of receptor biosynthesis and the level of translatable mRNA from the receptor rise, in parallel, prior to the peak in receptor accumulation and maximum binding, demonstrating that the aggregation stage expression of cAMP binding sites and receptor protein involves a rise in *de novo* synthesis of the cAMP receptor.

Our characterization of this signal-transducing receptor in *Dictyostelium* opens the possibility for numerous interesting studies. The system is accessible to genetic analysis by conventional mutagenesis and DNA-mediated transformation (25). Furthermore, the expression of the receptor is strongly developmentally regulated and apparently controls the expression of a number of other developmentally regulated proteins. Thus, not only does this system provide a model for investigation of chemotaxis and activation of adenylate cyclase, it offers a unique opportunity to define the role of transmembrane signaling events in the control of development.

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