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Purification of the Surface cAMP Receptor in *Dictyostelium**

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We have previously identified and demonstrated reversible ligand-induced modification of the major cell surface cAMP receptor in *Dictyostelium discoideum*. The receptor, or a subunit of it, has been purified to homogeneity by hydroxylapatite chromatography followed by two-dimensional preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purification was monitored by following $^{32}P_i$ incorporated by photoaffinity labeling with 8-azido-[^{32}P]cAMP or by *in vivo* labeling with $^{32}P_i$. Two interconvertible forms of the receptor, designated R (M_r 40,000) and D (M_r 43,000), co-purified. Two-dimensional peptide maps of independently purified and ^{125}I-iodinated R and D forms of the receptor were nearly identical but did have several distinct peptides. The estimated 6000-fold purification required is consistent with the number of cell surface binding sites assuming there are not multiple binding sites/polypeptide. In the accompanying article we report the generation of a monospecific polyclonal antiserum which has helped to further elucidate the physical properties and developmental regulation of the cAMP receptor.

The cAMP receptor serves several roles in the development of *Dictyostelium discoideum* (1-3). It binds extracellular cAMP, stimulating chemotaxis toward aggregation centers (4) and the cAMP signaling response (5) which relays the chemotactic signal (for review, see Ref. 6). The cAMP receptor also appears to regulate its own expression and that of several other aggregation stage genes as well as multiple prespore and prestalk genes later in development (reviewed in Ref. 7).

The aggregation of several million amoebae is coordinated by spiral and concentric waves of cAMP which emanate from aggregation centers and sweep through a cell monolayer at 6-min intervals (8). The leading edge of each wave provides a gradient that orients chemotactically sensitive cells toward the center. Each cell moves up the gradient for several minutes and then, due to adaptation, becomes transiently unresponsive to cAMP. As the wave passes, the local cAMP concentration falls; the cells become resensitized in time to respond to the subsequent cAMP wave (6).

Using the photoaffinity label 8-N_3-[^{32}P]cAMP,[1] we have recently identified a surface cAMP binding protein (1) that has the pharmacological specificity and developmental regulation of the receptor that mediates chemotaxis and cAMP signaling. The cAMP receptor was shown to undergo a reversible cAMP-dependent modification (2, 3) reflected in a change from higher electrophoretic mobility (M_r 40,000) before stimulation (R form) to lower electrophoretic mobility (M_r 43,000) following cAMP stimulation (D form). This modification is associated with an 8-fold increase in the level of phosphorylation (3, 10). The kinetics and cAMP dose dependence of this modification are closely correlated with the kinetics and cAMP dose dependence of adaptation (9). In fact, in cells undergoing spontaneous oscillations in cAMP synthesis, the receptor oscillates between the R and D forms with the same period (6 min) as the cAMP oscillations (2).

In this paper we report the purification of both forms of the cAMP receptor using hydroxylapatite chromatography and preparative SDS-PAGE. In the accompanying paper, a specific polyclonal antiserum directed against the purified cAMP receptor is employed to study the biochemical properties and developmental regulation of expression of the receptor.

MATERIALS AND METHODS

Cell Growth and Development—AX-3 cells were grown and developed as described previously (11, 12). For large-scale preparations, 30 liters of vegetative cells at approximately 5×10^6/ml were harvested using the Sharples T-1 continuous flow centrifuge at 16,000 rpm. Cells were allowed to flow in by gravity at a flow rate of 2.2 liters/min. The cell pellet was resuspended in 30 liters of development buffer (DB): 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, pH 6.8), recentrifuged, and resuspended at 4×10^7/ml in DB. Cells were then shaken (400 ml/4-liter flask) at 150 rpm and the medium adjusted to 50 nM cAMP every 6 min from 1 to 5 h of development. At 5.5 h of development, cells were centrifuged at 2500 rpm for 5 min in a Sorvall GS-3 rotor, washed once in DB, and resuspended in DB at 10^8/ml. [^{3}H]cAMP binding was determined at this point as described (13). To induce the R form of the receptor, cells were treated with 5 mM caffeine for 30 min. To induce a shift to the D form of the receptor, cells were then treated with 10 mM DTT and 10 μM cAMP for 12 min. In either case, concanavalin A was added at 100 μg/ml for 2 min prior to harvesting the cells.

Preparation of SDS-solubilized Membranes—The subsequent steps will be described for a typical purification from 1 liter of cells at 10^8/ml, although this purification has been carried out over 60 times at volumes from 1 to 2500 ml of cells with similar results. After concanavalin A treatment, 1 liter of cells was centrifuged at 2500 rpm for 5 min in a Sorvall GS-3 rotor at 4 °C. The pellets were resuspended in 1 liter of 98% saturated ammonium sulfate at 4 °C and centrifuged at 7000 rpm for 10 min in the same rotor at 4 °C. The pellets were then resuspended in 80 ml of ammonium sulfate and added to 4 liters of stirring receptor buffer (RB): 20 mM Tris, pH 7.5, 5 mM EDTA, 5 mM dithiothreitol (DTT), and a protease inhibitor mixture as described previously (1)) at 4 °C, which causes cell lysis. The cell lysate was centrifuged at 10,000 rpm for 30 min at 4 °C in GS-3 rotors. The pellets were dissolved in 1 liter of SDT (1.5% SDS, 5 mM DTT, 25 mM Tris, pH 6.8) and frozen. Once solubilized in 1.5% SDS, the receptor was stable at room temperature for at least 3 days and at −20 °C for up to 6 weeks.

Labeling the cAMP Receptor—For phosphorylation of the cAMP receptor, aggregation stage cells were washed twice in 10 mM MES-NaOH, pH 6.2, and resuspended at 10^8/ml (10). Five ml of cells were shaken with 5 mCi of $^{32}P_i$. To produce the R form, 5 mM caffeine was

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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; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid.

added at the same time as the $^{32}P_i$. To produce the D form, 10 mM DTT and 10 μM cAMP were added after 15 min. After 28 min in $^{32}P_i$, ConA was added at 100 μg/ml for 2 min to both sets of cells. Cells were then added to 20 ml of 98% saturated ammonium sulfate at 4 °C, centrifuged at 7000 rpm for 10 min in a Sorvall SS-34 rotor, washed once more in ammonium sulfate, and lysed by resuspension in 20 ml of RB. This lysate was centrifuged at 10,000 rpm for 10 min (Sorvall SS-34) at 4 °C. The pellets were resuspended in 20 ml of RB and centrifuged again. These pellets were then solubilized in 5 ml of sample buffer (25 mM Tris, pH 6.8, 1.5% SDS, 2.5% DTT, 5% glycerol, and bromphenol blue) and frozen. Photoaffinity labeling of the R and D forms with 8-N_3-[^{32}P]cAMP has been described (1, 2, 9).

Hydroxylapatite Columns—Hydroxylapatite columns were run in SDS using a modification of a previously described method (14). The columns were run in 2.5 × 20-cm Bio-Rad econo-columns which were connected to a peristaltic pump. A layer of sand (10 g) was added to cover the bed support, and 100 ml of a 50% slurry of hydroxylapatite, equilibrated and defined in 25 mM Tris, pH 6.8, 0.1% SDS, 5 mM DTT, was added to each column. For 1 liter of SDS-solubilized membranes, two 50-ml columns were run in parallel since a maximum of 10 column volumes of membranes can be loaded per column.

The SDS-solubilized membranes were thawed and filtered through Whatman No. 3MM paper in a Buchner funnel. ^{32}P-Labeled membranes were added at 1–2 ml/liter of unlabeled membranes. The sample was applied to the column at room temperature at a rate of 5–8 min/column volume (*i.e.* 12–20 ml/min for two 50-ml columns). The column was washed in 5 column volumes of 10 mM sodium phosphate, pH 6.4, 0.1% SDS, 5 mM DTT followed by 5 column volumes of 0.2 M sodium phosphate, pH 6.4, 0.1% SDS, 5 mM DTT. The column was then washed with 10 column volumes of 0.2 M sodium phosphate, pH 6.4, 5 mM DTT. A linear gradient of sodium phosphate, pH 6.4 (6 column volumes of 0.2 M and 6 column volumes of 0.6 M), 0.05% SDS, 5 mM DTT was applied at a flow rate of 1 column volume/15 min. Once the gradient was completed, another 6 column volumes of 0.6 M sodium phosphate, pH 6.4, 0.05% SDS, 5 mM DTT was applied.

The eluate was collected in fractions of 1–2 column volumes which were kept at 0 °C during collection. After 1 h, greater than 90% of the receptor co-precipitated with the SDS. (The sodium phosphate did not precipitate.) The precipitated fractions were centrifuged at 7500 × *g* for 20 min in a Sorvall HS-4 rotor, and the supernatant was discarded. The precipitated fractions were stored at −20 °C. Small aliquots of each fraction (2%) were run on SDS-PAGE/autoradiography to localize the receptor. The fractions containing receptor (the initial one or two fractions containing receptor were excluded due to significant contamination) were warmed to room temperature, which causes the precipitates to redissolve, yielding a volume of 5% the original volume. The fractions were pooled and immediately reprecipitated at 0 °C for 30 min in 50-ml centrifuge tubes, then centrifuged at 18,000 × *g* in a Sorvall SS-34 rotor, which further concentrated the sample to 0.5% of the original volume. These pellets were diluted 1:1 with 25 mM Tris, pH 6.8, 5% glycerol, 2.5% DTT, and bromphenol blue.

Preparative SDS-PAGE—SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (15) except that the concentration of bisacrylamide was varied. Gels with 10% total acrylamide, in which the concentration of bisacrylamide was 0.01, 0.25, or 0.6–1.0%, are designated L gels, N gels, or H gels, respectively. The pooled concentrated fractions were loaded onto a 3-mm thick L gels (no more than 5 ml/gel). Gels were run at 50 V (approximately 16 h), dried without fixation at 60 °C on Whatman No. 3MM paper, and autoradiographed overnight. The autoradiograph was aligned with the gel and the receptor band, which was the most intensely phosphorylated band at this step, was excised with a razor blade. The dry gel slice was cut into 2–3-mm pieces which were shaken overnight at room temperature in a 20-ml vial containing 10 ml of 0.05% SDS and 0.05% ammonium bicarbonate. The next day, the buffer was removed and the elution repeated. The eluates were pooled and filtered through glass wool. Typically 70–80% of the ^{32}P-labeled protein elutes from the gel. The eluate, which was stable at room temperature for up to 4 days, was lyophilized and resuspended in 0.5 ml of water or 25 mM Tris, pH 6.8, 5% glycerol, 2.5% DTT. An aliquot (25 μl) of the eluted sample was analyzed on a 1.5-mm H gel and silver stained (16) to evaluate purity (as in Fig. 3).

The sample was further purified by SDS-PAGE on a preparative H gel. A maximum of 2.5 ml of eluate from the preparative L gel was loaded onto a 1.5-mm thick H gel and electrophoresed at 50 V. The gel was then either silver stained as above and the receptor band excised with a razor blade or the protein was transferred to nitrocellulose as described in the accompanying article (3).

Recovery of Receptor and Total Protein Determination—The recovery of the receptor at each step of the purification was determined by Western blotting using the antibody described in the following article by scanning autoradiographs of the ^{32}P-labeled receptor, by inclusion of a tracer of previously ^{125}I-iodinated purified receptor, and, at later steps in the purification, by scanning the receptor band in silver-stained gels. The determination of total protein was complicated by the presence of numerous substances (such as amino acid analog protease inhibitors) at each step of the purification which interfere with standard protein assays. The most reliable determination of the relative amount of total protein was by SDS-PAGE of each fraction followed by Coomassie Brilliant Blue or silver staining and optical scanning of the entire gel lane. Total protein in whole cells (in phosphate buffer) was assayed by the method of Lowry *et al.* (17), and a standard curve was generated from the optical densities of a silver-stained gel of various concentrations of total cellular protein. Aliquots from each step of the purification were electrophoresed on the same gel, and the total protein was approximated from the respective optical densities of each lane.

Electroelution—The eluate from the preparative L gel was ^{125}I-iodinated using chloramine T as described (18) to a specific activity of approximately 18 Ci/μg and was applied to a Bethesda Research Laboratories 1100PG preparative electrophoresis system which allows collection of fractions from the bottom of the gel. As proteins electrophoresed out the bottom of the gel, they are carried by a stream of buffer which is pumped to a fraction collector as described in the Bethesda Research Laboratories 1100PG instruction manual. A glass cylinder was substituted for the plastic gel tube supplied, and a 2-cm high × 1.3-cm diameter H gel was cast. The stacking gel was 4% acrylamide as described by Laemmli (15). The ^{125}I-iodinated protein mixture (0.5 ml containing approximately 20 μg of protein) in sample buffer was loaded and electrophoresed at 150 V (6 mA) using electrophoresis buffer as described (15). The flow rate was 0.22 ml/min. Fractions (2.2 ml) were collected until three peaks in radioactivity eluted; fractions from each peak were pooled, lyophilized, and redissolved in water at 10% the original volume. Aliquots (2.5 μl) from each peak and the starting material (50 μl) were electrophoresed on a 1.5-mm H gel, silver stained, and autoradiographed. Recovery was estimated by scanning an autoradiograph from a 30-min exposure. ^{125}I-Iodinated R form was purified similarly.

Two-dimensional Peptide Maps—^{125}I-iodinated R and D forms, purified and eluted as described above, were subjected to further SDS-PAGE (H gel). The gel was fixed without staining in 25% isopropyl alcohol/10% acetic acid for 2 days, dried, and autoradiographed for 30 min. The receptor band was cut out from each lane, and the gel piece was digested in 0.5 ml of trypsin or chymotrypsin at 100 μg/ml in 50 mM ammonium bicarbonate, pH 7.8, at 37 °C overnight (19). The next day, another 0.5 ml of protease solution was added, and the digestion was continued for 6 h. The supernatants were taken through three cycles of lyophilization and resuspension in H_2O in siliconized glass tubes, resuspended in acetic acid:formic acid:water, 150:15:80, and subjected to electrophoresis on 10 × 130-mm thin-layer cellulose sheets followed by chromatography (19). The TLC sheets were dried and autoradiographed.

RESULTS

Labeling the cAMP Receptor—The cAMP receptor was labeled both by photoaffinity labeling with 8-N_3-[^{32}P]cAMP (Fig. 1, *lanes 1* and *2*) and by *in vivo* phosphorylation with $^{32}P_i$ (Fig. 1, *lanes 3* and *4*). Receptors were labeled in the R form (*lanes 1* and *3*) or in the D form (*lanes 2* and *4*). To label the R form, one set of cells was treated with caffeine, which blocks spontaneous oscillations in cAMP synthesis and ensures that a basal state is attained with a maximum of receptors in the R form. A second set of cells was stimulated with 10 μM cAMP, which maximizes the fraction of receptors in the D form (9). Although the cAMP receptor is a minor membrane protein, the photolabeled and phosphorylated R and D forms of the receptor are readily apparent. The phosphorylated R and D forms represent the same polypeptide as the respective photoaffinity labeled forms of the receptor since all co-purify to homogeneity and co-immunoprecipitate (3).

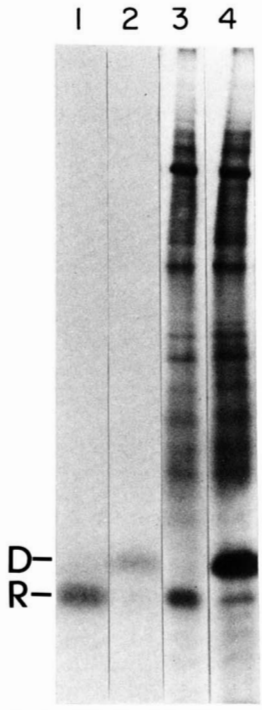

FIG. 1. **Specific photoaffinity labeling and *in vivo* phosphorylation of the cAMP receptor.** Sets of cells were prepared in which the receptor was in the R or D form. Intact cells were specifically photoaffinity labeled with 8-N_3-[^{32}P]cAMP or labeled *in vivo* with $^{32}P_i$. Membranes were prepared and proteins separated by SDS/PAGE (L gels). *Lanes 1* and *2* are membranes from 8-N_3-[^{32}P]cAMP photoaffinity-labeled cells; *lanes 3* and *4* are membranes from $^{32}P_i$-labeled cells. *Lanes 1* and *3* are from unstimulated cells (R form); *lanes 2* and *4* are from cells stimulated for 15 min with 10 μM cAMP (D form).

Although the receptor is less than 0.02% of the total protein, it is one of the most intensely phosphorylated membrane proteins; several moles of phosphate/mol of receptor are found after cAMP stimulation (3). While recovery of the D form from cells to membranes is 50–60% that of the R form, the higher level of phosphorylation of the D form makes it a useful tracer for the subsequent purification steps.

Hydroxylapatite Column—Cells (approximately 10^{11}) at the aggregation stage of development (5–6 h) were stimulated with cAMP to maximize the fraction of receptors in the D form. Membranes were isolated and solubilized in SDS (approximately 1 mg/ml total protein) and applied to two hydroxylapatite columns with a tracer of ^{32}P-labeled membranes. Aliquots from each fraction were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 2*A*). The relative optical density of total protein in each lane was plotted in Fig. 2*C* (*closed circles*). Fig. 2, *A* and *C*, shows that greater than 90% of the membrane proteins flow through the column and most of the retained protein elutes in 0.2 M sodium phosphate (fractions 15–20). The remaining protein bound to the hydroxylapatite elutes mostly in the 0.2–0.4 M range of the gradient (fractions 30–36), and less than 1% of the total protein elutes in the latter half of the gradient.

In order to visualize the proteins in the latter region of the gradient, fractions were electrophoresed on parallel gels, silver stained, and autoradiographed. The cAMP receptor was the major phosphoprotein in the autoradiograph (Fig. 2*B*, *right*) and represented approximately 10% of the total protein in these fractions (Table I) as determined by scanning the silver-stained gel (Fig. 2*B*, *left*).

To confirm the position of the receptor peak, fractions from the entire column profile were Western blotted using anti-receptor antiserum as described in the accompanying paper. Staining of the receptor was found only in the latter half of the phosphate gradient (Fig. 2*C*, *inset*). The autoradiographs were scanned and the relative optical density was plotted (Fig. 2*C*, *open circles*). Most of the receptor eluted (fractions 38–49) after greater than 99% of the total protein had eluted from the column.

Preparative SDS-PAGE—Fractions 40–49 were pooled and electrophoresed on a preparative L gel. The major phosphorylated protein was eluted from the dried gel. When an aliquot was analyzed by SDS-PAGE on an H gel, three major bands were revealed by silver staining (Fig. 3, *lane 1*). The predominant band was identified as the receptor since it was the only phosphorylated band in autoradiographs. Scanning of the silver-stained gel indicated that the receptor represented 59% of the total protein, while in other preparations, the purity varied from 50 to 90%. The yield of the receptor was approximately 50 μg. In addition to the contaminants (M_r 25,000 and 45,000), two minor bands are seen with apparent molecular mass 2-fold and 3-fold greater than the receptor. These bands are aggregates of the receptor which can be produced by heating pure receptor and by prolonged storage of pure receptor at −20 °C. These aggregates are also phosphorylated and specifically immunoprecipitated and stained in Western blots by anti-receptor antibody. Fig. 3, *lane 2*, shows the R form of the receptor, purified from cells treated with caffeine, at this stage of the purification. The major silver-stained band is of lower apparent molecular weight although the spectrum of contaminants is similar.

The H gels were used preparatively for the final purification step. The sample eluted from the preparative L gel was electrophoresed on a 1.5-mm preparative H gel. This gel was either silver stained or transferred to nitrocellulose; in either case, the cAMP receptor band was excised and used directly as an immunogen (3). The receptor could also be transferred to derivatized glass fiber filters recently described for use in protein sequencing (20).

Overall Purification—The overall purification of the cAMP receptor is summarized in Table I. By these data, the cAMP receptor represents approximately 0.02% of the total cellular protein, which corresponds to approximately 2×10^5 molecules/cell. Cells at 5–6 h of development typically have 1–1.5 $\times 10^5$ surface cAMP binding sites/cell, which is consistent with the data in Table I assuming that the receptor does not have multiple binding sites/polypeptide.

The most significant step in the purification is the hydroxylapatite column, which achieved a 300-fold purification (Table I, Fig. 2) but also incurred a considerable loss. The recovery from the column chromatography step, based on Western blots of loaded and eluted samples, was 6%. In 33 independent purifications, the recovery ranged from 6 to 35%, as estimated from recovery of ^{125}I-labeled receptor, recovery of ^{32}P-labeled receptor, and by Western blotting. The receptor recovered from the hydroxylapatite column does not represent a subpopulation of receptors since when purified ^{125}I-labeled receptor (also prepared by hydroxylapatite chromatography) was added back to membranes as a tracer and rechromatographed on hydroxylapatite, the same recovery was found. By adding purified ^{125}I-labeled receptor to membranes as a tracer, the points at which receptors were lost were localized. It was determined that less than 10% of the ^{125}I-labeled material initially applied to the column remained bound after the gradient. The majority of the losses appear to be due to degradation. Up to 50% of the ^{125}I counts flowed through the column during application of the sample, although no receptor

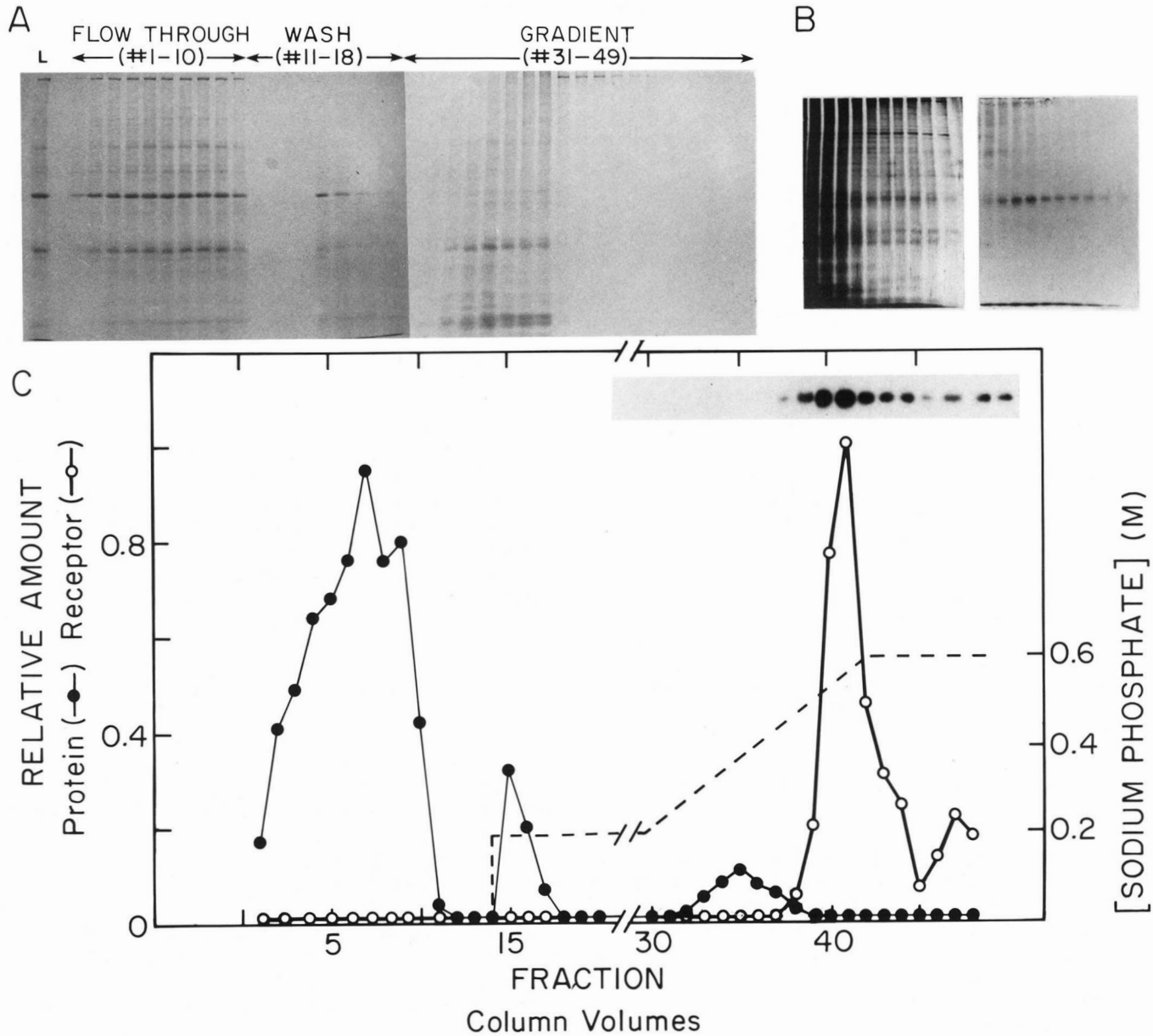

FIG. 2. **Hydroxylapatite column chromatography purification step.** *A*, Coomassie stain of SDS/PAGE (N gel) analysis of proteins loaded and eluted from hydoxylapatite column (fractions 30–49 were concentrated 10-fold). *B, left* is silver stain of SDS/PAGE of fractions 38–47; *right* is autoradiograph of silver-stained gel. *C*, plot of optical density scan of Coomassie-stained proteins (●); optical density scan of specific antiserum staining of receptor in Western blot of fractions 1–20 and 30–49 (○); *inset* shows Western blot of fractions 30–49. Each fraction represents 1 column volume.

TABLE I
Overall purification

Purification step	Recovery	Total protein	Recovery/ total protein	Purification
	%	μg	%/μg	*-fold*
1. Cells	100	8.4×10^6	11.9	1.0
2. Membranes	35	1.4×10^6	25.0	2.1
3. Hydroxylapatite column	2.2	294	7,480	629
4. L gel	3.7	102	36,300	3,050
5. H gel	3.7	54	68,500	5,756

or receptor fragments were detected in these fractions by SDS-PAGE autoradiography or by Western blotting, suggesting that the receptor that did not adsorb to the column was completely degraded. Furthermore, after elution from the column, the receptor is highly unstable, and the precipitation and preparation for electrophoresis must be performed rapidly. This may explain why the apparent recovery from the preparative L gel (3.7%) is slightly higher than was observed on the analytical gels used to assess recovery from the column (2.2%). Since the determinations for Table I required further manipulations of the samples prior to electrophoresis, the receptor in the column eluate was likely further degraded relative to the other samples.

Electroelution from Preparative SDS-PAGE—The H gels were also run as preparative tube gels to obtain soluble purified receptor. The eluates of the preparative L gels were lyophilized, radioiodinated with ^{125}I, and electrophoresed on preparative tube H gels (1.0% bisacrylamide). Fractions were collected as they eluted from the bottom of the gel (as described under "Materials and Methods"). Fig. 4 shows the

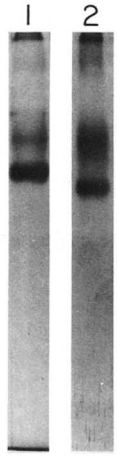

FIG. 3. **Silver stain of purified receptor.** Eluate from initial preparative SDS/PAGE (L gel) was run on analytical H gel. *Lane 1* shows the D form of receptor purified from cells stimulated with cAMP. *Lane 2* shows the R form of receptor purified from cells treated with caffeine.

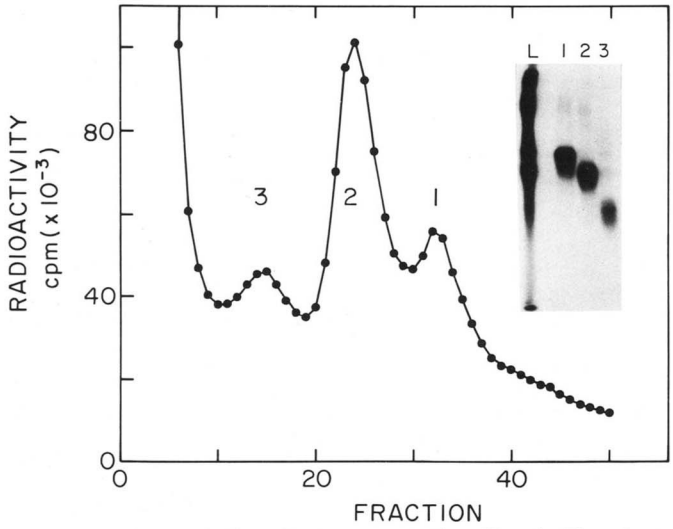

FIG. 4. **Electroelution from preparative H gel.** The eluate from a L gel was ^{125}I-iodinated and applied to a preparative tube H gel (1.0% bisacrylamide). Radioactive peaks were pooled, lyophilized, resuspended at 10% volume, again subjected to SDS/PAGE (H gel), and autoradiographed. *Lane L* shows the iodinated sample prior to purification. *Lanes 1* and *3* show separated contaminants. *Lane 2* shows the purified cAMP receptor.

profile of eluted radioactivity for the purification of ^{125}I-labeled D form, beginning with the dye front and continuing until the receptor (*peak 2*) and the slower mobility contaminant (*peak 1*) had eluted. The *inset* to Fig. 4 shows an autoradiograph of samples pooled from each peak. *Lane L* shows the ^{125}I-iodinated starting material in which several iodinated bands are evident (in shorter exposures). *Lane 2* shows the D form of the receptor, which represents greater than 99% of the iodinated protein. On shorter exposures, the receptor appeared as a sharp band with no other bands evident. The faint band (less than 1%) in *lane 2* is an aggregate of the receptor as described above. *Lanes 1* and *3* show the principal contaminants which are the same as those revealed by silver staining (Fig. 3, *lane 1*). The recovery of highly purified receptor was 60–70% as determined by scanning the autoradiograph from shorter exposures.

Two-dimensional Peptide Maps—Parallel preparations of the ^{125}I-labeled R and D forms of the receptor were purified

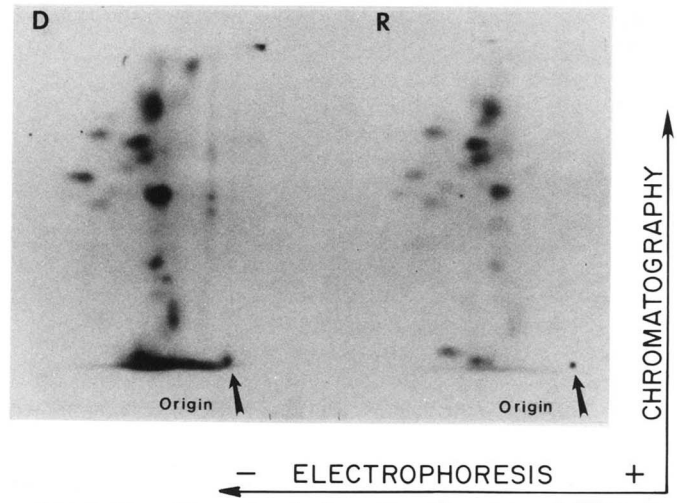

FIG. 5. **Two-dimensional chymotryptic maps of ^{125}I-iodinated R and D forms of purified receptor.** Purified ^{125}I-iodinated D (*left*) and R (*right*) forms of the receptor were digested with chymotrypsin, subjected to electrophoresis and thin-layer chromatography as indicated, and autoradiographed.

as described above. These highly purified proteins were again subjected to SDS-PAGE autoradiography. The bands were excised from the gel and digested with chymotrypsin. The released peptides were subjected to two-dimensional thin layer electrophoresis chromatography as described under "Materials and Methods." Fig. 5 shows the maps of the ^{125}I-labeled D form (*left*) and R form (*right*). The maps of the two forms of the receptor were nearly identical. Only two peptides appeared to differ between the two maps. Although maps of trypsin-released peptides differed from the chymotryptic maps, the R and D forms showed the same degree of similarity.

DISCUSSION

The cAMP receptor has been purified to homogeneity by hydroxylapatite chromatography in SDS (14) followed by sequential preparative SDS-PAGE. Hydroxylapatite, which separates the receptor from greater than 99% of the total protein, allows a separation of SDS-solubilized proteins based on characteristics other than molecular weight so that when combined with SDS-PAGE, a powerful purification is possible. The final steps of the purification are based on the fortuitous observation that the electrophoretic mobilities of both forms of the receptor are anomalously affected by changes in the concentration of bisacrylamide. This allows significant purification by sequential SDS-PAGE with gels of differing concentrations of bisacrylamide.

Several lines of evidence strongly support that the polypeptide purified is the cAMP receptor or a subunit of it. It has been previously shown that the cAMP receptor that mediates chemotaxis and cAMP signaling is specifically photoaffinity labeled by 8-N_3-[^{32}P]cAMP (1, 2, 21–23). Both the R and D forms of this photolabeled protein are purified to homogeneity by the protocol described here. The phosphorylated R and D forms are equivalent to the respective photolabeled proteins since they co-purify to homogeneity. After elution from the preparative L gels, no band co-migrating with the D form of the receptor is present if the starting cells are not stimulated with cAMP; and no band co-migrating with the R form of the receptor is present if the cells are stimulated with cAMP. The pattern of contaminants is the same regardless of cAMP stimulation. This observation suggests that only a protein that shifts its electrophoretic mobility in response to cAMP stimulation of intact cells is purified and strongly argues

against the artifactual purification of another protein that comigrates with the D form of the cAMP receptor. Finally, as reported in the accompanying paper, when the purified D form is used as an immunogen, antibodies are generated that specifically and quantitatively immunoprecipitate the R and D forms of the photoaffinity and *in vitro* $^{32}P_i$ and [^{35}S] methionine labeled cAMP receptor.

The purity of the receptor in the final step of purification, isolation from the H gel (either by electroelution or by excision), is indicated by several criteria. The iodinated protein resolves as a single band in the H gel even on prolonged exposure; the peptide maps of the two forms of the receptor are nearly identical even though they were isolated from different regions of the polyacrylamide gel; no other protein is seen migrating in the position of the D form of the receptor when the purification is carried out with cells that have not been stimulated with cAMP (as above); and when injected into rabbits, the antiserum generated is highly specific for the cAMP receptor (3).

Meyers-Hutchins and Frazier (24) have reported the identification and purification of a membrane-bound cAMP binding protein from *Dictyostelium* that has an M_r of 70,000. Its pharmacological specificity parallels that of the chemotaxis receptor. It is possible that the protein purified here is a fragment of the M_r 70,000 protein reported by those authors. However, the *in vitro* translated receptor recognized by the antiserum described in the following paper has an M_r of 37,000, which is close to the M_r of the R and D forms of the receptor reported here. Bands are frequently observed at M_r 85,000 (as well as at higher multiples of receptor M_r) at various stages of purification, but these bands represent aggregates of the receptor at M_r 43,000 since the intensity of the aggregate bands increases upon boiling the purified protein and also accumulates after prolonged storage of the completely purified protein at −20 °C.

Since the 8-N_3-[^{32}P]cAMP photoaffinity-labeled protein is purified following denaturation in SDS, it is likely to contain the cAMP binding site. The same purified polypeptide contains basal and cAMP-dependent phosphorylation sites. Since the 8-N_3-[^{32}P]cAMP attachment site is accessible to the extracellular milieu, while the phosphorylation sites must be exposed to the cytoplasmic side of the membrane, the polypeptide purified here must also contain a transmembrane domain. In the following paper, the receptor is shown to remain associated with membranes in aqueous buffer and is solubilized in Triton X-100, further supporting that the receptor is an integral membrane protein (3). Because membranes are initially solubilized in SDS, any noncovalently associated subunits are expected to be lost, and thus a subunit of the intact receptor may have been purified.

There is strong evidence that the R and D forms of receptor are interconvertible forms of the same protein rather than distinct proteins whose cAMP binding affinities and phosphorylation states are reciprocally altered by cAMP stimulation. Two-dimensional peptide maps of the ^{125}I-labeled R and D forms are nearly identical. The possibility that the few peptides that do differ are the sites of ligand-induced phosphorylation is currently being investigated.

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