

## Desensitization of G-protein-coupled Receptors

AGONIST-INDUCED PHOSPHORYLATION OF THE CHEMOATTRACTANT RECEPTOR *cAR1* LOWERS ITS INTRINSIC AFFINITY FOR cAMP\*

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**Agonist-induced phosphorylation of G-protein-coupled receptors has been shown to facilitate the desensitization processes, such as receptor internalization, decreased efficiency of coupling to G-proteins, or decreased ligand affinity. The lowered affinity may be an intrinsic property of the phosphorylated receptor or it may be the result of altered interactions between the modified receptor and downstream components such as G-proteins or arrestins. To address this issue, we purified *cAR1*, the major chemoattractant receptor of *Dictyostelium discoideum* by a strategy that is independent of the ligand binding capacity of the receptor. To our knowledge, this represents the first successful purification of a chemoattractant receptor. The hexyl-histidine-tagged receptor was solubilized from a highly enriched plasma membrane preparation and purified by Ni<sup>2+</sup>-chelating chromatography. The protocol offers a simple way to purify 100–500 µg of a G-protein coupled receptor that can be targeted to the plasma membrane of *D. discoideum*. The *K<sub>d</sub>* value for the purified *cAR1* was about 200 nM, consistent with that of receptors that are not coupled to G-proteins in intact cells. In contrast, the affinity of phosphorylated *cAR1*, purified from desensitized cells, was about three times lower. Treatment of the phosphorylated receptor with protein phosphatases caused dephosphorylation and parallel restoration of higher affinity. We propose that ligand-induced phosphorylation of G-protein-coupled receptors causes a decrease in intrinsic affinity and may be useful in maintaining the receptor's sensitivity at high agonist levels. This affinity decrease may precede other processes such as receptor internalization or uncoupling from G-proteins.**

G-protein-coupled receptors (GPCRs)<sup>1</sup> are involved in a wide variety of important biological processes including vision, olfaction, chemotaxis, and immune response. It is remarkable that the receptors for such diverse stimuli all share the same topological feature of seven-membrane spanning segments. It is believed that these segments cluster to form the binding pocket. Upon agonist binding, the receptors undergo conforma-

tional changes, activating intracellularly coupled G-proteins, which proceed to interact with the downstream effectors (1). Despite extensive pharmacological studies on certain representative GPCRs, detailed biochemical and biophysical characterization of most of these receptors is still lacking. With the exception of rhodopsin, the extreme difficulties in purifying most GPCRs have hindered studies of the structures and functions of these proteins.

A physiologically important property of GPCRs is their tendency to desensitize during exposure to agonist. Desensitization mechanisms include “down-regulation” or reduction of receptor number, “sequestration” or apparent shielding of the receptors from interacting ligands, and “uncoupling” from G-proteins. Agonist-induced receptor phosphorylation, usually carried out by G-protein-coupled receptor kinases, may contribute to each of these processes. In the case of the β-adrenergic receptor and rhodopsin, the two most extensively characterized GPCRs, phosphorylation is proposed to promote the association of arrestins and subsequent uncoupling from G-proteins and internalization (2–4). Desensitization may also be accompanied by a lowered affinity of the phosphorylated receptor as in the cases of *cAR1*, angiotensin II receptor, D2 dopamine receptor (5–7), and possibly the yeast pheromone receptor (21). It has been speculated that the lowered affinity is due to receptor uncoupling from G-proteins and consequent coupling to arrestins, but recent evidence suggests that receptor-arrestin complexes also display high affinity (8). It is possible that agonist-induced phosphorylation may lower the intrinsic affinity of the receptor.

To investigate the intrinsic properties of a phosphorylated GPCR, we purified *cAR1*, the major chemoattractant receptor of the social amoeba, *Dictyostelium discoideum*. *cAR1* is coupled to the heterotrimeric G-protein G2, which transmits the activation signal downstream to mediate actin polymerization, chemotaxis, calcium uptake, cell-cell signaling, and differentiation (9). We developed a purification protocol which, unlike previous GPCR purification schemes, does not rely on the ligand binding capacity of the receptor. In this procedure, a specialized plasma membrane subdomain highly enriched in receptor was isolated (10). After detergent extraction, the solubilized receptor was applied to a Ni<sup>2+</sup> column and purified in a single step. Tens of micrograms of purified, active *cAR1* are obtained from a liter of cell culture. The agonist affinity of the final purified receptor is similar to that of the binding sites on cells lacking G-proteins. To our knowledge, this represents the first successful attempt to purify a chemoattractant receptor to near homogeneity. In principle, this protocol can be extended to the purification of any GPCR that can be targeted to the plasma membrane of *D. discoideum* cells.

Using this purification procedure, we have found that the lower affinity displayed by phosphorylated receptors after ag-

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<sup>1</sup> The abbreviations used are: GPCR, G-protein-coupled receptor; *cAR1*-H6, hexyl-histidine-tagged wild-type *cAR1*; cm1234-H6, hexyl-histidine-tagged non-phosphorylatable *cAR1*; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

onist pretreatment *in vivo* is an intrinsic property of the modified proteins. When this phosphorylation was blocked by substituting serines 303 and 304, the major phosphorylation sites of cAR1, with alanine and glycine, the mutant receptor failed to display lowered affinity after similar agonist pretreatment (5). Additionally, protein phosphatase treatment of the phosphorylated receptor led to its dephosphorylation and a corresponding enhanced ligand affinity. This suggests that receptor phosphorylation itself, independent of other interacting components or downstream processes, may directly contribute to the desensitization.

#### EXPERIMENTAL PROCEDURES

**Vectors and Constructs**—C-terminal hexyl-histidine-tagged cAR1 constructs were created by polymerase chain reaction. The N-terminal primer, GCCGGAAGATCTTATTAAAAAATGGGTCCTTTAGATGGAAATC, contains a *Bgl*II site (first underline) at the 5' end, followed by the *Dictyostelium* consensus ribosomal binding site (italicized) and the N-terminal residues of cAR1 (second underline). The C-terminal primer, CGAGGCGTAGCTAGCTGGTGGATTATTTCCCTTGACCATTGTGTC, contains the last six residues of cAR1 sequence (italicized), followed by two prolines (underlined) and a *Nhe*I site. Two constructs were made to create the hexyl-histidine-tagged wild-type cAR1 (abbreviated as cAR1-H6), the wild-type cDNA sequence of cAR1 was used as template. To create hexyl-histidine-tagged non-phosphorylatable form of cAR1 (abbreviated as cm1234-H6), a mutant cAR1 sequence in which all C-terminal serine and threonine residues were substituted (cm1234, Ref. 12) was used. A modified pBluescript (Stratagene) containing a hexyl-histidine tag was created as follows: the *Eco*RV site was replaced with a *Bgl*II site. After digestion with *Bgl*II and *Bam*HI, a double-stranded filler fragment containing sequentially *Bgl*II and *Nhe*I sites followed by a six-histidine sequence and an in-frame stop codon and a *Bam*HI site was digested with *Bgl*II and *Bam*HI and then cloned into the modified vector through *Bam*HI and *Bgl*II sites. The sequence for the top strand of the filler (from 5' to 3') is as follows: GATCTCGCTCTGCTAGCCACCATCACCATCACCAATAAAG. The polymerase chain reaction products were gel-purified, digested with *Bgl*II and *Nhe*I, and then cloned into compatible sites of the above mentioned pBluescript. The final fragments containing the assembled cAR1 followed by a six-histidine tag and stop codon was released by *Bgl*II/*Bam*HI digestion and cloned into pB18, a *D. discoideum* integration expression vector; or released by *Bgl*II/*Not*I digestion and cloned into pMC34, an extrachromosomal expression vector. Under both conditions the tagged sequence is downstream of the *D. discoideum* actin 15 promoter and expressed throughout the growth and developmental stages.

**Cell Lines and Growth Conditions**—The expression vectors encoding His-tagged cAR1 were transformed into *car*I<sup>-</sup>/*car*3<sup>-</sup> cells (12). Clones were grown up and further characterized by cAMP binding assay. The clone giving the highest binding was selected. Cells are maintained in a Petri dish in HL-5 medium (12) plus 20 µg/ml G418. Cells are washed off the plate into large axenic cultures and grown up by shaking at 200 rpm at 22 °C to a density of 5–8 × 10<sup>6</sup>/ml.

**Intact Cell Binding Assays**—cAMP binding of intact cells in either phosphate buffer or in saturated ammonium sulfate was carried out essentially as described (12). Briefly, for assay in phosphate buffer, 100 µl of cells at 10<sup>8</sup> cells/ml were incubated with various concentrations of [<sup>3</sup>H]cAMP (NEN Life Science Products Inc.) at 4 °C for 3 min and centrifuged at 12,000 × *g* for 2 min to remove unbound ligands. For ammonium sulfate assay, cells were suspended in saturated ammonium sulfate and [<sup>3</sup>H]cAMP was added. One wash with saturated ammonium sulfate was carried out. Background binding was obtained by adding unlabeled cAMP.

**CHIFF Preparation**—CHIFF was prepared as described previously (10). Briefly, cells were harvested and washed once in DB (10). The cell pellet was resuspended in DB to 5 × 10<sup>7</sup>/ml and shaken at 120 rpm at 22 °C for 4–6 h. Cells were harvested again and washed with TEB (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EDTA). They were finally resuspended in TEBP (TEB plus various protease inhibitor) to 2 × 10<sup>8</sup>/ml. CHAPS powder was added at 20 mg/ml. After gentle mixing, the lysed cells were kept on ice for 3–5 min and then sucrose crystals were added to 55% final concentration. They were loaded under a 20–45% step sucrose density gradient and centrifuged at 150,000 × *g* for 12 h. The CHIFF band was collected and washed once, then resuspended to 5 × 10<sup>8</sup> cell equivalent/ml in TBP (TEBP without the EDTA) with 30% sucrose and then stored frozen at -70°.

**Purification of Hexyl-histidine-tagged cAR1 Over Ni<sup>2+</sup>-chelating Sepharose Column**—CHIFF was thawed and solubilized in 1–2%

Lubrol PX for 2–4 h with mixing at 4 °C at a cell equivalent density of 3–4 × 10<sup>8</sup>/ml. After centrifugation at 100,000 × *g* for 30 min, the supernatant was recovered. NaCl and imidazole were added at final concentrations of 200 and 2 mM, respectively. About 40 ml of this supernatant was first batch incubated with 0.6 ml (sedimented volume) of metal-chelating Sepharose resin (Pharmacia) precharged with 50 mM Ni<sup>2+</sup> according to the manufacturer's suggestion. After 2–4 h incubation, the resin was spun down and loaded into a 5-ml Bio-Rad econocolumn. The supernatant was further absorbed over the column by gravity flow (for about 1 h). After washing the column with more than 20 ml of wash buffer (40 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM imidazole, and 0.1% Lubrol-PX), the column was further washed with buffer containing 15 mM nonylglucoside (40 mM Tris, 200 mM NaCl, 2 mM imidazole, and 15 mM nonylglucoside) to exchange the original Lubrol. This was found to be necessary since our result showed that nonylglucoside preserved receptor binding activity more consistently than Lubrol-PX. cAR1 was eluted in four steps of increasing concentrations of imidazole: 25, 50, 100, and 250 mM. Each step consisted of 4 column volumes of elution solution.

**Preparation of Basal and Maximally Phosphorylated Hexyl-histidine-tagged cAR1**—The procedure was essentially as described (5). cAR1-H6 cells suspended in DB were shaken at 200 rpm for 20 min with either 4 mM caffeine or 10 mM dithiothreitol plus 10<sup>-5</sup> M cAMP to convert cAR1 into either the basal (unphosphorylated) or desensitized (phosphorylated) forms, respectively. The efficiency of this treatment was monitored by analyzing the treated sample on SDS-PAGE and visualizing positions of the cAR1 band on the gel by immunoblotting. Basal state corresponded to a faster migrating form; the phosphorylated state corresponded to a slower migrating form. The cells were then lysed and processed for cAR1 purification through CHIFF as detailed above.

**cAMP-binding Analysis of CHIFF and Purified cAR1**—The major cAR1 fractions of Ni<sup>2+</sup> column elutions were pooled and further characterized for cAMP binding through equilibrium dialysis (13). The binding reaction typically contained 200 ng of pure protein, 15 mM nonylglucoside, 40 mM Tris-HCl, 2 mg/ml bovine serum albumin, and 10% sucrose. For background controls, 1,000-fold excess unlabeled cAMP was added to both sides of the chamber, the differential between the two sides was taken as the background. For CHIFF binding, no detergent was present and 5–10 mM dithiothreitol was added to inhibit phosphodiesterase activity. Usually the reaction was stopped after 6–8 h of mild rocking as this time was tested to be sufficient for equilibrium establishment. Ten different [<sup>3</sup>H]cAMP concentrations in the range of 1–5,000 nM were used to generate the binding curve. Computer modeling program ORIGIN (Microcal Software, Inc., Northampton, MA) was employed to fit the binding data and obtain the number of affinity states and value of dissociation constant (*K<sub>d</sub>*). Other binding assays, such as spin column assay and a polyethylene glycol precipitation assay, also confirmed the activity of the receptor preparations. But these methods were not satisfactory for quantitative analysis. The equilibrium dialysis method proved to be the most reproducible method. We confirmed the integrity of cAMP throughout the incubation process by monitoring its chromatographic behavior. Little phosphodiesterase activity was detected in the purified preparations of receptor.

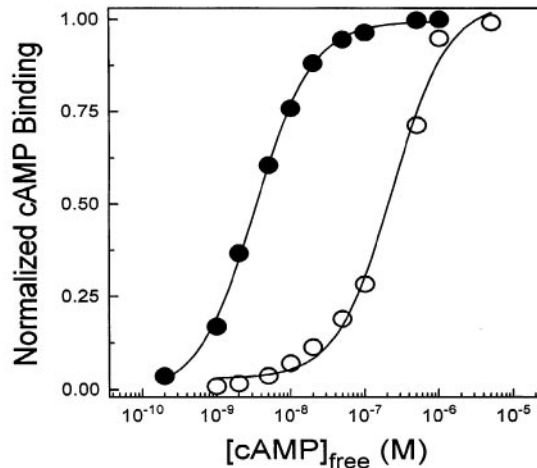
**Dephosphorylation of Phosphorylated cAR1**—CHIFF prepared from desensitized cells were resuspended in 1 × NEB III buffer at a cell equivalent density of 1–2 × 10<sup>9</sup>/ml. 2 units of alkaline phosphatase (New England Biolabs) was added and dephosphorylation carried out at 37 °C for 20–30 min. Gel Shifting assay indicated that this treatment was sufficient to dephosphorylate 80–90% of cAR1. The CHIFF membrane was recovered by centrifugation and resuspended in TEB for cAMP binding assay.

#### RESULTS

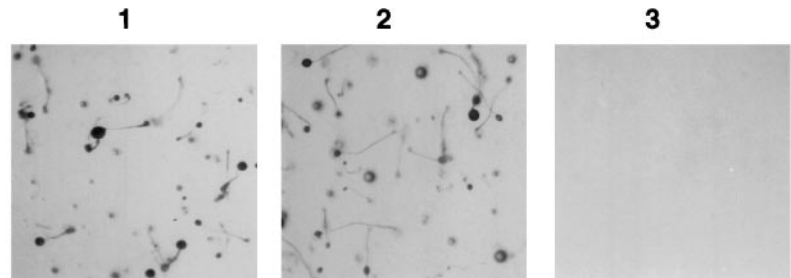
**C-terminal Hexyl-histidine-tagged cAR1s Are Fully Functional**—Several lines of evidence indicated that the C-terminal hexyl-histidine fusion did not interfere with the functional properties of the receptor. To avoid any potential receptor heterogeneity caused by variable extents of phosphorylation, we initially used a hexyl-histidine-tagged cAR1 in which all serines and threonines in the C-terminal domain were substituted (cm1234-H6). After transformation into *car*I<sup>-</sup>/*car*3<sup>-</sup> cells, transformed clones that expressed 3-fold higher surface cAMP-binding sites (3 × 10<sup>5</sup> sites/cell) than optimally developed wild-type cells were isolated. As shown in Fig. 1A, under physiological conditions in phosphate buffer, most of the receptors displayed an average affinity of 200–300 nM (*K<sub>d</sub>* = 235 ± 40 nM), and in the presence

FIG. 1. A, hexyl-histidine-tagged receptor displays the same cAMP-binding profiles as wild-type receptor in both phosphate buffer and ammonium sulfate. cm1234 cAR1, tagged at the C terminus with hexyl-histidine (cm1234-H6), was overexpressed in cAR1-null cell line (RI9).  $K_d$  values in both phosphate buffer (*open circles*) and ammonium sulfate (*solid circles*) were assessed as described under "Experimental Procedures." Ten different ligand concentrations were used. The dissociation constant ( $K_d$ ) values are derived by computer modeling method (ORIGIN). For ammonium sulfate,  $K_d$  was  $3.5 \pm 0.2$  nM, for phosphate buffer,  $K_d$  for the predominant population (>90%) of receptors is  $235 \pm 42$  nM. B, histidine-tagged cAR1 rescues development of cAR1-null cells. Vegetative state cm1234-H6 cells were washed in DB once and then plated on non-nutrient agar (1). Untagged cm1234 cAR1-rescued RI9 cells (2) and vector transformed RI9 cells (3) were also plated to offer positive and negative controls. After 24 h, pictures were taken for each plate to assess their developmental conditions. C, histidine-tagged receptor on CHIFF displays similar cAMP binding affinity as receptors on cell membranes. Purified CHIFF from His-tagged cAR1 cells was isolated and resuspended in TB buffer. cAMP binding was assayed by equilibrium dialysis. Ten cAMP concentrations were used to create the binding curve (1, 2, 5, 10, 20, 50, 100, 200, 500 nM, 1  $\mu$ M, and 5  $\mu$ M). For each point, about 2–3  $\mu$ g of CHIFF was used which contains about 100 ng of cAR1 (5% of total CHIFF proteins).

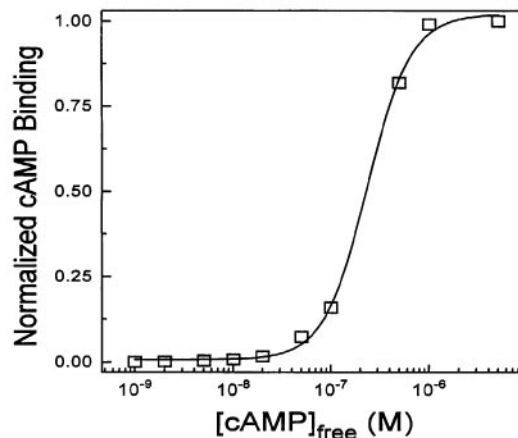
A



B



C



of ammonium sulfate,<sup>2</sup> the affinity increased about 50-fold ( $K_d = 3.5 \pm 0.3$  nM). These values are essentially the same as those previously reported for untagged receptors (11). To assess the functional properties of the tagged receptor, we plated the transformants on non-nutrient agar and observed the developmental properties of the cells.<sup>3</sup> As shown in Fig. 1B, cm1234-H6

rescued the development of *car1*<sup>-</sup>/*car3*<sup>-</sup> cells as efficiently as the untagged cm1234, indicating that the C-terminal hexyl-histidine does not interfere with the functions of receptor. As expected, the vector transformed *car1*<sup>-</sup>/*car3*<sup>-</sup> cells showed no development.

We have previously shown that wild-type cAR1 is quantitatively localized to a specialized plasma membrane fraction designated CHIFF (10). Our strategy was to use CHIFF as an intermediate purification step, providing a 500-fold enrich-

<sup>2</sup> The binding in phosphate buffer corresponds to binding at physiological conditions. Ammonium sulfate has been shown to convert the receptors into a single high affinity state ( $K_d$  3–5 nM), so bindings obtained at 20–30 nM cAMP in ammonium sulfate reflect the total binding of cells.

<sup>3</sup> During nutrient deprivation, the *D. discoideum* cells start a developmental program whereby central cells secrete cAMP, attracting surrounding cells through chemotaxis. The surrounding cells in turn secrete cAMP to attract more distal cells. Eventually tens of thousands of

cells will aggregate to form a mound. The four cAMP receptor proteins, cAR1–cAR4, are the cell surface receptors responsible for sensing the pulses of cAMP and activating downstream events. cAR1 is the major type expressed during the initial stage of aggregation. In its absence, cells fail to aggregate.

ment, and then to solubilize and purify the hexyl-histidine-tagged receptor by metal affinity column chromatography. We first determined that the tagged receptor was also enriched in CHIFF and its activity remained intact. Our results indicated that cm1234-H6 displayed the same localization in CHIFF as wild-type cAR1 (data not shown), showing that the histidine tag does not interfere with the normal targeting of receptor to the specialized plasma membrane domains.

The ligand binding activity of CHIFF membranes was characterized by equilibrium dialysis. Computer analysis indicated that a single affinity form was present, with a  $K_d$  of  $250 \pm 65$  nM (Fig. 1C). This value was close to the  $K_d$  of the predominant receptor population at the cell surface ( $K_d = 235$  nM, Fig. 1A) and indicated that the agonist binding sites remain essentially unchanged during the CHIFF preparation process.

**Solubilization and Purification of Hexyl-histidine-tagged cAR1 by  $Ni^{2+}$  Column Chromatography**—cm1234-H6 cAR1 was solubilized and purified to near homogeneity by chromatography on a  $Ni^{2+}$ -chelating column. We have previously shown that CHIFF proteins can be efficiently extracted by the non-ionic detergent Lubrol-PX (9). We resuspended the CHIFF sample to a density of  $2\text{--}4 \times 10^8$  cell equivalents/ml and added 1% Lubrol-PX. It is crucial to carry out solubilization at this density; at higher densities, CHIFF fragments tend to aggregate, preventing efficient solubilization. Under these conditions, more than 90% of the receptor was typically extracted. The solubilized fraction was supplemented with NaCl and imidazole to prevent nonspecific absorption and then chromatographed over the  $Ni^{2+}$  column. After extensive washes, the bound receptor was eluted with four steps of increasing imidazole concentrations: 25, 50, 100, and 250 mM (Fig. 2). cAR1 emerged from the column in two peaks, about 25% eluted at 100 mM and the remainder eluted at 250 mM. This ratio varied slightly between experiments. The purified receptor displayed two major molecular weight forms according to gel migration positions, corresponding to a monomeric (indicated by "M") and a dimeric form (indicated by "D"). The second peak, emerging from the column at 250 mM imidazole, was enriched in the dimer form.

Samples taken from the three stages of the purification process were examined by SDS-PAGE followed by silver staining (Fig. 2C). Comparisons of protein profiles between intact cell, CHIFF, and final purified samples revealed a significant purification. Gel scanning showed that in both peaks eluted from the column the purity of receptor was over 80%. Latter experiments suggested that the purity could be further enhanced by prolonging the 25 mM wash step (data not shown).

**cAMP Binding Activity and Receptor Protein Co-fractionates on  $Ni^{2+}$  Column**—Next we determined whether the column-purified receptor retained cAMP binding activity. We assayed the cAMP binding activity of each column fraction by equilibrium dialysis. Specific bindings were obtained from these fractions (Fig. 3A, *hatched bars*). Comparison between this profile and the previous cAR1 protein elution profile (Fig. 3A, *inset*) clearly demonstrated a direct correspondence between the levels of binding activity and receptor protein, suggesting that the purified cm1234-H6 is responsible for the observed binding.

**Purified Receptors in Detergent Solution Display a Similar cAMP Affinity as Receptors on Cell Surface**—Having established that the purified receptor is still active, we quantitatively characterized its affinity by equilibrium dialysis. For these studies, we pooled both elution peaks containing cAR1, representing over 90% of the eluted receptors (Fig. 2A, fractions 9–15). Specific binding data at 10 different cAMP concentrations were obtained. The curve was best fit by a single affinity state with a dissociation constant of  $188 \pm 39$  nM (Fig. 3B). This value is similar to that of cAR1 on CHIFF membranes

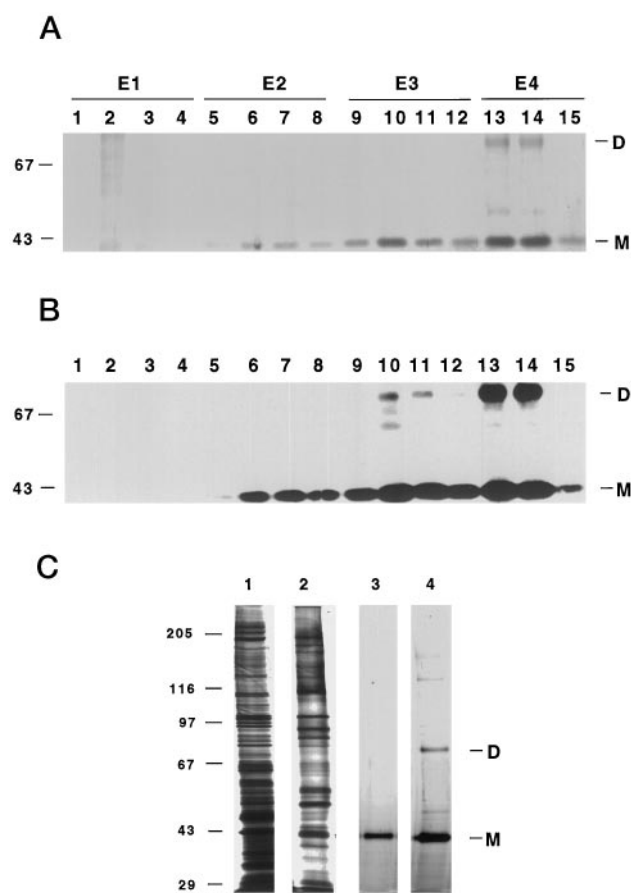


FIG. 2. Solubilization and purification of cm1234-H6 cAR1 over  $Ni^{2+}$  column. cm1234-H6 CHIFF preparation were solubilized with Lubrol-PX and the extracts were loaded onto  $Ni^{2+}$  chelating column. After extensive washes with 20–40 column volumes of binding buffer, bound receptors were eluted off the column by four wash steps of increasing imidazole concentration (25, 50, 100, and 250 mM in binding buffer). Each step consists of four column fractions. The elution was shown in both: A, silver staining profile; and B, cAR1-immunoblotting profile. Lanes 1–4, 25 mM elution; 5–8, 50 mM elution; 9–12, 100 mM elution; and 13–15, 250 mM elution. C, sample purities at different stage of purification (silver staining). Lane 1, whole cells; 2, CHIFF; 3 and 4, purified receptor (two peaks). For lanes 1 and 2, same total proteins were loaded. Monomer and dimer forms of cAR1 are indicated by "M" and "D," respectively.

and on the surface of cells lacking G-proteins (22). These data suggest that the purified preparation consists of a homogeneous population of receptors not coupled to G-proteins.

The data from a representative purification were tabulated (Table I) to illustrate the recoveries of protein, [ $^3H$ ]cAMP binding activity and changes in specific activity in this experiment. Step 1, which involves purifying CHIFF from cells, yielded approximately 50% of the receptor protein and 60% of cAMP binding activity. Since the CHIFF fraction contains less than 0.2% of the cellular protein, the specific activity of receptors increased nearly 500-fold during this step. Step 2, the purification of receptors from solubilized CHIFF, yielded 40% recoveries of both receptor protein and cAMP binding activity. The cumulative recoveries for the whole procedure were about 18% for the protein and 25% for binding activity. The overall fold of purification was about 7,000. The specific activity of the final purified sample was about  $3 \times 10^4$  pmol/mg, corresponding to  $1.8 \times 10^{16}$  sites/mg. The theoretical specific activity of pure cAR1 is calculated to be  $1.6 \times 10^{16}$  sites/mg, assuming one binding site per cAR1 molecule. These data suggest that the purified receptors in detergent solution are fully active, although these calculations were based on several measurements

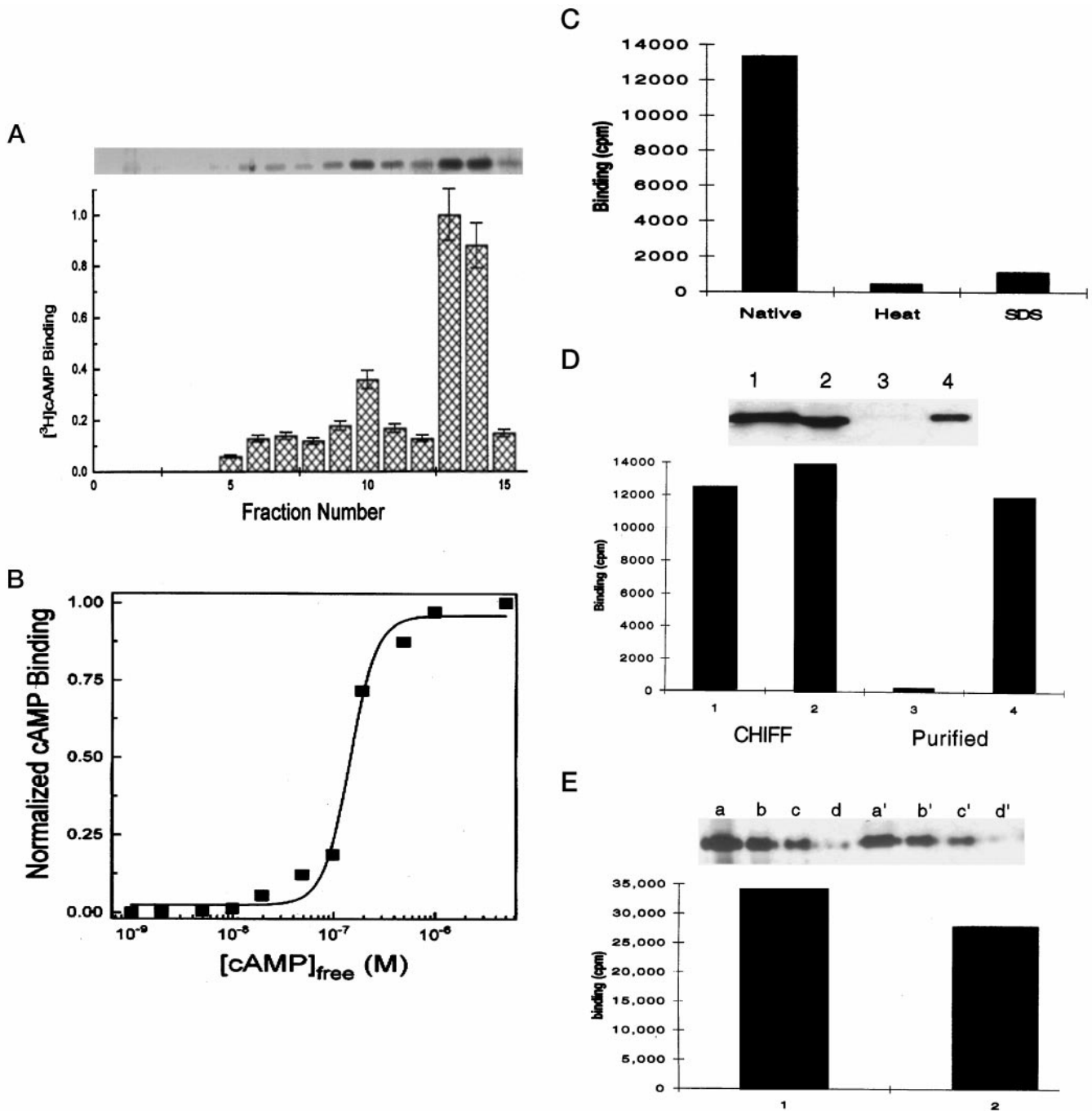


FIG. 3. *A*, cofractionation of cAMP binding activity with His-cAR1 over  $\text{Ni}^{2+}$  column. To establish that the solubilized cAR1 is active, equilibrium dialysis binding was performed on each of the  $\text{Ni}^{2+}$  column elution fractions (Fig. 2A; see "Experimental Procedures" for binding conditions). The final ligand concentration is 100 nM [ $^3\text{H}$ ]cAMP for all fractions. Since fraction 13 displays the highest level of cAMP binding, this fraction was given 100% value. All the other fractions were quantified as a relative percentage value of this fraction. Bindings were plotted as *hatched* columns. The *upper inset* shows the corresponding cAR1 immunoblot profile. Each lane was aligned with its respective column of binding. *B*, ligand affinity of purified cm1234-H6 receptor. Final purified His-tagged cAR1 was assayed for cAMP binding through equilibrium dialysis. Ten different cAMP concentrations were used: 1, 2, 5, 10, 20, 50, 100, 500 nM and 1 and 5  $\mu\text{M}$ . Computer modeling was used to assess the  $K_d$  value at  $188 \pm 39$  nM. *C*, specific cAMP binding of purified receptor is sensitive to heat and SDS denaturations. Equal amounts of purified His-cAR1 was directly used in the equilibrium dialysis binding assay (*Native*), or first treated with heating at 95 °C for 5 min (*Heat*), or treated with 1% SDS for 10 min (SDS), and then used for the binding assay. Assay was performed as previously and specific bindings were plotted. *D*, the observed cAMP bindings is specifically due to the receptor. CHIFF samples were prepared from WT cAR1 (non-histidine-tagged) expressing cells or hexyl-histidine-tagged cAR1-expressing cells. They were solubilized and purified over  $\text{Ni}^{2+}$  column. The purified samples and the original CHIFFs were assayed for cAMP binding through equilibrium dialysis (*bar graph*). 1, CHIFF with WT cAR1; 2, CHIFF with His-cAR1; 3, column-purified sample from 1; 4, column-purified sample from 2. These four samples were also loaded on SDS-PAGE for cAR1 immunoblotting (*upper inset*). *E*, recoveries of receptor protein and binding activity through the last purification step (purification of receptor from CHIFF through  $\text{Ni}^{2+}$  column). CHIFF was prepared from His-cAR1 cells and divided into two equal aliquotes: the first one was saved on ice (sample 1) while the second one was subjected to extraction and purification of receptor (sample 2). Equal fractions of samples 1 and 2 were assayed for cAMP binding (*bar graph*) and cAR1 immunoblotting (*upper inset*) to compare the recovery rates of activity and protein, respectively. For immunoblotting, a dilution series consisting of four decreasing concentrations (a, b, c, and d for sample 1; a', b', c', and d' for sample 2) were used to better assess the recovery.

TABLE I  
Two-step purification of cAR1

Protein concentrations were assayed by Bio-Rad microBCA assay (detergent compatible). When measuring the final purified receptor, we used both bovine serum albumin and purified oxaloglutarate transporter protein as standards (both gave similar results). This purification process used  $10^{10}$  cm1234-H6 cells (1 liter fully grown culture). Total ligand binding of whole cells was obtained by ammonium sulfate binding assay at 20 nM cAMP concentration (4 times the  $K_d$  level). Overall bindings for both CHIFF and pure cAR1 preparations were evaluated by equilibrium dialysis at 100 nM cAMP concentration, and then calculating the saturation binding values by extrapolation.

	Total protein	cAR1 content	cAMP-binding	Activity recovery	Specific activity	Purification
			<i>nmol sites</i>			<i>-fold</i>
Intact cells	620 ± 32 (mg)	90 μg <sup>a</sup>	2.4 ± 0.2	100%	3.9 ± 0.3 pmol/mg	
CHIFF	780 ± 40 (μg)	43 ± 3 <sup>b</sup> (47%)	1.5 ± 0.2	62%	1,923 ± 86	493
Purified cAR1	20 ± 2 (μg)	16 ± 2 (18%)	0.6 ± 0.1	25%	30,000 ± 1,500	7,600

<sup>a</sup> The cAR1 content of intact cells was determined from the total number of surface cAMP-binding sites. We assume one binding site per receptor ( $M_r$  43,000). This assumption was verified by quantitative immunoblot comparing the receptor level in whole cells with known quantities of purified receptors.

<sup>b</sup> The cAR1 content of CHIFF was determined by SDS-PAGE. The gel was silver-stained and scanned. The receptor abundance in CHIFF is usually in the range of 5–7%. The final purified sample was analyzed similarly.

which all have margins of error. We routinely purify about 10–20 μg of pure active receptor from  $10^{10}$  cells, which corresponds to 1 liter of axenic culture. We can conveniently grow and harvest 30 liters of cells, which would correspond to about half a milligram of purified cAR1. The current limitation in the procedure is the requirement of a separate ultracentrifugation run to purify CHIFF from each 5 liters of culture.

*The cAMP Binding as Measured by Equilibrium Dialysis Depends on Native Receptors*—To control for the specificity of the equilibrium dialysis binding assay, we performed several experiments. Purified receptors were divided into three equal sets: the first was not treated, the second was heat-denatured (95 °C for 5 min), and the third was treated with 1% SDS at room temperature for 10 min. The three sets were then assayed in parallel for [<sup>3</sup>H]cAMP binding. As shown in Fig. 3C, only the first set displayed binding activity. The other two sets showed only very low activity, indicating that the observed binding was due to a native protein.

We carried out a further test to confirm that the binding activity was specifically due to the receptor. We used parallel sets of cells, overexpressing two versions of cAR1 which did or did not contain the hexyl-histidine tag. CHIFF were prepared from both cell lines and then both samples were solubilized and chromatographed on Ni<sup>2+</sup> columns. Presumably both receptors would fractionate with CHIFF, but only the hexyl-histidine-tagged receptor will be retained and purified by the column. At each stage, cAMP binding was assayed by equilibrium dialysis (Fig. 3D). At the CHIFF stage (1 and 2) both cell lines displayed comparable specific binding; while at the purified stage (3 and 4), only the His-tagged receptor preparation displayed binding. The immunoblot showed that CHIFFs from both cell lines contained cAR1 (*inset, lanes 1 and 2*), but only the hexyl-histidine-tagged receptor was purified (*inset, lanes 3 and 4*), demonstrating that cAMP binding was specifically due to purified receptor.

We also carried out an additional experiment to further quantitate the step from CHIFF to purified receptor. We prepared CHIFF from cm1234-H6 cells and divided it into two equal aliquots. The first was saved (CHIFF) while the second was subjected to extraction and purification (purified). Equal fractions of each sample were assayed both for cAMP binding by equilibrium dialysis and by immunoblotting to quantitate the recovery of binding activity and receptor protein, respectively. For immunoblotting, a dilution series of four decreasing concentrations of samples were applied to better assess the recovery. As shown in Fig. 3E, the recoveries of binding activity

and receptor protein were about 78 and 69%, respectively. This observation indicates that there was no significant inactivation of the receptor during the solubilization and purification process.

*Phosphorylated Receptors Have an Intrinsically Lower Affinity for [<sup>3</sup>H]cAMP*—To address whether the lowered affinity of phosphorylated receptors on the cell surface (5) was intrinsic to the modified protein or due to interactions with other proteins, we purified the unphosphorylated and phosphorylated forms of receptors in parallel. We used the cell line expressing hexyl-histidine-tagged wild-type receptor (cAR1-H6). The cells were pretreated with excess cAMP to induce maximal phosphorylation or with caffeine to ensure complete dephosphorylation of the receptor (“Experimental Procedures”). Binding assays were performed on these two sets of cells to determine their respective affinities. As shown in Fig. 4A, consistent with previous studies, the cAMP pretreated set displayed a decreased affinity compared with the untreated control set ( $K_d$  for pretreated cells: 728 ± 76 nM;  $K_d$  for untreated cells: 220 ± 60 nM). The total number of binding sites was the same for both sets of cells. These observations also demonstrated that hexyl-histidine tagging did not interfere with the normal desensitization properties of receptor.

It has been previously shown that upon detergent lysis, cAR1 from cAMP-pretreated cells was stably maintained in its phosphorylated state, and phosphorylation did not alter the localization of receptor to the CHIFF fraction (10). Thus we were able to use CHIFF to purify both forms of receptors. CHIFF preparations were obtained for both untreated and cAMP-pretreated cells and tested for ligand binding by equilibrium dialysis (Fig. 4B). The two forms of cAR1-H6 essentially retained the same affinities as in intact cells. The 3-fold difference in affinities between the two forms persisted; the CHIFF preparation from cAMP-pretreated or resting cells displayed  $K_d$  values of 812 ± 69 and 279 ± 50 nM, respectively. We have shown previously that G-proteins are absent from CHIFF (10). This indicates that the lowered affinity of phosphorylated receptors could *not* be due to their uncoupling from G-proteins.

Subsequently, the two forms of receptors were extracted from CHIFFs and purified in parallel over Ni<sup>2+</sup> columns. Similar fractionation profiles were obtained for both forms and were essentially the same as for the cm1234-H6 receptors except that the dimer form was less abundant. The pure fractions were pooled, analyzed by SDS-PAGE, and silver stained (Fig. 5A). The phosphorylated form displayed a lower mobility, consistent with previous results (12), indicating that the extent

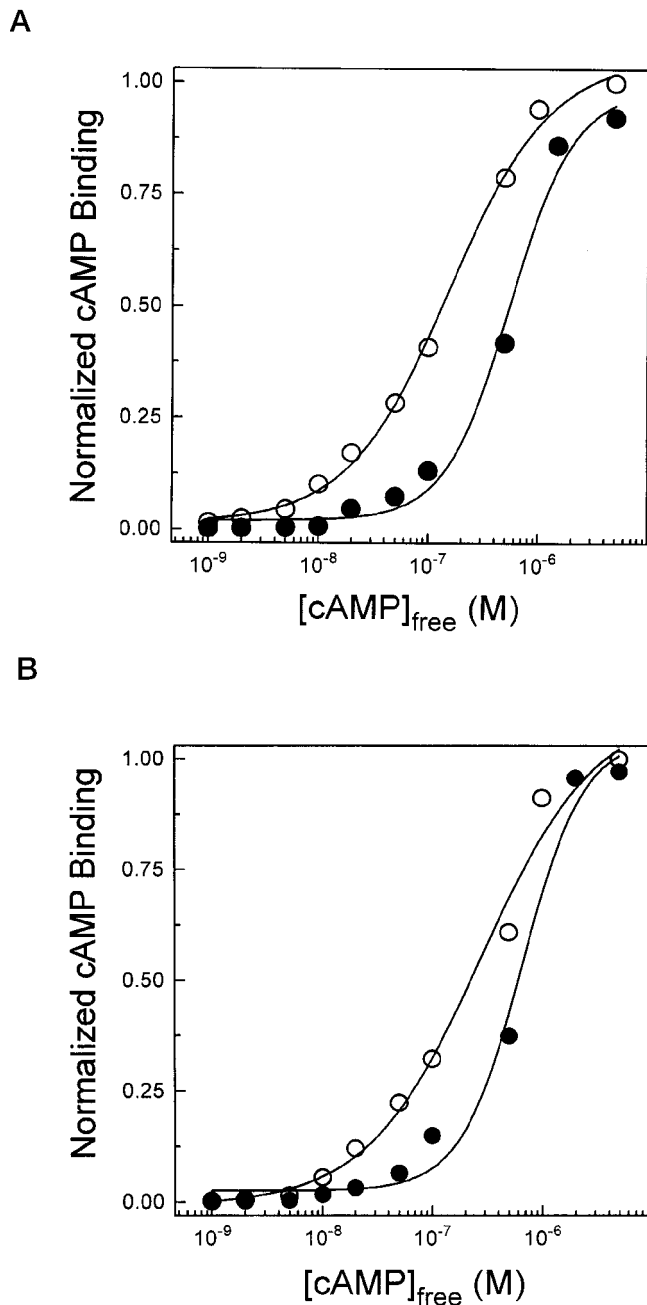


FIG. 4. A, ligand affinities of basal and desensitized cAR1-H6 cells. cAR1-H6 cells (hexyl-histidine-tagged WT cAR1) were pretreated with caffeine or excess cAMP to basalize or desensitize the receptors as under "Experimental Procedures." cAMP binding was carried out for both sets as described before. 10-point binding assay was performed to determine the binding curve and  $K_d$  values were derived by computer fitting program:  $220 \pm 60$  nM for basalized cells (predominant population, open circles),  $728 \pm 76$  nM for desensitized cells (solid circles). B, ligand binding profiles of CHIFF from basal and desensitized cAR1-H6 cells. CHIFF from both basalized and desensitized cAR1-H6 cells were prepared and assayed for ligand binding as described in the legend to Fig. 1C. Ten-point bindings were performed to determine the respective  $K_d$  values:  $279 \pm 50$  nM for unphosphorylated CHIFF (open circles),  $812 \pm 69$  nM for phosphorylated CHIFF (solid circles).

of phosphorylation was fully preserved during the purification. The ligand binding properties of these preparations were characterized by equilibrium dialysis (Fig. 5B). Computer fitting yielded predominantly a single site for both forms. The  $K_d$  values were essentially the same as those in intact cells and CHIFF. The purified phosphorylated receptors displayed a  $K_d$  of  $882 \pm 70$  nM, three times higher than the unmodified recep-

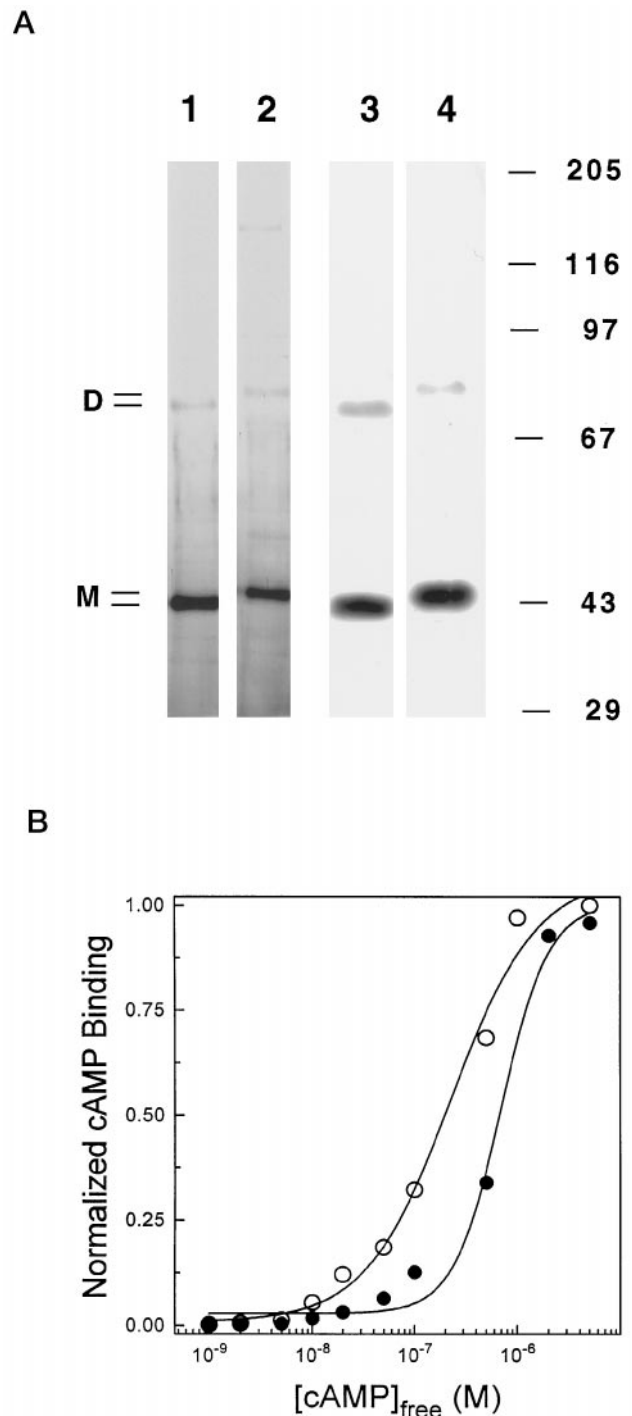


FIG. 5. A, purification of cAR1-H6 receptor in both unphosphorylated and phosphorylated form. cAR1-H6 cells were treated before detergent lysis to induce receptor phosphorylation or dephosphorylation (as in Fig. 4). The two forms of cAR1 were purified in similar fashion from CHIFF over the  $Ni^{2+}$  column. The pooled major elution fractions were shown for each form. Lane 1, unphosphorylated receptor; 2, phosphorylated receptor; 3 and 4, corresponding cAR1 immunoblotting profile of 1 and 2. B, phosphorylated receptor has intrinsically lower ligand affinity. The phosphorylated and unphosphorylated forms of purified cAR1 from A were assayed for cAMP affinities through equilibrium dialysis.  $K_d$  values were derived from computer modelings:  $249 \pm 63$  nM (open circle) for unphosphorylated receptor,  $882 \pm 70$  nM for phosphorylated form (solid circle). This experiment was repeated twice with consistent findings.

tors which had a  $K_d$  of  $249 \pm 63$  nM. The  $K_d$  values for both forms of purified receptors closely reflect their original cell surface values, suggesting that the decreased ligand affinity of

TABLE II

Comparisons of cAMP affinity values of receptor at different stages of purification

Data shown are mean  $\pm$  S.D. of at least two experiments. Data were analyzed by computer modeling methods (ORIGIN).

Dissociation constant $K_D$	Intact cell	CHIFF	Pure receptor
<i>nM</i>			
Unphosphorylated	220 $\pm$ 60	279 $\pm$ 50	249 $\pm$ 63
Phosphorylated	728 $\pm$ 76	812 $\pm$ 69	882 $\pm$ 70

phosphorylated cAR1 is an intrinsic property of the receptor itself and does not require associated proteins. The receptor affinities during the purification process are summarized in Table II.

**Receptor Dephosphorylation Enhanced Its Ligand Affinity**—To demonstrate that phosphorylation, not other possible concurring modifications, directly accounts for the lowered affinity of the phosphorylated receptor, we dephosphorylated the receptor with different protein phosphatases and then determined whether removal of phosphates lead to enhanced receptor affinity. Various phosphatases, including protein phosphatase 1,  $\lambda$ -phosphatase, and alkaline phosphatase (calf intestinal) dephosphorylated cAR1 efficiently (Fig. 6A). Alkaline phosphatase displayed the highest efficiency (lanes 10–13): a 10-min incubation led to about 80% dephosphorylation according to the electrophoretic mobility shifting assay, while protein phosphatase 1 and  $\lambda$ -phosphatase required several hours to achieve the same result. Control samples (mock-treated with buffer) incubated at 37 °C for 0 and 18 h showed minimal self-dephosphorylation (lanes 9 and 14).

[<sup>3</sup>H]cAMP binding assays were performed on the alkaline phosphatase-treated samples in comparison with untreated control samples (Fig. 6B). In one experiment, untreated controls showed a ligand binding of 17,000 cpm, the mock-treated controls showed somewhat lowered binding at 8,700 cpm, indicating that incubation of CHIFF at 37 °C for 20 min lead to partial reduction in cAMP binding. The samples treated with alkaline phosphatase displayed an enhanced binding of 42,000 cpm, 2–3-fold higher than the untreated control. The real increase may be even higher since 37 °C incubation leads to partial reduction of receptor ligand binding.

#### DISCUSSION

Our current study demonstrates that lowered affinity of receptor induced by agonist pretreatment is an intrinsic property of the phosphorylated receptor. That is, phosphorylation within the C-terminal domain of the receptor (at serines 303 and 304) imposes a lower affinity. Removal of these phosphate groups caused a 2-fold increase in cAMP binding capacity. This *in vitro* enhancement is consistent with the observed affinity difference between unphosphorylated and phosphorylated receptor. These results argue that phosphorylation, rather than other putative accompanying modifications, directly causes the decreased affinity. This suggests that phosphorylation *per se* could directly lead to receptor desensitization through reduced ligand occupancy.

It has been proposed for other GPCRs that the ligand-binding site resides within a pocket formed by the seven hydrophobic transmembrane segments (1). To explain the relatively higher affinity of the basal form of receptor and the lower affinity of the phosphorylated receptor, we propose that under resting conditions, there is minimal contact between the C-terminal domain and intracellular loops of the receptor (which underline the ligand binding pocket). After agonist binding and phosphorylation, the C-terminal domain may undergo a conformational change and interact with the loop regions, thus

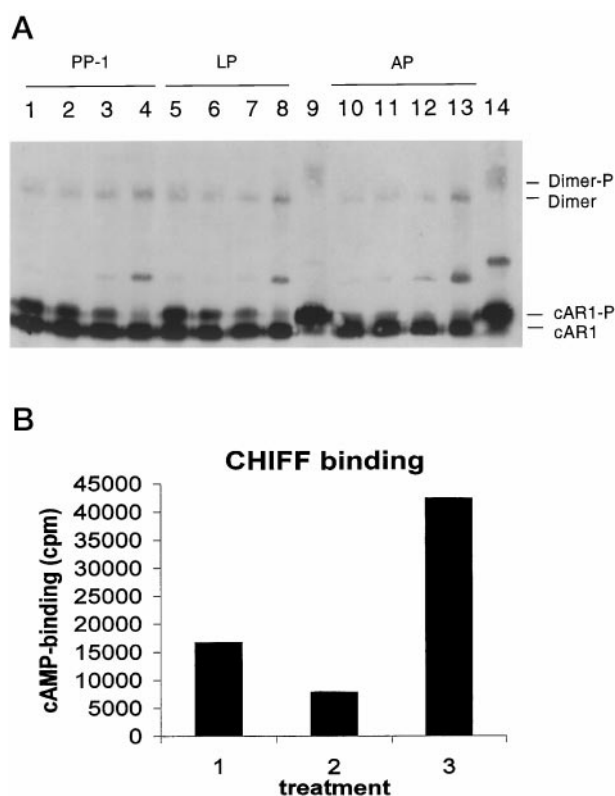


FIG. 6. A, dephosphorylation of cAR1 by different protein phosphatases. CHIFF with phosphorylated WT-cAR1 was resuspended in appropriate buffers and treated for increasing times with protein phosphatase 1 (PP-1: lanes 1, 0.5 h; 2, 1 h; 3, 2 h; 4, 18 h),  $\lambda$ -phosphatase (LP: lanes 5, 0.5 h; 6, 1 h; 7, 2 h; 8, 18 h), and alkaline phosphatase (AP: lanes 10, 15 min; 11, 30 min; 12, 1 h; 13, 2 h). Lanes 9 and 14 are control samples treated with buffer at 37 °C for 0 and 18 h, respectively. cAR1-P and Dimer-P indicate phosphorylated cAR1 monomer and dimer, respectively. B, dephosphorylation of cAR1 enhances its cAMP affinity. Phosphorylated WT-cAR1 was dephosphorylated by alkaline phosphatase and tested for cAMP-binding by equilibrium dialysis. Column 1, untreated phosphorylated cAR1 control; 2, phosphorylated cAR1 treated with buffer at 37 °C for 20 min; 3, phosphorylated cAR1 treated with alkaline phosphatase at 37 °C for 20 min.

imposing the lowered affinity. Although other models are possible, two lines of observation favor this proposal. First, truncation of the C-terminal domain yields a receptor with high affinity that is resistant to agonist-induced affinity reduction (14), suggesting that the higher affinity of unphosphorylated receptor does not require the C-terminal domain. Second, the current study demonstrates that completely purified phosphorylated receptors display the lowered affinity, ruling out the need for interacting proteins in this process.

Lowered affinity displayed by phosphorylated GPCRs has not been widely recognized as a major cause of desensitization. In *in vitro* reconstitution studies with unphosphorylated or phosphorylated receptors, the phosphorylated receptors were found to have reduced coupling to G-protein (15), but comparisons between the affinities of two forms of receptors were typically not carried out. In *in vivo* studies, receptor internalization, sequestration, or down-regulation often occur during or immediately after receptor phosphorylation, thus making direct assessment of receptor affinity after phosphorylation impractical. Hence, the processes such as internalization or sequestration are usually considered the major contributing factors to desensitization while altered affinity is not. Possible decreases in receptor affinity can only be observed in situations where internalization/sequestration is delayed or does not occur. Alternatively, the phosphorylated and unphosphorylated



receptors can be purified so their affinities can be directly compared. We propose that such unrecognized affinity decreases may be a typical consequence of GPCR phosphorylation and could directly contribute to receptor desensitization.

The purification scheme we have developed is in principle applicable to any GPCR or membrane protein that can be expressed in *D. discoideum* as long as that protein is quantitatively enriched in the minor detergent-resistant plasma membrane fraction, CHIFF. The CHIFF purification step provides a 500-fold purification, which makes the subsequent fractionation on the Ni<sup>2+</sup> column very effective. Direct loading of whole cell extracts on the column yielded little purification (data not shown). Purification schemes for other GPCRs have often included ligand affinity chromatography as a major enrichment step (16–18). In contrast, our procedure is independent of ligand binding. We required such a scheme since we wished to purify various forms of receptors, including those with decreased binding activities. In the current study we have used it to purify the phosphorylated and unphosphorylated forms of cAR1 even though they have different affinities. Furthermore, we have constructed many mutant forms of cAR1 which are specifically deficient in ligand binding, G-protein coupling, signal transduction, or desensitization and they have been analyzed *in vivo* (12). This purification scheme which is applicable to both wild-type receptor and all the mutant forms will enable us to dissect the structural differences between these mutants and identify the structural determinants for each property of the receptor.

It has been reported that certain GPCRs display dimeric and higher oligomeric forms when solubilized in non-ionic detergents and run on SDS-PAGE (19). We often detected cAR1 dimer on SDS-PAGE in both whole cell extracts and plasma membrane samples. The dimers are not formed through disulfide bonds since they are resistant to 100 mM dithiothreitol treatment (data not shown). Since the dimers may be only partially resistant to SDS, we speculate that the apparent dimers on SDS-PAGE represent a residual portion of the dimers originally present in non-ionic detergents. Several lines of evidence further confirm the dimerization of cAR1 in non-ionic detergents. First, cAR1 solubilized in non-ionic detergents migrates as two forms on sucrose velocity gradients (data not shown). The more rapidly sedimenting form contained a higher proportion of apparent dimers on SDS-PAGE. Second, unlike the monomers which eluted at both 100 and 250 mM imidazole concentrations, the apparent dimers only eluted at the 250 mM imidazole range in the Ni<sup>2+</sup> column profile (Fig. 2), consistent with the anticipated tighter binding two histidine tags would confer. Third, we performed the following co-immunoprecipitation test to confirm the presence of receptor dimers (data not shown). A cAR1-GFP (green fluorescence protein, Ref. 20) fusion construct was transformed into wild-type cells such that the cells would co-express both wild-type cAR1 and cAR1-GFP fusion proteins. Detergent extracts from these cells were prepared and immunoprecipitated with anti-GFP antibody. Wild-type cAR1 as well as cAR1-GFP was found in the final immunoprecipitate, demonstrating the existence of mixed dimers. It is difficult to determine the physiological relevance of dimerization from biochemical analysis. Our results suggest that the dimer and monomer forms do not differ dramatically in ligand affinity (since only one affinity form was present for the whole preparation). Further analysis will require a complete purification of each of the two forms.

In *D. discoideum*, many cAR1-mediated responses desensitize, and their time courses closely reflect that of receptor phosphorylation. Conversely, the resensitization of these processes closely follows the dephosphorylation of cAR1 (14). This

suggests that receptor phosphorylation is the central step in desensitization. However, we have shown definitively that receptor phosphorylation is *not* essential for termination of receptor-mediated responses. Cells expressing only cm1234, the non-phosphorylatable form of cAR1, display the normal kinetics in attenuation of adenylate cyclase and other cAR1-mediated responses. This observation indicates that a mechanism(s) other than receptor phosphorylation operates to terminate multiple receptor-mediated pathways. However, the cells expressing cm1234 do recover more quickly from adaptation after removal of the stimulus, suggesting that both phosphorylation-dependent and independent mechanisms are functioning redundantly in mediating adaptation.<sup>4</sup>

Although the phosphorylated receptors have a lower affinity for agonist, they are not necessarily signaling deficient. On the contrary, we speculate that this affinity decrease may be important for fine tuning the responses that chemoattractant receptors mediate. As cells initially move toward a source of attractant, the attractant concentration is low, so the higher affinity of the unmodified receptors is needed. As the cells approach the source, a higher ligand concentration will be encountered. As the concentration nears saturation level, cells carrying receptors that cannot be modified and hence cannot assume a lower affinity will be unable to distinguish the directionality of the gradient; whereas cells with receptors whose affinity can be adjusted downwards by phosphorylation will be able to detect a larger range of chemoattractant concentrations. In summary, the agonist-induced phosphorylation of the chemoattractant receptor which lowers affinity may be useful in maintaining the receptor's sensitivity at high agonist levels.

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