THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 15, Issue of April 14, pp. 8667–8672, 1995
Printed in U.S.A.

Agonist-induced Loss of Ligand Binding Is Correlated with Phosphorylation of cAR1, a G Protein-coupled Chemoattractant Receptor from *Dictyostelium**

(Received for publication, December 27, 1994)

Michael J. Caterina‡, Peter N. Devreotes, Jane Borleis, and Dale Hereld§

From the Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The parallel agonist-induced phosphorylation, alteration in electrophoretic mobility, and loss of ligand binding of a guanine nucleotide-binding regulatory protein (G protein)-coupled chemoattractant receptor from *Dictyostelium* (cAR1) depend upon a cluster of five C-terminal domain serine residues (Caterina, M. J., Hereld, D., and Devreotes, P. N. (1995) *J. Biol. Chem.* 270, 4418–4423). Analysis of mutants lacking combinations of these serines revealed that either Ser^{303} or Ser^{304} is required; mutants lacking both serines are defective in all of these responses. Interestingly, several mutants, including those substituted at only Ser^{299}, Ser^{302}, or Ser^{303} or at non-serine positions within the third cytoplasmic loop, displayed an unstable mobility shift; the alteration was rapidly reversed upon cAMP removal. These mutants also exhibited subnormal extents of loss of ligand binding, which is assessed after removal of the ligand.

For the wild-type receptor, we found that the stability of phosphorylation depends upon the concentration and duration of agonist pretreatment. This suggests that, following phosphorylation of Ser^{303} or Ser^{304}, cAR1 undergoes a further transition ($EC_{50} \approx 140$ nM, $t_{1/2} \approx 4$ min) to a relatively phosphatase-resistant state. We used this insight to show that, under all conditions tested, the extent of loss of binding is correlated with the fraction of cAR1 in the altered mobility form. We discuss possible relationships between cAR1 phosphorylation and loss of ligand binding.

Receptors that couple to G proteins[1] undergo a series of changes upon agonist binding, including serine/threonine phosphorylation of cytoplasmic domains as well as a rapid, reversible decrease in ligand binding (reviewed in Ref. 1). The latter has been attributed in some cases to a reduction in binding affinity (32) and in others to internalization of receptors (often referred to as "sequestration"). The exact relationship between phosphorylation and reduction of binding is unclear, and may vary among different G protein-coupled receptors. Studies of acetylcholine, gastrin-releasing peptide, and cAMP receptors indicate that phosphorylation and decreases in ligand binding are coordinately regulated (2–5, 32). In contrast, the sequestration of β_2-adrenergic receptors is preserved despite inhibiting receptor phosphorylation or eliminating putative phosphorylation sites by mutagenesis (1, 6). In addition, in this latter system, it has been proposed that, while phosphorylation does not appear to be a prerequisite for sequestration of the β_2-adrenergic receptor, sequestration is necessary for receptor dephosphorylation (7).

cAR1, the chemoattractant cAMP receptor of *Dictyostelium*, is a G protein-coupled receptor which, upon nutrient depletion, coordinates the aggregation of 10^5 individual amoebae into a multicellular structure (Ref. 9; reviewed in Ref. 8). Like other members of the seven transmembrane domain receptor family, cAR1 exhibits both agonist-induced phosphorylation and loss of ligand binding (LLB) (10–13). While cAR1 internalization can occur under some circumstances (14), we have shown that LLB results largely from a reduction in binding affinity (32).

The cAR1 C-terminal cytoplasmic domain contains 18 serine residues organized into four clusters. Agonist binding results in the addition of 3–4 phosphates to serines within two of these clusters. The majority of this phosphorylation occurs within cluster 1 and results in a marked reduction in cAR1 electrophoretic mobility. A mutant in which the five serines of cluster 1 were substituted by site-directed mutagenesis exhibits reduced cAMP-stimulated phosphorylation and fails to undergo the electrophoretic mobility shift (15). Furthermore, this mutant is markedly impaired in cAMP-induced LLB (32). In contrast, elimination of the other 13 serines of the cytoplasmic domain (clusters 2, 3, and 4) had little effect on either the mobility shift or LLB. These findings suggest that phosphorylation of cluster 1 and LLB are related.

In the present study, we describe a detailed mutational analysis of cluster 1 and identify the specific residues required for each of these processes. We analyzed both the onset and reversal of these two processes as exhibited by mutant and wild-type cAR1s under a variety of cAMP pretreatment conditions. Our results indicate that LLB and phosphorylation of specific residues within cluster 1 are coordinately regulated upon the introduction and removal of cAMP and suggest a causal relationship between these two processes.

MATERIALS AND METHODS

Site-directed Mutagenesis of Serine Cluster 1—Using site-directed mutagenesis and three previously described oligonucleotides (15), Ser^{299} and Ser^{308} were independently substituted with Ala and $Ser^{302}Ser^{303}Ser^{304}$ was replaced with Gly-Ala-Gly. Alternatively, the same replacements of Ser^{302}, Ser^{303}, and Ser^{304} were made independently using the degenerate antisense oligonucleotide, 5′-GTACCACGACXTGYACXATATGGTG-3′ (where *X*

* This work was supported in part by National Institutes of Health Grant GM34933 (to P. N. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Medical Scientist Training Program Grant GM07309.

§ Supported by a fellowship from The Damon Runyon-Walter Winchell Cancer Research Fund. To whom correspondence should be addressed: Dept. of Biological Chemistry, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-955-4699; Fax: 410-955-5759.

[1] The abbreviations used are: G proteins, guanine nucleotide-binding regulatory proteins; cAR, cAMP receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; (R_p)-cAMPS, cyclic adenosine 3′,5′-monophosphorothioate, (R_p)-isomer; PAGE, polyacrylamide gel electrophoresis; LLB, loss of ligand binding.

TABLE I
Definition of cAR1 cluster 1 mutants

Cluster 1 mutants used in this study and the amino acid substitutions in each are indicated. In the mutants, preserved wild-type residues are indicated by dashes (–). A, alanine; G, glycine; S, serine.

Receptor	Residue no.				
	299	302	303	304	308
Wild-type	S	S	S	S	S
Mutants					
cm1	A	G	A	G	A
mut1	A	–	–	–	–
mut2	A	–	–	–	A
mut4	–	G	A	G	–
mut14	–	G	–	–	–
mut15	–	–	A	–	–
mut16	–	–	–	G	–
mut17	–	G	A	–	–
mut18	–	–	A	G	–
mut19	–	G	–	G	–

= T or C and *Y* = A or C). The cluster 1 mutants are summarized in Table 1.

Cell Lines Expressing cAR1 Mutants—Mutated cAR1 cDNAs, subcloned into an expression vector (either pB18 or pJK1) (15), were introduced into AX-3 cells or cells lacking cAR1 (JB4 cells) (16) by electroporation as described previously (17). Similar results were obtained when wild-type cAR1 and selected mutants were expressed in each cell type. Clonal, stably transformed cell lines were selected and maintained in HL-5 medium (18) containing 20 μg/ml G418 (Life Technologies, Inc.). Cells expressing wild-type cAR1 (MC36 cells) and mutant cm1 are described elsewhere (15, 16). Previously described third intracellular loop mutants (16) used in this study and their amino acid substitutions (in parentheses) are as follows: A3 (R184L, Y185N, Y187S); A5 (T182S, Y187H); A22 (V188F, V189L); A42 (V189D); A53 (T182S, V188T, V189R); A60 (T186A, Y187D); and A62 (R184C, V188A, V189A).

Induction and Measurement of the Electrophoretic Mobility Shift—Cells, grown as shaken suspensions to densities of ~5 × 10^6 cells/ml, were washed with PB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 6.1) prior to each experiment. Washed, growth-stage cells were stimulated with the indicated concentrations of cAMP (22 °C, shaking at 200 rpm, durations indicated in legends) in the presence of 10 mM DTT to inhibit phosphodiesterase (19). Where indicated, 5 mM caffeine was added to inhibit endogenous cAMP production (20). Aliquots of cells were combined with sample buffer (21), mixed, and stored at −20 °C. These samples were subjected to SDS-PAGE on 10% or 12% low bispolyacrylamide gels (22), transferred to polyvinylene difluoride filters (Immobilon, Millipore), blocked with 3% bovine serum albumin, and immunoblotted using cAR1 C terminus-specific antiserum and chemiluminescence as described elsewhere (15). Quantitation of immunoblots was performed using a digitizing scanner (Logitech) and SigmaScan/Image software (Jandel).

Induction and Measurement of LLB—Unless indicated otherwise, washed, growth-stage cells were stimulated with cAMP as described for the induction of the electrophoretic mobility shift. Unless indicated otherwise, cells were then diluted 10-fold in ice-cold PB, pelleted by centrifugation at 0–4 °C (Sorvall SS34 rotor, 2000 rpm, 3 min), washed three times with ice-cold PB, and resuspended at 10^8 cells/ml. Binding of 16 nM [^{3}H]cAMP was then measured in PB by either a sedimentation assay (23) or by centrifugation through silicone oil (24) as indicated in the figure legends.

Reversal of LLB and Mobility Shift—In order to synchronize the reversal of mobility shifting and LLB for cell samples treated at various cAMP concentrations, the initial two PB washes sometimes contained cAMP and dithiothreitol, as indicated in the figure legends. Reversal time is therefore defined as beginning with the initiation of the third wash, which was always with PB alone. By this convention, the 20 min time point illustrated in the figures occurs immediately after completion of the fourth wash and resuspension of the cells.

RESULTS

Phosphorylation of the Central Three Serines of Cluster 1 Causes the Electrophoretic Mobility Shift—We have previously demonstrated that agonist-induced phosphorylation of cAR1 within the five serines of cluster 1 (Ser^{299}, Ser^{302}, Ser^{303}, Ser^{304}, and Ser^{308}) causes a discrete shift to a lower electrophoretic mobility form (15). This is illustrated in Fig. 1 by the inability of mutant cm1, which lacks these five serines, to undergo the transition in mobility. To identify the specific serine residues required, we selectively substituted the serines of cluster 1 as indicated in Table I. As shown in Fig. 1, when the flanking residues (Ser^{299} and Ser^{308}) were both substituted (*mut2*), the ability of the receptor to exhibit the mobility shift was unaffected. In contrast, substitution of the central three serine residues (Ser^{302}, Ser^{303}, and Ser^{304}) in *mut4*, eliminated the shift. The failure of cm1 or mut4 to shift is not related to levels of expression (Fig. 1) or due to impaired binding (data not shown). These results indicate that one or more of the central three serines, but not the flanking serines, mediate the electrophoretic mobility shift.

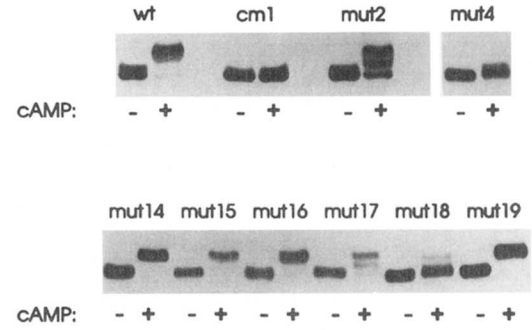

FIG. 1. **Electrophoretic mobility shift of cAR1 serine cluster 1 mutants.** Cells expressing wild-type (*wt*) or mutant cAR1s were incubated without (−) or with (+) cAMP as indicated (15 min, 22 °C, shaken at 200 rpm). Caffeine (5 mM) and DTT (10 mM) were added to inhibit cAMP synthesis and degradation, respectively. Samples of whole cells stimulated with 1 μM cAMP (*wt*, *cm1*, and *mut2*) or CHAPS-extracted cells (15) stimulated with 10 μM cAMP (all others shown) were analyzed by SDS-PAGE on 12% low bisacrylamide gels and immunoblotting with anti-cAR1 antiserum.

We then mutated the central three serines in various combinations. We found that each could be mutated individually without impairing the electrophoretic mobility shift (Fig. 1, *mut14*, *mut15*, and *mut16*). Thus, none of these serines is essential for this property. The mobility shift was markedly impaired when both Ser^{303} and Ser^{304} were mutated (Fig. 1, *mut18*). Furthermore, the preservation of either Ser^{303} or Ser^{304} by itself (Fig. 1, *mut17* and *mut19*) resulted in receptors which shifted like wild-type cAR1. These results suggest that either Ser^{303} or Ser^{304} can be phosphorylated and cause the shift. The small fraction of shifted receptor in the cAMP-stimulated mut18 sample suggests that Ser^{302} can also mediate the shift but with lower efficiency than either Ser^{303} or Ser^{304}. Occasionally, wild-type and mutant receptors having intermediate electrophoretic mobilities upon cAMP-stimulation are observed (*e.g. mut2* and *mut17* in Fig. 1). These possibly represent underphosphorylated forms.

cAMP-induced LLB of Mutants and Its Relationship to the Electrophoretic Shift—We next assessed the abilities of these cluster 1 mutants to undergo LLB in order to further evaluate the relationship between this property and receptor phosphorylation. As shown in Fig. 2, mutants which did not undergo the electrophoretic mobility shift (*e.g. cm1* and *mut4*) were also impaired in LLB. However, among the mutants which displayed the electrophoretic mobility shift, a spectrum of LLB behaviors was observed, ranging from those with normal LLB (*e.g. mut16*) to those with dramatically impaired LLB (*e.g. mut15* and *mut17*).

Wild-type cAR1 phosphorylation and the resulting mobility shift are readily reversible at 22 °C but very slowly reversible at 4 °C (11, 25). Since measurement of LLB requires prior removal of the cAMP stimulus by repeated washing, it was conceivable that instability of the shifted forms of mutants

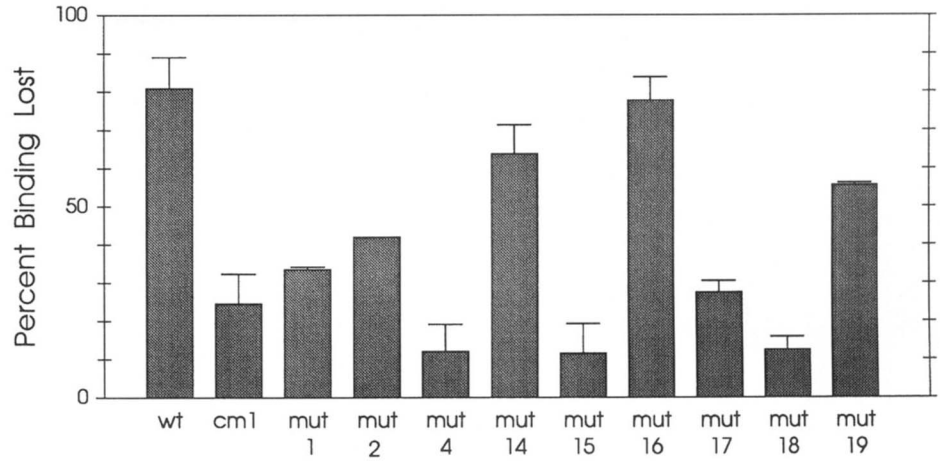

FIG. 2. **Loss of ligand binding by cAR1 mutants.** Cells expressing wild-type (*wt*) or mutant cAR1s were incubated without or with cAMP (10^{-5} M, 15 min), washed, resuspended to 10^8/ml, and analyzed for LLB by the sedimentation assay described under "Materials and Methods." Plotted for each cell line is the percentage of binding exhibited by untreated cells which is lost upon cAMP pretreatment. Values represent the means ± range of triplicate determinations from two independent experiments. mut2 was analyzed in only one experiment.

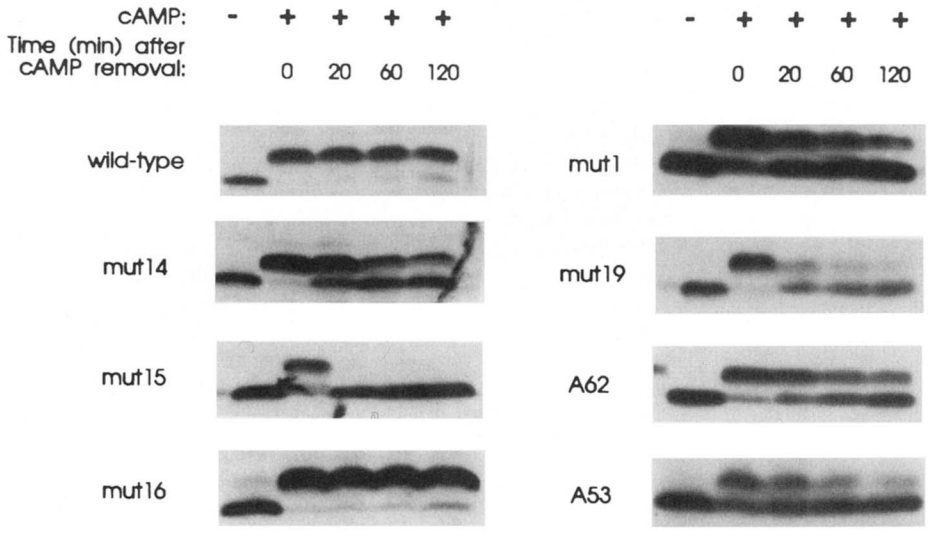

FIG. 3. **Stability of the electrophoretic mobility shift of cAR1 mutants.** Cells expressing wild-type or mutant cAR1s were incubated with cAMP (10^{-5} M, 15 min) washed four times with ice-cold PB, resuspended to 5×10^7/ml, and incubated on ice for the indicated times. Aliquots of cells were transferred to sample buffer before the addition of cAMP (−), following the 15 min stimulation and at 20, 60, and 120 min after the initiation of the third PB wash. (See "Materials and Methods" for explanation of time scale.) Cluster 1 mutants are defined in Table I. *A62* and *A53* are third intracellular loop mutants (16). The data shown are representative of at least two independent experiments.

such as mut15 explained the apparent incongruity of their LLB and shifting properties. To examine this possibility, the shifted forms of selected mutants were assessed at various times at 4 °C following cAMP removal, including the time at which we routinely measure LLB. As shown in Fig. 3, the reversal of the mobility shift for wild-type cAR1 is extremely slow at 0 °C. Nearly all of the receptor remained shifted 120 min after cAMP removal. Quantitation and extrapolation (not shown) of these data indicated that the half-time for reversal exceeds 5 h. In contrast, some of the cluster 1 mutants exhibit an increased rate of conversion of the shifted to the unshifted form. Mutation of Ser^{303} (mut15) dramatically reduced the stability of the shifted form ($t_{1/2} < 20$ min). Mutation of either Ser^{299} (mut1) or Ser^{302} (mut14) had a moderate destabilizing effect ($t_{1/2} \approx 60$–120 min). Surprisingly, mutation of Ser^{304} (mut16), one of the two serines able to mediate the shift, had no apparent effect on stability. Mutants substituted at two cluster 1 serines exhibited stabilities consistent with those observed for single-serine substitutions. Those lacking Ser^{303} (mut17 and mut18) were predictably unstable ($t_{1/2} < 20$ min, data not shown). The intermediate stability of mut19 ($t_{1/2} \approx 20$ min) can be attributed to mutation of Ser^{302}.

In light of the instabilities of the shifted forms of cluster 1 mutants, we revised our strategy and compared the extent of LLB with the residual mobility shift of each mutant at the time of the LLB measurement. As demonstrated in Fig. 4, this analysis now revealed a significant correlation between the extent of LLB and the fraction of receptors in the shifted state, suggesting an interrelationship of these two properties.

These observations prompted us to re-examine the mobility shift of cAR1 third intracellular loop mutants which we had previously found to be impaired in LLB (16). As with the cluster 1 mutants, we found that the lower mobility forms of some of these mutants were less stable than that of wild-type cAR1 (*e.g. A53* and *A62*, Fig. 3). Inclusion of data from these mutants in Fig. 4 further strengthened the correlation between LLB and the electrophoretic shift.

cAMP Concentration-dependent Stabilization of the Shifted Form of Wild-type cAR1—To further evaluate this correlation and establish its relevance to the wild-type receptor, we performed a detailed analysis of the stability of the mobility shift in wild-type cAR1 and its relationship to LLB. We first sought to assess whether the stabilization of the lower mobility form of wild-type cAR1 depended on cAMP concentration. We therefore treated cells expressing wild-type cAR1 with either 10^{-7} M or 10^{-5} M cAMP, removed the cAMP, and monitored the stability of the lower mobility receptor form. As illustrated in Fig. 5*A*, each concentration elicited a complete conversion from unshifted to shifted forms. Upon removal of cAMP and incubation at 4 °C, however, the stabilities of the shifted forms differed markedly. While receptors treated with the higher cAMP concentration remained completely shifted for at least 2 h, those treated at the lower concentration exhibited a time-dependent decay to the higher mobility form ($t_{1/2} \approx 60$ min). Thus, as we found for the mutants, electrophoretically shifted wild-type receptors can exhibit markedly different stabilities. Their relative stabilities depend upon the cAMP concentration with which they were stimulated.

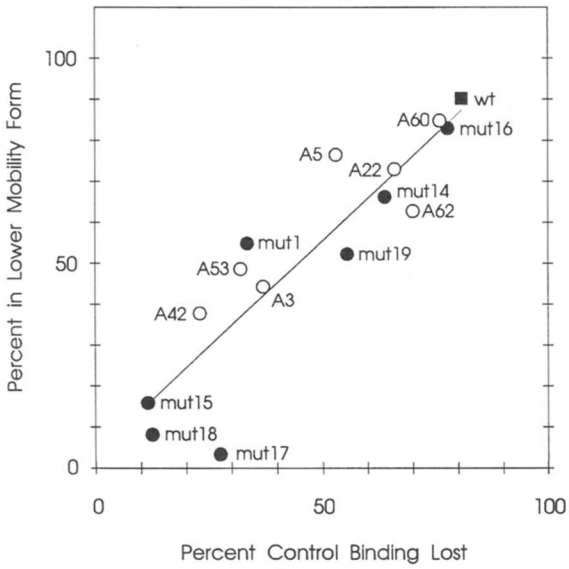

FIG. 4. **Correlation between cAMP-induced loss of ligand binding and electrophoretic mobility shift in cAR1 mutants.** The percentage loss of cAMP binding exhibited by wild-type cAR1 (*wt, filled square*), cluster 1 mutants (*filled circles*) and third intracellular loop mutants (*open circles*) immediately following pretreatment with cAMP (10^{-5} M, 15 min) and four washes with ice-cold PB (20-min time point in Fig. 3) is plotted against the percentage of receptor in the lower mobility form, determined under the same conditions. LLB data for wild-type cAR1 and cluster 1 mutants is taken from Fig. 3 and that for the third intracellular loop mutants is from Caterina *et al.* (16). Mobility shift data was obtained by scanning the 20-min time point from autoradiographs generated as in Fig. 3 and represents the mean determination from two independent experiments. LLB and mobility shift for a given mutant were usually assayed in separate experiments but at the same time after cAMP removal. Comparison of data points with the illustrated correlation line (generated by linear regression) yields $r^2 = 0.81$, $\alpha < 0.001$. Separate analyses of the third loop mutants ($r^2 = 0.78$, $\alpha < 0.01$) and cluster 1 mutants ($r^2 = 0.81$, $\alpha < 0.01$) reflected similarly strong correlations (not depicted).

To evaluate the kinetics of the stabilization, we completely converted receptors to the mobility-shifted form with 10^{-7} M cAMP and then exposed the cells for various times to 10^{-5} M cAMP (Fig. 5*B*). As before, receptors exposed to only the lower cAMP concentration exhibited an unstable mobility shift. Those exposed to the higher concentration acquired stability with a $t_{1/2}$ of approximately 4 min.

Correlation between Shifting and LLB of the Wild-type cAR1—Previously reported EC_{50} values for the cAR1 mobility shift are up to 10-fold lower than those for LLB (11, 13, 25, 32). To assess whether this apparent disparity could be explained by instability of shifted receptors, we undertook a detailed analysis of the cAMP concentration dependence of cAR1 mobility shifting prior to and at times following cAMP removal. As shown in Fig. 6*A*, the concentration of cAMP required to convert half of the cAR1 molecules to the shifted form, or EC_{50}, is 7 nM. Due to instability at low cAMP concentrations, the apparent EC_{50} values observed 20 and 150 min after cAMP removal and incubation at 4 °C are 30 and 140 nM, respectively. We also assessed the concentration-dependence of LLB at the 20- and 150-min time points. The EC_{50} values for LLB increased with time and closely paralleled those of the mobility shift (Fig. 6*B*).

Lastly, we examined the relationship between the electrophoretic mobility shift and LLB of wild-type cAR1, by evaluating the kinetics of both processes. As shown in Fig. 7, the kinetics of the electrophoretic mobility shift and LLB, induced by 10^{-5} M cAMP and assessed immediately after cAMP removal, were nearly identical. These findings, taken together with the results of the concentration-dependence experiments, extend the correlation observed between LLB and phosphorylation in cAR1 mutants to the wild-type receptor.

DISCUSSION

We have previously shown that agonist binding induces the addition of 3 or 4 mol of phosphate per mol of cAR1 to serine residues of the C-terminal cytoplasmic domain. Phosphoryla-

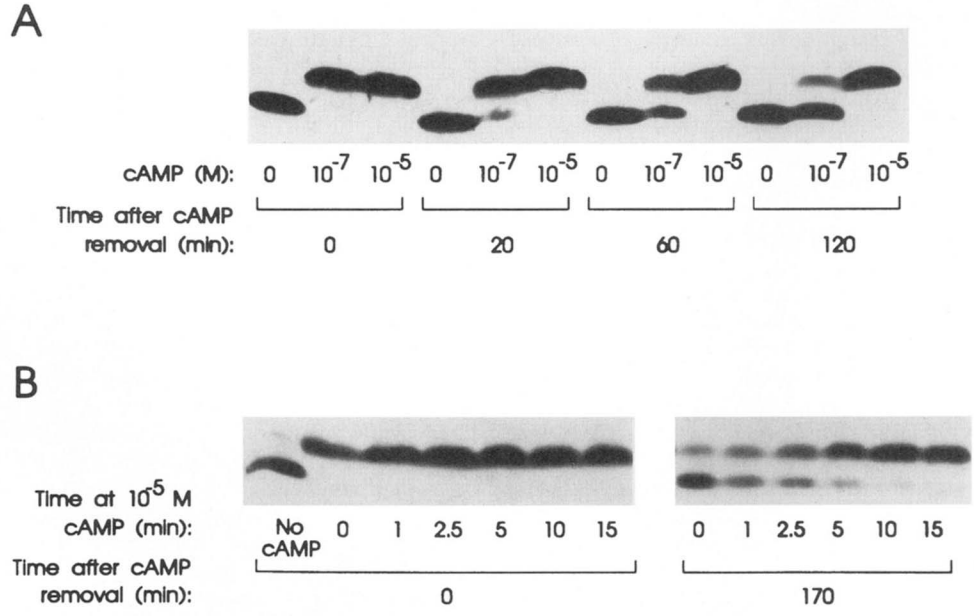

FIG. 5. **Concentration-dependent stability of the mobility shifted form of wild-type cAR1.** *A*, cells expressing wild-type cAR1 were incubated without (0 M) or with 10^{-7} M or 10^{-5} M cAMP (15 min, 22 °C), washed twice with ice-cold PB containing 10 mM DTT (for the unstimulated sample) or ice-cold PB containing 10^{-7} M cAMP and 10 mM DTT, then twice with ice-cold PB alone, resuspended to 5×10^7/ml, and incubated on ice. Cells were combined with sample buffer at the indicated times after the initiation of the third wash and analyzed by SDS-PAGE on 10% low bisacrylamide gels and immunoblotting with cAR1-specific antiserum as described under "Materials and Methods." *B*, time-dependent stabilization of the lower mobility receptor form. Cells expressing wild-type cAR1 were incubated with 10^{-7} M cAMP (15 min, 22 °C) after which the cAMP concentration was adjusted to 10^{-5} M cAMP for the indicated period of time. (*0* indicates no further cAMP addition.) Cells were then washed twice with ice-cold PB containing cAMP (10^{-7} M) and 10 mM DTT, then twice with ice-cold PB alone, resuspended to 5×10^7/ml, and incubated on ice

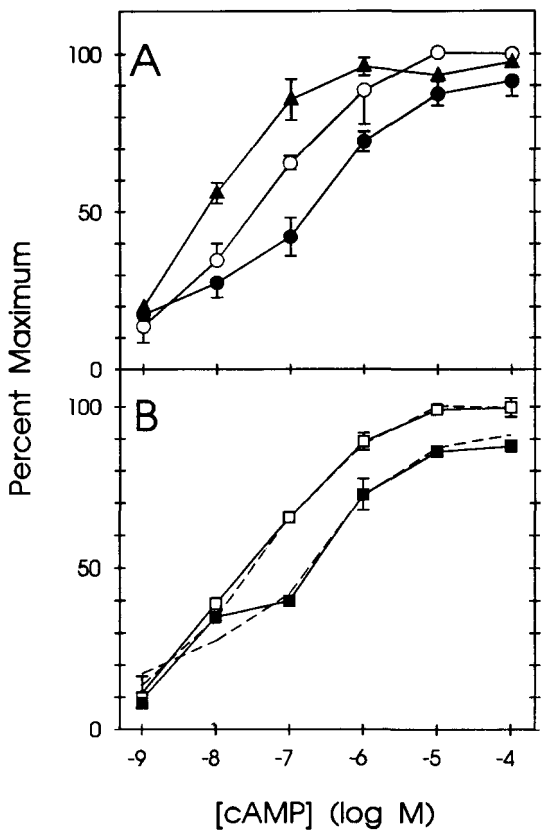

FIG. 6. **Concentration-dependence of wild-type cAR1 mobility shift and LLB.** *A*, cells expressing wild-type cAR1 were treated 15 min with a range of cAMP concentrations (0 to 10^{-4} M) in the presence of caffeine and DTT. Cells treated at concentrations below 10^{-7} M were washed twice with ice-cold PB containing DTT and the same concentration of cAMP. Cells treated with 10^{-7} M cAMP and above were washed twice with ice-cold PB containing 10^{-7} M cAMP and DTT. All cells were then washed twice with ice-cold PB alone, resuspended to 10^8/ml, and incubated at 4 °C with shaking (200 rpm) for an additional 130 min. Cells were combined with sample buffer immediately after the incubation with cAMP (*filled triangles*) and at 20 min (*open circles*) or 150 min (*filled circles*) after the initiation of the third wash. Samples were subjected to SDS-PAGE on 10% low bisacrylamide gels, immunoblotted with cAR1-specific antiserum, and the fraction of receptor in the lower mobility form determined as described under "Materials and Methods." These values were then normalized to the percentage of shifting observed at 20 min in cells treated with 10^{-4} M cAMP (90%). Data shown represent the mean ± range of two independent experiments. *B*, binding to 10 nM [^{3}H]cAMP was assessed for the cells described in *Panel A*, 20 min (*open squares*) or 150 min (*filled squares*) after the initiation of the third wash. Binding, measured by the silicone oil method, was normalized to that of untreated cells. In order to allow a comparison between LLB and the mobility shift (*dashed lines*, reproduced from *Panel A*), binding data were expressed as a percentage of LLB observed at the 20 min time point in cells treated with 10^{-4} M cAMP (86%). Data shown represent the mean ± range from two independent experiments performed in triplicate. The 20 min LLB data for these two experiments have previously been presented elsewhere (32).

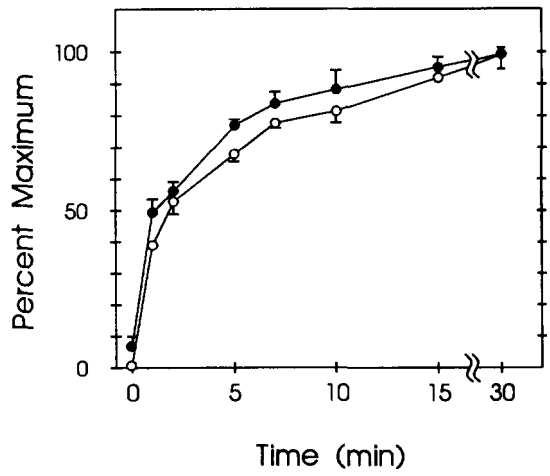

FIG. 7. **Kinetics of wild-type cAR1 mobility shift and LLB.** Kinetics of LLB (*open circles*) and the mobility shift (*filled circles*). Washed, transformed cells expressing wild-type cAR1 were incubated without or with cAMP (10^{-5} M, 15 min) washed four times with PB, and resuspended to 10^8/ml. The percentage of receptor in the lower mobility form and the percentage of control binding lost upon cAMP pretreatment were simultaneously determined as in Fig. 6. To allow a direct comparison of kinetics, data were normalized to those values obtained for cells exposed to a 15 min cAMP stimulus (81% LLB, 92% mobility shift). Data shown represent the means ± range from two experiments performed in triplicate. The LLB data from these experiments have previously been reported elsewhere (32).

tion within cluster 1, which includes Ser^{299}, Ser^{302}, Ser^{303}, Ser^{304}, and Ser^{308}, accounts for two-thirds of the total and causes a marked decrease in cAR1's electrophoretic mobility (15). In this report, we show that the lower electrophoretic mobility form can result from the phosphorylation of either Ser^{303} or Ser^{304}. Presumably, one or both of these two serine residues are targets of phosphorylation in the wild-type receptor.

We have also previously demonstrated that agonist-induced LLB is largely due to a reduction in the binding affinity of cAR1 for cAMP. While under some circumstances receptor internalization can result from cAMP treatment, this process need not occur for LLB to be observed. Furthermore, like the electrophoretic mobility shift, LLB is markedly impaired in mutant receptors lacking all five serines of cluster 1 (32). Some of the substitutions of individual cluster 1 serines, described in the present report, initially appeared to break this correlation between LLB and the mobility shift. For example, substitution of Ser^{303} yielded a mutant, mut15, which exhibited the electrophoretic mobility shift but virtually no LLB. Careful examination, however, revealed that the lower mobility form of this mutant is abnormally unstable. When cells were washed free of ligand and incubated on ice, this receptor returned to the higher mobility form with a half-life of several minutes or less compared with hours for the wild-type receptor. Consequently, this and other similar mutant receptors are found predominantly in the higher mobility form at the time of LLB assay. The discovery of this instability prompted us to re-examine receptors with mutations in the third cytoplasmic loop that exhibit impaired LLB (16). Some of these mutants also displayed unstable mobility-shifted forms (*e.g.* A53). When these instabilities are taken into account, the extent of LLB is significantly correlated with the fraction of receptors in the lower mobility form at the time of LLB assay for all mutants examined. Dose-dependence and kinetics studies, presented in this report, revealed a similar correlation for the wild-type receptor.

This correlation implies that the phosphorylation-dependent electrophoretic shift and LLB are sequential processes. Phosphorylation might occur first and induce a low affinity conformation either allosterically or by promoting the binding of a protein such as an arrestin homolog. Alternatively, LLB might represent the adoption of a low affinity receptor conformation that is necessary but not sufficient for subsequent phosphorylation. This possibility is supported by the observation that

for 170 min. Cells were combined with sample buffer prior to the addition of cAMP (no cAMP), just prior to washing (0 min) or at 170 min after the initiation of the third wash. Data shown are representative of two independent experiments.

(R_p)-cAMPS induces LLB without causing the electrophoretic shift (26). Still, the LLB induced by (R_p)-cAMPS is unstable (reversing within minutes at 4 °C),[2] suggesting that phosphorylation might stabilize the low affinity state. Interestingly, the G protein-coupled receptor kinase GRK2 has been shown to facilitate the sequestration of mammalian m2 muscarinic receptors (3), suggesting that similar relationships between receptor phosphorylation and agonist binding alterations may exist in other systems.

We identified a variety of factors which affect the stability of the electrophoretically shifted form of the receptor. These include the dose and time of cAMP pretreatment as well as mutations within serine cluster 1 and the receptor's third cytoplasmic loop. For the wild-type receptor, low cAMP concentrations ($\sim 10^{-7}$ M) induce the electrophoretic mobility shift. As described above, phosphorylation of either Ser^{303} or Ser^{304} could be sufficient to produce the shift. Upon cAMP removal, however, receptors phosphorylated under these conditions are rapidly dephosphorylated and return to the unshifted state with a $t_{1/2}$ of ~20 min at 4 °C. Saturating cAMP concentrations ($>10^{-6}$ M), in addition to causing the shift, trigger a process which dramatically increases the stability of the shifted form ($t_{1/2}$ of several hours at 4 °C). Assuming that the involved kinase ceases to phosphorylate the receptor after cAMP is removed, this stabilizing process must reflect either inactivation of the involved phosphatase or a change in the shifted receptor which impedes its dephosphorylation. We suspect the latter possibility is the case and that stabilization of shifted receptors might be the consequence of further phosphorylation of the receptor, beyond that required to cause the shift. Consistent with this possibility, ^{32}P-labeling experiments have shown that the specific radioactivity of *shifted* receptors increases ~2-fold during a response to saturating cAMP (11). Furthermore, we have previously shown that cAMP occupancy triggers the addition of up to 2 or 3 phosphates to cluster 1 serines (15).

The cAR1 mutations described in this report that impair stability of the shifted form might do so by diminishing the extent of receptor phosphorylation. For instance, mutations within cluster 1 might directly eliminate phosphorylation sites or, alternatively, might diminish phosphorylation or enhance the dephosphorylation of other cluster 1 residues. How mutations in the putative third intracellular loop might influence phosphorylation of serine cluster 1 is more puzzling. Perhaps juxtaposition of these two regions in the native receptor permits these mutations to affect the accessibility of cluster 1 to the kinase or phosphatase. Alternatively, mutations in the third loop could reduce the extent of receptor phosphorylation if, like β-adrenergic receptor kinase and rhodopsin kinase, the kinase responsible for cAR1 phosphorylation is activated by interaction with the receptor's intracellular loop domains (27–29).

It has been proposed that β_2-adrenergic receptors must be internalized in order to be dephosphorylated (7, 30). This appears not to be the case for cAR1 since we observed dephosphorylation at 4 °C, a temperature which should preclude vesicular traffic. Furthermore, under the conditions of our experiments, prior internalization is unlikely as nearly all cAR1 molecules remain on the cell surface following cAMP treatment (32).

Our findings suggest that an important role of phosphorylation might be to bring about (or maintain) a low affinity state of cAR1, possibly serving to broaden the sensitive range of cells to micromolar cAMP concentrations. Such high concentrations are transiently produced by aggregating cells and are thought to persist in multicellular structures (reviewed in Ref. 31). It is also tempting to speculate that loss of ligand binding reflects the interaction of cAR1 to another protein, perhaps one involved in the desensitization of G protein-mediated signaling. Whatever their functions might be, the concentration-dependent stabilization of cAR1 phosphorylation and LLB, described here, is likely to be most relevant to the multicellular stage. Experiments directed at distinguishing these possibilities are in progress.

Acknowledgment—We are grateful to Dr. Peter J. M. Van Haastert for valuable discussions.

[2] P. J. M. Van Haastert, personal communication.

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